UNIVERSITY „Ss. CYRIL AND METHODIUS" IN SKOPJE
FACULTY OF VETERINARY MEDICINE – SKOPJE

BOOK OF PROCEEDINGS

DAYS OF VETERINARY MEDICINE 2012

3rd International Scientific Meeting

Republic of Macedonia
2-4 September 2012

EXECUTIVE COMMITTEES OF DAYS OF VETERINARY MEDICINE 2012

Organizing Committee

Prof. Dr. Dine Mitrov, Prof. Dr. Velimir Stojkovski, Prof. Dr. Zehra Hajrulai-Musliu, Prof. Dr. Slavco Mrenoski, Prof. Dr. Vlatko Ilieski, Prof. Dr. Blagica Sekovska, Prof. Dr. Plamen Trojacanec, Prof. Dr. Igor Ulcar, Prof. Dr. Pavle Sekulovski, Prof. Dr. Toni Dovenski, Asst. Prof. Dr. Jovana Stefanovska, Asst. Prof. Dr. Lazo Pendovski, Asst. m-r Dean Jankuloski, Asst. m-r Ljupco Mickov, Asst. m-r Irena Celeska

International Scientific Committee

Prof. Dr. Marjan Kosec
University of Ljubljana, Slovenia
Prof. Dr. Jelka Zabavnik-Piano
University of Ljubljana, Slovenia
Prof. Dr. Dinko Dinev
Trakia University of Stara Zagora, Bulgaria
Prof. Dr. Aleksandar Pavlov
Trakia University of Stara Zagora, Bulgaria
Prof. Dr. Tomislav Dobranic
University of Zagreb, Croatia
Prof.Dr. Alen Slavica
University of Zagreb, Croatia
Prof. Dr. Andrej Kirbis
University of Ljubljana, Slovenia
Prof. Dr. Geert Opsomer
University of Gent, Belgium
Prof. Dr. Robert Farkas
University of Budapest, Hungary
Prof. Dr. Almedina Zuko
University of Saraevo, Bosnia and Herzegovina
Prof. Dr. Mehmed Muminovic
University of Saraevo, Bosnia and Herzegovina
Prof. Dr. Danijela Kirovski
University of Belgrade, Serbia
Prof. Dr. Miodrag Lazarevic
University of Belgrade, Serbia
Prof. Dr. Ivanco Naletoski
Joint FAO/IAEA Division, Vienna, Austria
Prof. Dr. Giovanni M. Lacalandra
University of Bari, Italy
Prof. Dr. Kiro R. Petrovski
University of Adelaide, Australia
Prof. Dr. Mustafa Atasever
Istanbul University, Turkey
Prof. Dr. Halil Gunes
Istanbul University, Turkey

Secretariat

Asst. Prof. Dr. Florina Popovska-Percinik, D-r Elizabeta Dimitrievska-Stojkovik Asst. m-r Aleksandar Dodovski, Asst. m-r Iskra Cvetkovik, Asst. m-r Ksenija Ilievska, Asst. m-r Kirili Krstevski, Asst. m-r Igor Dzadzovski, Asst. m-r Nikola Adamov, Asst. m-r Igor Esmerov, Asst. m-r Katerina Blagoevska, Asst. m-r Branko Atanasov, m-r Biljana Stojanovska – Dimzoska, Asst. Sandra Kostova, Ljupco Angelovski, Mirko Prodanov, Marija Ratkova, Sinisa Acevski, Branko Angelovski

Topics of the Days of Veterinary Medicine 2012

Animal Health
Food Safety and Veterinary Public Health
Animal Welfare and Genetics
Animal Reproduction

Editors:

Prof. Dr. Dine Mitrov
Assist. Prof. Dr. Lazo Pendovski

Published by:

Faculty for veterinary medicine – Skopje, Lazar Pop Trajkov 5/7, 1000 Skopje
Tel: ++389 2 3420 700 Fax: ++ 389 2 3114 619
www. fvm.ukim.edu.mk

CIP - Каталогизација во публикација
Национална и универзитетска библиотека "Св. Климент Охридски", Скопје
636.09(062)
DAYS of veterinary medicine 2012 : book of proceedings : 3rd International scientific meeting, 2-4 September, 2012 Republic of Macedonia / [editors Dine Mitrov, Lazo Pendovski]. - Skopje : Faculty of Veterinary medicine, 2012. - 308 стр. : граф. прикази ; 21 см
Текст на мак. и англ. јазик. - Библиографија кон трудовите
ISBN 978-9989-774-23-2
1. Mitrov, Dine [главен уредник] 2. Pendovski, Lazo [уредник]
а) Ветеринарна медицина - Собири
COBISS.MK-ID 91886090

Dear Colleagues,

On behalf of Organizing Committee, we are pleased to invite you, for active participation on **DAYS OF VETERINARY MEDICINE 2012**, which will be held from 02-04 September in Ohrid, Macedonia. The organizer is the Faculty of Veterinary Medicine at the University of Ss. Cyril and Methodius in Skopje.

The program offers unique opportunity for plenary lectures, scientific presentations and discussions about mutual interest for animal health, food safety, public health, animal welfare, genetics and animal reproduction. We believe that this meeting is an excellent opportunity for renewal of old and making new contacts between scientists, veterinary practitioners, farm veterinarians and official veterinarians.

Veterinary science has undergone tremendous development in all fields of research and gained ever-increasing importance in the management of many diseases. Faculty of Veterinary Medicine in Skopje continuously plays a fundamental role in these processes. These activities have generated significant results in various areas of the veterinary science and contributed greatly to the incorporation of science into every day veterinary practice.

The ancient town of Ohrid is ready to welcome all participants offering unique experience through a blend of beautiful lake, museums, old churches and above all traditional Macedonian food.

We look forward to seeing you in Ohrid for this unique and stimulating event!

Executive Committee
Days of Veterinary Medicine 2012

CONTENT

Topic: Animal Health

PLENARY LECTURE
EFFECT OF HEAT STRESS ON METABOLIC AND ENDOCRINE STATUS OF DAIRY COWS
Vujanac Ivan, Horvat Jožef, Šamanc Horea, Kirovski Danijela..... 11

PLENARY LECTURE
APPLICATION OF FLEXIBLE ENDOSCOPY IN CARNIVORES
Krstić Vanja..... 15

ORIGINAL ARTICLES
INFECTIOUS BURSAL DISEASE VIRUS: THE CURRENT SITUATION IN SERBIA, CONTROL AND PREVENTION
Velhner Maja, Miljković Biljana, Dobrosavljević Ivan..... 17

SERA AND YOLK HAETHCING EGGS` EXAMINATION ON SALMONELLA ENTERITIDIS ANTIBODIES
Miljković Biljana, Radojčić Marina, Velhner Maja, Ilic Živka, Dobrosavljević Ivan..... 21

ANTIBODY PATTERNS OF INFECTIOUS BRONCHITIS IN VACCINATED FLOCKS WITH CLINICAL SIGNS
Dodovski Aleksandar, Krstevski Kiril, Mitrov Dine, Naletoski Ivancho..... 25

EVALUATION OF VACCINATION COVERAGE AGAINST CLASSICAL SWINE FEVER IN FARM PIGS IN MACEDONIA AND POSSIBLE REASONS FOR THE POOR RESULTS
Djadjovski Igor, Krstevski Kiril, Mitrov Dine, Mrenoshki Slavcho, Acevski Sinisha, Cvetkovikj Iskra, Celeska Irena, Kirandjiski Toni, Naletoski Ivanco..... 28

MONITORING BAIT UPTAKE THROUGH TETRACYCLINE PRESENCE AND AGE STRUCTURE OF FOXES IN ORAL VACCINATION AGAINST RABIES CAMPAIGNS IN R. MACEDONIA
Cvetkovikj Aleksandar, Radeski Miroslav, Mrenoshki Slavcho, Kirandjiski Toni, Krstevski Kiril, Dzhadzhovski Igor, Gjurovski Ivica, Branko Angjelovski, Cvetkovikj Iskra, Florence Cliquet..... 30

MOST IMPORTANT FOOD-BORNE DISEASES OF DOGS CAUSED BY TICKS AND ITS CONTROL
Pavlovic Ivan, Petkovic Dragana, Kukovska Valentina, Stamenkovic Vojislav,Jovcevski Srdjan, Pavlovic Miloš, Jovcevski Stefan, Elezovic Milica..... 34

SEROLOGICAL EVIDENCE OF EHRLICHIA CANIS AND LEISHMANIA INFANTUM CO-INFECTION IN NATURALLY EXPOSED DOGS ON THE TERRITORY OF REPUBLIC OF MACEDONIA
Stefanovska Jovana, Kochevski Zoran, Iskra Cvetkovikj, Farkas Robert, Ivancho Naletoski, Mrenoshki Slavcho..... 38

COMPARISON OF THE ANESTHETIC EFFECTS OF XYLAZINE/KETAMINE, PROPOFOL AND ZOLETIL IN DOGS
Ilievska Ksenija, Trenkoska-Spasovska Pandorce, Trojacanec Plamen..... 40

CREATINE KINASE ACTIVITY IN DOGS WITH EXPERIMENTALLY INDUCED STAPHYLOCOCCUS AUREUS INFECTION
Zapryanova Dimitrinka, Mircheva Teodora, Lalev Damyan..... 44

EFFECT OF NEGATIVE ENERGY BALANCE ON IGF SYSTEM IN DAIRY COWS
Kirovski Danijela, Šamanc Horea, Vujanac Ivan, Prodanović Radiša, Đurić Miloje, Sladojević Željko..... 48

APPLICATION OF IRON CHELATOR DESFERIOXAMINE IN DOGS WITH MALIGNANT MAMMARY GLAND TUMORS TREATED WITH EPIRUBICIN
Todorova Irina..... 52

THE ROLE OF COMMUNICATIONAL SKILLS IN SUCCESSFUL MANAGEMENT OF VETERINARY PRACTICE
Sekovska Blagica, Tosevska-Apostolova Milica..... 56

COMPARISON OF RESULTS OBTAINED WITH TWO DIFFERENT ELISA KITS FOR DETECTION OF ANTIBODIES AGAINST CLASSICAL SWINE FEVER VIRUS
Krstevski Kiril, Dzadzovski Igor, Mitrov Dine, Mrenoshki Slavcho, Acevski Sinisa, Cvetkovikj Iskra, Naletoski Ivancho..... 59

BRUCELLA EPIDEMIOLOGY IN GEORGIA
Makaradze Levan, Mirtskhulava Merab, Giorgobiani Marina..... 62

UNILATERAL NEPHRECTOMY AND URETERECTOMY IN DOG: CLINICAL CASE
Trenkoska-Spasovska Pandorce, Ilievska Ksenija, Trojacanec Plamen..... 64

CASE REPORT: MODIFIED SEGMENTAL SPINAL FIXATION TECHNIQUE FOR TREATMENT OF LUMBAR FRACTURE IN DOG
Pavlovski Damjan..... 67

INTESTINAL ADENOCARCINOMA IN RAINBOW TROUT (*Oncorhynchus Mykiss*)
Gombač Mitja, Seničar Marija, Švara Tanja, Žižek Suzana, Hristovski Mišo, Pogačnik Milan..... 71

CANINE TRANSMISSIBLE VENERAL TUMOR – SURGICAL TREATMENT AND CHEMOTHERAPY: CASE REPORT
Atanaskova Petrov Elena, Nikolovski Goran, Ilievska Ksenija, Trojacanec Plamen, Celeska Irena, Velev Romel.....73

A CASE REPORT OF CANINE MONOCYTIC EHRLICHIOSIS IN 6 YEARS OLD MALE SIBERIAN HUSKY
Dimeski Z., Josheski M. and Trajanoska B..... 77

LABORATORY DIAGNOSTIC OF CAT LIP FIBROMA - CASE REPORT
Ulčar Igor, Pavlovski Damjan, Celeska Irena 83

RADIOLOGICAL DIAGNOSTIC OF LUNG METASTASIS WITH FEMUR OSTEOSARCOMA ORIGIN
Acevski Sinisha, Mitrov Dine, Krstevski Kiril, Dzadzovski Igor, Janevski Aleksandar, Velkovski Dimche..... 86

EFFECTS OF DIETARY VITASIL® ON GROWTH PERFORMANCE OF CARP (*Cyprinus caprio*)
Atanasoff Alexander, Ivanov Veselin, Nikolov Galin, Zhelyazkov Georgi, Petrova Biliana..... 90

Topic: Food Safety and Veterinary Public Health

PLENARY LECTURE
MODERN CHALLENGES IN FOOD HYGIENE/SAFETY AND THE RESPONSES FROM VETERINARY EDUCATION - EAEVE, EBVS AND ECVPH
Buncic Sava..... 97

PLENARY LECTURE
EFFECT OF MODIFIED ATMOSPHERE PACKAGING ON EXTENSION OF FOOD SHELF LIFE
Milijasevic Milan, Matekalo-Sverak Vesna, Babic Jelena.....102

ORIGINAL ARTICLES
RAW MILK FROM VENDING MILK MACHINE IN SLOVENIA: FOOD SAFETY CONTROL
Kirbiš A., Biasizzo M., Vadnjal S., Torkar K., Bauer M., Jevšnik M..... 106

ASSESSMENT OF CADMIUM INTAKE ASSOCIATED WITH LIVER AND KIDNEY CONSUMPTION IN SERBIA
Janković Saša, Antonijević Biljana, Ćurčić Marijana, Radičević Tatjana, Stefanović Srđan, Nikolić Dragica, Petrović Zoran.....108

HPLC/FL METHOD FOR HISTAMINE TESTING IN FISH
Stoilova Nadezhda, Peycheva M., Yankovska T..... 111

PFGE TYPING OF MAJOR FOODBORNE PATHOGENS AS TOOL FOR TRACKING THE SOURCES OF CONTAMINATION ALONG THE FOOD CHAIN
Jankuloski Dean, Sekulovski Pavle, Mojsova Sandra, Angelovski Ljupco, Prodanov Mirko, Ratkova Marija.....116

EFFECT OF IMPLEMENTATION OF HACCP SYSTEM ON DISTRIBUTION OF *LISTERIA MONOCYTOGENES* IN BULGARIAN FOODS
Daskalov Hristo, Daskalova Alexandra..... 120

DETERMINATION OF ZERANOL RESIDUES LEVELS IN BOVINE URINE WITH ELISA METHOD
Hajrulai-Musliu Zehra, Uzunov Risto, Dimitrieska-Stojkovik Elizabeta, Stojanovska-Dimzoska Biljana, Sekulovski Pavle, Stojkovski Velimir, Todorovic Aleksandra.....123

DETERMINATION OF TRENBOLONE IN CATTLE MEAT WITH ELISA METHOD
Uzunov Risto, Hajrulai-Muslu Zehra, Dimitrieska-Stojkovik Elizabeta, Stojanovska-Dimzoska Biljana, Sekulovski Pavle, Stojkovski Velimir, Todorovic Aleksandra.....127

ESTIMATION OF TIME OF SEMI-DECAYOF ^{137}Cs IN MUSHROOMS
Todorovik Aleksandra, Sekulovski Pavle, Dimitrieska Stojkovik Elizabeta, Hajrulai-Musliu Zehra, Stojanovska Dimzoska Biljana, Uzunov Risto.....132

INACTIVATION OF SALMONELLA TYPHIMURIUM ON POULTRY MEAT BY ELECTROLYZED WATER
Çilİplikçioğlu Güzin, Demirel Yağmur Nil, Şireli Ufuk Tansel.....136

FLUOROMETRIC VALIDATION PROCEDURE FOR DETERMINATION OF OCHRATIOXIN A IN WINE
Stojanovska-Dimzoska Biljana, Hajrulai-Musliu Zehra, Dimitrieska-Stojković- Elizabeta, Uzunov Risto, Todorovic Aleksandra, Sekulovski Pavle.....139

ANTIBIOTIC RESISTANCE OF *CAMPYLOBACTER* STRAINS ISOLATED FROM BROILERS IN MACEDONIA
Angelovski Ljupco, Sekulovski Pavle, Jankuloski Dean, Ratkova Marija, Prodanov Mirko, Mojsova Sandra 143

RISK FACTORS AFFECTING PRESENCE OF *CAMPYLOBACTER* SPP. IN POULTRY AND POULTRY MEAT
Vashin Ivan, Stoyanchev Todor.....146

OCCURANCE OF RESIDUES OF TETRACYCLINES IN RAW EWE'S MILK
Dimitrieska-Stojković Elizabeta, Stojanovska-Dimzoska Biljana, Hajrulai-Musliu Zehra, Stojković Goran, Prodanov Risto.....149

RESULTS FROM MONITORING THE EDIBLE ANIMAL TISSUES FOR RESIDUES OF SOME VETERINARY DRUGS
Dimitrieska-Stojković Elizabeta, Arsova Gordana, Hajrulai-Musliu Zehra, Stojanovska-Dimzoska Biljana, Uzunov Risto.....154

QUALITY CONTROL OF POTENTIATED SULFONAMIDE COMMERCIAL VETERINARY FORMULATIONS BY UV-SPECTROPHOTOMETRY
Mihajlović Jelena, Dimitrieska - Stojković Elizabeta, Velev Romel, Stojković Goran..... 159

COMPARISON OF SPECTROPHOTOMETRIC AND COMPLEXOMETRIC – SPECTROPHOTOMETRIC ASSAY FOR DETERMINATION OF OXYTETRACYCLINE IN VETERINARY DRUGS
Naumoska Marina, Dimitrieska-Stojković Elizabeta, Stojković Goran..... 165

APPLICATION OF ZERO- AND FIRST-ORDER DERIVATIVE SPECTROSCOPY IN THE QUALITY CONTROL OF VETERINARY DRUGS
Trajkovska Violeta, Dimitrieska-Stojković Elizabeta, Stojković Goran..... 170

ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS ON SOME FOOD BORNE PATHOGENIC AND SAPROPHITIC BACTERIA
Ratkova Marija, Sekulovski Pavle, Jankuloski Dean, Angelovski Ljupco, Mojsova Sandra, Prodanov Mirko..... 176

LEGISLATION AND HEALTH ASPECT OF NUTRITIONAL FEED SUPPLEMENTS
Crceva Nikolovska Radmila, Sekulovski Pavle, Prodanov Risto, Hajrulai Musliu Zehra, Angelovski Ljupco, Arsova Gordana, Nikolovski Aleksandar..... 179

CONTROL OF PROCES HYGIENE IN FERMENTED DAIRY PRODUCTS IN THE R. MACEDONIA
Prodanov Mirko, Ratkova Marija, Angelovksi Ljupčo, Mojsova Sandra, Jankuloski Dean, Sekulovski Pavle.....182

PHARMACOKINETICS OF THE FERROUS SULPHATE IN BROILER CHICKENS
Arnaudova-Matey Anna, Mehmedov Tanju.....185

GATIFLOXACIN RESIDUES IN CHICKEN MEAT, SKIN AND GIBLETS
Kyuchukova Ralica 189

Topic: ANIMAL WELFARE & GENETICS

PLENARY LECTURE
DEVELOPMENTS IN FARM ANIMAL WELFARE AS AFFECTED BY HANDLING, TRANSPORT AND SLAUGHTER
Anil Haluk.....193

PLENARY LECTURE
RELEVANCE OF GENETIC RESEARCH FOR MILK PRODUCTION AND UDDER HEALTH
Ogorevc Jernej, Prpar Sonja, Kunej Tanja and Dovc Peter..... 199

ORIGINAL ARTICLES
INFLUENCE OF TRANSPORTATION AND SLAUGHTER METHOD ON STRESS REACTION OF COMMON CARP (*CYPRINUS CARPIO* L.) FOR HUMAN CONSUMPTION
Daskalova Alexandra, Pavlov Alexander..... 204

TYPIFIED THE NERVOUS SYSTEM OF THE DOG IN ORDER TO PROPERLYSOCIALIZATION AND MODELING OF CERTAIN BEHAVIORS
Uzunova Krasimira, Todoroska Marina, Binev Rumen, Miteva Chonka, Yuri Mitev..... 207

THE EFFECT OF FIRST FREEDOM RESTRICTION ON BROILER WELFARE
Blagoevska Katerina, Dodovski Aleksandar, Blagoevski Andreja, Radeski Miroslav, Popovska-Percinik Florina, Ilieski Vlatko, Stojkovski Velimir, Mickov Ljupco, Esmerov Igor..... 212

ASSESSING THE WELFARE OF DAIRY CATTLE USING OUTCOME BASED MEASURES AND HUMAN – ANIMAL RELATIONSHIP IN DIFFERENT HOUSING SYSTEMS
Radeski Miroslav, Ilieski Vlatko..... 216

ACTH CELLS AFTER CHRONIC EXPOSURE OF RATS TO HIGH AMBIENT TEMPERATURE: MORPHOLOGICAL STUDY
Popovska-Percinic Florina, Ajdzanovic Vladimir, Trifunovic Svetlana, Ilieski Vlatko, Pendovski Lazo, Blagoevska Katerina, Milosevic Verica..... 223

MULTI-DIMENSIONAL SCALING ANALYSIS OF GENOME-WIDE SNP DATA ON ITALIAN SHEEP BREEDS REVEALS A STRONG PHYLOGEOGRAPHIC GRADIENT
Ciani Elena, D'andrea Mariasilvia, Lasagna Emiliano, Napolitano Francesco, Carta Antonello, Matassino Donato, Crepaldi Paola, Ciampolini Roberta, Bordonaro Salvatore, Modesto Paola, Macciotta Nicolò p.p., Ajmone Marsan Paolo, Portolano Baldassarre, Kompan Drago Consorzio Biovita.....226

MICROSATELITE GENOME CHARACTERIZATION OF THE GRAY WOLF IN REPUBLIC OF MACEDONIA
Esmerov Igor, Branko Atanasov, Nikola Adamov, Katerina Blagoevska, Stojkovski Velimir.....229

POTENTIALS OF MOLECULAR GENETICS FOR IMPROVEMENT OF LIVESTOCK PRODUCTION IN REPUBLIC OF MACEDONIA
Adamov Nikola, Pendovski Lazo, Esmerov Igor, Mickov Ljupco, Adamov Mihajlo..... 234

MAGNETIC RESONANCE IMAGING APPLICATION IN RABBIT LIVER ANATOMY. CORRELATION WITH CADAVER CROSS-SECTIONAL CUTS
Stamatova-Yovcheva Kamelia, Dimitrov Rosen, Chaprazov Zvetan, Russenov Anton, Yovchev David.....239

DISTRIBUTION OF MAST CELLS IN THE PORCINE GALL BLADDER
Stefanov Ivaylo, Vodenicharov Angel.....243

RENAL VEIN BRANCH PATTERNS IN PIG KIDNEYS
Pendovski Lazo., Ilieski Vlatko., Petkov Vladimir., Popovska-Percinic Florina., Tososka Lazarova Dobrila.....247

Topic: ANIMAL REPRODUCTION

PLENARY LECTURE
CAUSES OF ABORTION IN SMALL RUMINANTS
Nektarios D. Giadinis.....255

PLENARY LECTURE
MANAGEMENT OF EQUINE REPRODUCTION DURING THE BREEDING SEASON (FRESH, COOLED AND FROZEN SEMEN: WHAT, WHEN AND HOW)
Lacalandra GM, Nicassio M,..... 258

ORIGINAL ARTICLES

ACTIVITY OF NADH-TETRAZOLIUM REDUCTASE IN BULL SEMEN AFTER CRYOPRESERVATION IN RELATION TO THEIR MOTILITY
Stefanov Rossen, Chervenov Mihail, Georgiev Boyko, Kacheva Dimitrina, Taushanova Paulina, Kistanova Elena, Sabev Milko..... 262

DISTRIBUTION OF THE SPERMATOZOA SUBPOPULATIONS IN CRYOPRESERVED BOAR SEMEN
Mickov Ljupco, Dovenski Toni, Petkov Vladimir, Atanasov Branko, Adamov Nikola, Nikolovski Martin, Blagoevska Katerina, Tomeska-Mickova Svetlana..... 265

UTEROSCOPY EXAMINATIONS OF UTERINE DISORDERS IN DAIRY COWS
Pavlović Miloš, Pavlović Vojislav, Vakanjac Slobodanka, Pavlović Ivan, Jakić-Dimić Dobrila..... 272

ULTRASOUND OBSERVATION OF OVARIAN DYNAMIC IN DAIRY COWS WITH TRUE POSTPARTUM ANOESTRUS AFTER TREATMENT WITH DIFFERENT DOSES OF eCG
Atanasov Branko, Mickov Ljupco, Esmerov Igor, Trojacanec Plamen, Ilievska Ksenija, Adamov Nikola, Dovenski Toni..... 282

VAGINAL FOLD PROLAPSE AND TRANSMİSSİBLE VENEREAL TUMOR RELATED TO OVARIAN REMNANT SYNDROME İN A BITCH
Turna Yılmaz Özge, Uçmak Melih, Kırşan İsmail..... 285

TRANSMISSIBLE VENEREAL TUMOR IN A BITCH WITH OVARIAN REMNANT SYNDROME
Evkuran Dal Gamze , Töre Merih Duygu..... 287

OVARIAN SEX CORD TUMOR WITH ANNULAR TUBULES IN A DOG
İbrahim Firat , Hande Özyogurtcu, Sinem Özlem Apaydin, Hayri Ekici..... 290

ABSTRACTS

KINETICS OF THE RESIDUE LEVELS OF GATIFLOXACIN IN POULTRY MEAT AT STORAGE
Kyuchukova Ralica and Pavlov Aleksandar..... 293

SPECIES DISTRIBUTION OF METHICILLIN RESISTANT STAPHYLOCOCCI ISOLATED FROM ANIMALS, ENVIRONMENTAL SAMPLES AND STAFFS
Arzu Funda Bağcıgil, Serkan İkiz, Özlem Güzel, Çağla Parkan Yaramış, Atila Ilgaz..... 293

THE EVALUATION OF BLOOD ELECTROLYTES, SYSTOLIC BLOOD PRESSURE AND ELECTOCARDIOGRAPHIC FINDINGS IN CATS WITH AZOTEMIA
M.Ali Sağır, Alev Akdoğan Kaymaz, Alper Bayrakal..... 294

RABIES AND CSF CONTROL (SITUATION) IN KOSOVO
Skender Muji, Ardita Jahja, Bajram Batusha..... 294

RHEOLOGICAL PARAMETERS AND CHANGES IN THE PERIPHERAL LYMPHOCYTE MEMBRANE IN DIABETIC DOGS
Alev Akdoğan Kaymaz, Işıl Albeniz, Şule Tamer..... 295

VENTRICULAR SEPTAL DEFECT IN FIVE DOGS
Sinem Ülgen and Utku Bakırel..... 295

THE URINE BLADDER STONE IN FIVE-MONTH TABBY CAT
Alev Akdoğan Kaymaz, Taner Bahçeli, Kürşat Özer..... 296

COMPARATIVE INVESTIGATIONS ON THE DISTRIBUTION AND NUMBER OF MAST CELLS IN THE PELVIC PART OF THE MALE PIG`S URETHRA AFTER DIFFERENT STAININGS
Kostadinov G., Vodenicharov A., Kostadinova L..... 296

HOUSING FACTORS EFFECTING ON MEAT TYPE POULTRY BONE STRENGTH
Jahja Ardita, Mestani Nuridin, Alltane Kryeziu..... 297

ASSESSING THE WELFARE OF LAYING HENS IN CONVENTIONAL CAGE HOUSING SYSTEMS
Prodanov Mirko, Sekulovski Pavle, Ilieski Vlatko..... 297

EFFECTS OF DIFFERENT TRANSPORT TEMPERATURES ON IN VITRO DEVELOPMENT OF QUEEN OOCYTES
Ozen Banu Ozdaş, Alper Baran, Asiye Izem Sandal, Gul Bakirer, Cagatay Tek, Sinem Ozlem Enginler, Mehmet Can Gunduz, Guven Kasikci and Kemal Ak..... 298

UTERINE PROLAPSE IN A COCKATIEL RELATED TO CHRONIC EGG LAYING
Sinem Özlem Enginler, Gamze Evkuran, Esra Çalışkan, Hayri Ekici..... 298

STUDY OF THE CRITERIA FOR DEVELOPMANT OF STAFF IN THE FIELD OF VETERINARY SCIENCE BEFORE AND AFTER THE LAW OF DEVELOPMENT OF THE ACADEMIC STAFF IN REPUBLIC OF BULGARIA
Kostadinova Laska..... 299

INDEX OF AUTHORS..... 301

ANIMAL HEALTH

Plenary Lecture UDC: 636.2.034

EFFECT OF HEAT STRESS ON METABOLIC AND ENDOCRINE STATUS OF DAIRY COWS

Vujanac Ivan[1], Horvat Jožef[2], Šamanc Horea[1], Kirovski Danijela[3]

[1]Department of Farm Animal Disease, Faculty of Veterinary Medicine University of Belgrade, Belgrade, Serbia
[2]Veterinary Institute Subotica, Subotica, Serbia
[3]Department of Physiology and Biochemistry, Faculty of Veterinary Medicine University of Belgrade, Belgrade, Serbia

ABSTRACT
Stress is organism's response to changed environmental demands. Heat stress is extensively investigated due to world global warming. In dairy cows heat stress has negative impact on milk production, reproductive performances and health. It is of strong importance to investigate all the physiological mechanisms that are provoked by heat stress in dairy cows in order to prevent negative effect of heat stress on cow's organism. Metabolic and endocrine status of cows is dramatically changed during heat stress. Thyroid hormone concentration decreased which is considered as mechanism of adaptation to increased environmental temperature. Cortisol concentration increases while insulin concentration decreases in the condition of heat stress. Depression of insulin concentration is probably the consequence of decreased feed intake during hot environmental conditions. Glucose concentration is decreased due to its increased utilization in peripheral tissue. Simultaneously, fatty acid oxidation is decreased which is probably the main mechanism of metabolic adaptation of organism to heat stress. Namely, oxidation of glucose release less heat than oxidation of fatty acid and therefore is more desirable metabolic pathway in the condition of high environmental temperature. Urea concentration is usually increased during heat stress due to increased protein catabolism which supplies gluconeogenesis process with additional amounts of amino acids.
As a conclusion, it was widely accepted that decreased milk production during hot summer months is only a consequence of decreased feed intake at heat stressed cows. But, new research in the field proved that the decrease in milk production during heat stress is not fully in accordance with depressed feed intake but is partly a consequence of metabolic and endocrine adaptations to high environmental temperature. Understanding the borderline when those mechanisms of adaptations become risk factors for health of dairy cows will enable to define procedures that may prevent negative effect of heat stress on dairy cows health.
Key words: heat stress, dairy cows

INTRODUCTION

Effect of heat stress on production, reproduction and health of dairy cows is extensively investigated. Heat stress is a consequence of strong impact of high enviromental temperature and humidity on organism. The combination of the effects of temperature and humidity is measured by the temperature humidity index (THI) (NRC, 1971; McDowell et al., 1976). There are a number of equations that have been used to calculate THI. One equation is: THI = (Tdb + Twb) x 0.72 + 40.6 (Tdb - Dry bulb temperature; Twb – wet bulb temperature).

According to McDowell (1976), if the THI values are lower than 70 than the conditions for cow`s organism are optimal. If the values are between 72 and 76 conditions are considered to be stresogenic for cows. If the values are higher than 78 conditions are considered to be severely stresogenic for cows due to the fact that high yielding dairy cows may not maintain its body temperature.

In order to maintain homeostasis during heat stress, body initiates mecahnisms of adaptations (Collier et al., 2006). In the cases when mechanism of adaptations are disturbed, health disorders, reproductive problems and decreased production occurs (Baumgard et al., 2006).

Understanding a mechanism of metabolic and endocrine adaptations to heat stress in dairy cows open a wide range of possibilities to find a methods for preventing the stresogenic effect of heat stress on dairy cows.

Effect of heat stress on endocrine status of dairy cows

Adaptation of endocrine system to increased environmental temperatures includes changes in hormonal concentrations as well as changes in responsiveness of tissues on hormones action. It especially refers on the concentrations of thyroid hormones, prolactin, growth hormone, glucocorticoids and mineralocorticoids. For example, prolaction concentrations are changed in accordance with season. Corticotrophin realizing hormone (CRH) stimulates somatostatin secretion in hypothalamus which depresses secretion of thyroid stimulating hormone (TSH) and growth hormone from pituitary. Thus, somatostatin by the mechanism of negative feedback loop ("down regulation"), affects oxidative and termogneric processes that are under control of thyroid hormones and growth hormone. It is known that application of thyroid releasing hormone (TRH) to high yielding cows during summer lead to higher increase of T_4 concentrations than if application is done during spring or winter season. It clearly indicates that there are seasonal variations in feedback loop control mechanism. Therefore there is scientific opinion that climate conditions may have impact on mechanisms of regulation of metabolic status of dairy cows.

Heat stress and adrenal cortex function

Activation of peptic region in the condition of heat stress stimulates hypothalamus to release CRF which stimulates pituitary gland to secrete ACTH. ACTH leads

to increase synthesis of glucocorticosteroids (mainly cortisol). Increased cortisol concentration in blood is one of the main defensive mechanisms of the body against stress including heat stress. Namely, cortisol initiates mechanism of physiological adaptations in body which help organism to overcome stress provoked by high environmental temperature (Christison and Johnson, 1972). Cortisol concentration increase 20 minutes after the start of acute heat stress and reach a peak 2 hours later (Christison and Johnson, 1972). This increase in cortisol concentration leads to increased concentrations in glucose that is main energetic precursor for adequate adaptation of cows to heat stress.

In a contrast to acute stress reaction when cortisol concentration increases, cortisol concentration changes differently during chronic heat stress. Decreased cortisol concentration during heat stress was established by Christison and Johnson, 1972; Correa – Calderon et al., 2004 and Habeeb et al., 1992, while Wise et al. (1988) and Johnson et al (1991) did not established any change in cortisol concentrations during chronic heat stress. Muller at al. (1994) established that cows in the stalls with a roof in the Mediterranean climate has lower cortisol level during summer season as well as depressed body temperature and respiratory rate during warmest part of the day. They hypothesized that cows are good adaptive to heat stress if they have lower cortisol concentration but are non adaptive if their cortisol concentration remain high during chronic heat stress.

Heat stress and thyroid hormone function

Pituitary gland secretes TSH which stimulates thyroid gland to synthesize and secrete T_4 and T_3. Thyroid hormones affect many cellular functions but especially termogenic ones that produce about 50 % of body total metabolic basal energy (Habbeb et al., 1992; Johnson et al., 1988). In the condition of acute heat stress thyroid gland activity is depressed (Habbeb et al., 1992). It was established that in the heat stress condition production of thyroid hormones decreases up to 25 % (Beede and Collier, 1986). Changes in thyroid gland activity during heat stress condition are in accordance with decreased body metabolic activity, amount of feed intake, body gain and milk production (Beede and Collier, 1986).

The response of thyroid gland to heat stress is slower than response of adrenal gland. Therefore, several days are needed for the thyroid hormone concentrations to be established on lower level. For reestablishment of thyroid gland activity longer period of time is needed.

Decreased milk production during summer season is partly a consequence of decreased thyroid hormone synthesis or, more precisely, decreased conversion of T_4 to T_3, which leads to decreased metabolic energy production.

Scott et al. (1983) established negative correlation between thyroid hormone concentration and body temperature in cows. During night, environmental temperature decrease and therefore have beneficial effect on thyroid hormone concentration maintenance. It indicates that cooling of the cows that are exposed to heat stress during day may improve their metabolic status.

Decreased activity of thyroid gland indicate on response of organism on adaptation to environmental conditions, while increased cortisol concentration indicate on strong impact of heat stress meaning that cows with increased cortisolemia are under strong influence of heat stress while cows with decreased thyroid hormone concentrations in blood are under moderate influence which initiate mechanism of adaptations.

Heat stress and endocrine pancreas function

Endocrine pancreas function in the heat stress conditions were examined using loading tests. Results showed that basal insulin levels as well as levels established during arginine, butirate and glucose loading tests were higher in the cows exposed to heat stress than in the cows under normal environmental conditions (Itoh et al., 1998). Similar results were presented by Sano et al (1988) who obtained experiments on non lactating sheep. Wheelock et al. (2006 and 2010) showed that insulin response of heat stressed cows is more appropriate than in cows that were not under stress condition. On the contrary, Denbow et al (1986) established lower insulin concentration in the blood of cow during summer than during spring and winter season. Itoh et al (1998) have established lower insulin concentration in non lactating cows exposed to heat stress. Authors did not offer a answer to the question why is insulin response in non lactating cows different than in lactating cows. One of the possible answer is that the lactating cows have lower insulin secretion under optimal enviromental conditions due to homeorhetic mechanisms while in the heat stress condition dietary uptake is depresses leading to depressed milk production. Due to that, differences in insulin concentrations during loading tests obtained during summer or winter season should be considered as a cosequence of changes in secretory capacity as well as metabolic realighment.

Effect of heat stress on metabolic status of dairy cows

In the conditions of high milk production organism needs high amount of organic and inorganic component. Some of those components are components of milk and some are precursors for milk synthesis. During lactation digestive system is under greatest challenge due to the fact that high yielding cow ingests 18 to 22 kg of dry matter per day which must be completely digested and absorbed. In order to settle all those requirements, new technologies in feeding regiment are applied. Nevertheless it is very hard to achieve adequate supply of dairy cows with dry matter, especially during summer season when appetite of cow is depressed. Most sensitive period is early lactation period, when energy requirements are dramatically changed. Namely, hormone concentrations are changed in order to increase supply of mammary gland with necessary amount of organic and inorganic precursors.

New investigations showed that metabolic profile in heat stressed dairy cows is very different than metabolic profile of cows that are restrictively feed (Wheelock et al., 2006; Shwartz et al., 2009). When the cows are in the condition of physiological negative energy balance (NEB) the main characteristic of metabolic profile is increased blood NEFA concentration (Bauman and Currie, 1980). Cows that are under heat stress are in the state of NEB but NEFA concentration is not increased as in cows under optimal environmental condition (Baumgard et al., 2006; Rhoads et al., 2009). High NEFA concentration is the main metabolic pathway that

spears glucose in the conditions of decreased feed intake (Bauman and Currie, 1980). The fact that cows that are exposed to heat stress can not use this mechanism shows that heat stress directly affect their energy metabolism and indicate on their metabolic nonadaptiblity.

Baumgard and Roads (2007) postulated hypothesis that the better way of adaptation of cows to heat stress would be if they, instead beta oxidation of fatty acid use oxidation of glucose since that in this metabolic process lesser heat is produced. When deficit of energy occurs in the dairy cows due to decrease feed intake, insulin concentration decrease as well as response of peripheral tissue to insulin (Bauman and Currie, 1980). Insulinemia is low at early lactating dairy cows and maintained low until energy balance is achieved. During that period the growth hormone concentration is highest and insulin concentration is lowest allowing optimal use of metabolic energy precursors for body stores.

Wheelock et al (2006; 2010) established that insulin concentration was within physiological values in cows that were underfed. But in cows exposed to heat stress insulin concentration was significantly higher. Higher insulin concentration may be a cause of low NEFA concentration in blood of the cows exposed to heat stress. Due to strong antilypolitic action of insulin, it may be supposed that high insulin concentration during heat stress lead to increased use of glucose as energy source instead of NEFA (Wheelock et al., 2010).

In accordance to that are a results that shows lower glucose concentration in heat stress exposed cows (Itoh et al., 1998). Koubkova et al (2002) established significant increase in glucose concentrations at the bigining of heat stress (increase from 2.98 to 3.35 mmol/L), which thay explained with the fact that there is haemoconcentration at that period of heat stress. Thereafter they established significant decrease in glucose concentration to the value of 2.91 mmol/L. Decreased glucose concentration was established during cooling of the heat stressed cows (2.82 mmol/L). Others established increase glucose concentration during cooling of heat stressed cows (Aboulnaga et al., 1989).

Sesonal variation of urea concentrations is discribed by meny authors (Peterson and Waldern 1981, Rasooli et al., 2004). There is agreement among authors that urea concentration in blood is higher during summer compared to winter season probably due to pasture feeding as well as due to lost of extracellualar fluid during hot summers.

Koubkova et al (2002) established significant oncrease of urea concentration in the condition of heat stress (6.64 and 7.13 mmol/L) compared to optimal values (4.08 and 4.84 mmol/L). They also established that increase of urea concentration was combined with decrease in glucose concentration. It may be a consequence of increased protein catabolism due to increase need for gluconeogeneses precursors. Acute heat stress, which has duration of at least three days, leads to increase protein catabolism, decrease total protein blood concentration and increase non protein nitrogen and creatinin in blood serum. Ronchi et al. (1997) found decreased glucose concentration and increased urea and creeatin concentration in heat stressed cows as a consequence of increase muscle protein catabolism that provide better energy supply for organism.

CONCLUSION

As a conclusion, it was widely accepted that decreased milk production during hot summer months is only a consequence of decreased feed intake at heat stressed cows. But, new research in the field proved that the decrease in milk production during heat stress is not fully in accordance with depressed feed intake but is partly a consequence of metabolic and endocrine adaptations to high environmental temperature (Vujanac et al., 2012). Understanding the borderline when those mechanisms of adaptations become risk factors for health of dairy cows will enable to define procedures that may prevent negative effect of heat stress on dairy cows health.

ACKNOWLEDGEMENTS:

This work was supported by Ministry of Science and Technology, Republic of Serbia, Project Grant No 31003.

REFERENCES:

1. Aboulnaga AI, Kamal TH, El Masry KA, Marai IF, 1989, Short-term response of spray cooling and drinking cold water for improving milk production of heat stressed Friesian cows, In: Proceedings of the third Egyptian British Conference, Alexandria, Egypt, Vol 2, 607–612.
2. Bauman E, Currie W, 1980, Partitioning of nutrients during pregnancy and lactation. A review of mechanisms involving homeostasis, J Dairy Sci, 63, 1514.
3. Baumgard LH, and Rhoads RP, 2007, The effects of hyperthermia on nutrient partitioning, 69th Proc Cornell Nutr Conf, Cornell University, Ithaca, NY, 93–104.
4. Baumgard LH, Odens LJ, Kay JK, Rhoads RP, Van Baale MJ, and Collier RJ, 2006, Does negative energy balance (NEBAL) limit milk synthesis in early lactation?, Proc Southwest Nutr Conf, 181–187.
5. Beede DK, Collier RJ, 1986, Potential nutritional strategies for intensively managed cattle during heat stress, J Anim Sci, 62, 543–550.
6. Christison GI, Johnson HD, 1972, Cortisol turnover in heat-stressed cows, J Anim Sci, 53, 1005–1010.
7. Collier RJ, Dah GE l, VanBaale MJ, 2006, Major advances associated with environmental effects on dairy cattle. J Dairy Sci 89, 1244-1253.
8. Correa-Calderon, A, D Armstrong, D Ray, S DeNise, M Enns, and C Howison, 2004, Thermoregulatory responses of Holstein and Brown Swiss heat-stressed dairy cows to two different cooling systems, Int, J, Biometeorol, 48, 142–148.
9. Denbow CJ, Perera KS, Gwazdauskas FC, Akers RM, Pearson RE, and McGilliard ML, 1986, Effect of season and stage of lactation on plasma insulin and glucose following glucose injection in Holstein cattle, J Dairy Sci, 69, 211–216.
10. Habeeb AAM, Marai IFM, Kamal TH, 1992, Heat stress, Phillips C, Pigginns D, (Eds.), Farm Animals and the Environment, CAB International, Wallingford, UK, 27–47.
11. Itoh F, Obara Y, Rose MT, Fuse H, and Hashimoto H, 1998, Insulin and glucagon secretion in lactating cows during heat exposure, J Anim Sci, 76, 2182–2189.
12. Johnson H D, R Li, W Manalu, K J Spencer-Johnson, B A Becker, R J Collier and C A Baile, 1991, Effects of somatotropin on milk yield and physiological responses during summer farm and hot laboratory conditions, J Dairy Sci, 74, 1250–1262.

13. Koubkova M, Knížková I, Kunc P, Härtlová1 H, Flusser J, doležal O, 2002, Influence of high environmental temperatures and evaporative cooling on some physiological, hematological and biochemical parameters in high-yielding dairy cows, Czech J. Anim. Sci., 47, (8): 309–318.
14. Mc Dowell RE, Hooven NW, Camoens JK, 1976, Effects of climate on performance of Holsteins in first lactation, J Dairy Sci, 59, 965–973.
15. Muller CJC, Botha JA, Coetzer WA, Smith WW, 1994, Effect of shade on various parameters of Friesian cows in a Mediterranean climate in South Africa, 2. Physiological responses, South Afric, J Anim Sci, 24, 56–60.
16. National Research Council, 1971, A guide to environmental research on animals, Natl. Acad. Sci., Washington, DC.
17. Peterson RG, DE Waldern, 1981, Repeatabilites of serum Contituens in Holstein-Frisians Affected by Feeding, Age, Lactation, and Pregnancy, J dairy Sci, 64, 5, 822-831
18. Rasooli A, Nouri M, Khadjeh G H and Rasekh A, 2004, The influences of seasonal variations on thyroid activity and some biochemical parameters of cattle, Iranian Journal of Veterinary Research, University of Shiraz, Vol. 5, No. 2, Ser. No. 1Q 1383
19. Rhoads ML, Rhoads RP, VanBaale MJ, Collier RJ, Sanders SR, Weber WJ, Crooker BA, and Baumgard LH, 2009, Effects of heat stress and plane of nutrition on lactating Holstein cows: I, Production, metabolism, and aspects of circulating somatotropin, J Dairy Sci, 92, 1986–1997.
20. Ronchi B, Bernabucci U, Lacetera NG, Nardone A, 1997, Effects of heat stress on metabolic-nutritional status of Holstein cows, Zoot Nutr Anim, 23, 3–15.
21. Sano H, Lee SR, Yamazaki F, Orlandi M, Sasaki Y, and Tsuda T, 1988, Effects of dietary propionate and heat exposure on glucose disappearance rate and insulin secretion in sheep, Jpn J Zootech Sci, 59,1019–1026.
22. Scott IM, Johnson HD, Hahn GL, 1983, Effect of programmed diurnal temperature cycles on plasma thyroxine level body temperature, and feed intake of Holstein dairy cows, Int J Biometeorol, 27, 47–62.
23. Shwartz G, Rhoads ML, VanBaale MJ, Rhoads RP, and Baumgard LH, 2009, Effects of a supplemental yeast culture on heatstressed lactating Holstein cows, J Dairy Sci, 92, 935–942.
24. Vujanac I, Kirovski D, Šamanc H, Prodanović R, Lakić N, Adamović M, Valčić O, 2011, Milk production in high yielding dairy cows under different anviromental temperatures, Large Animal Review, 18, 1, 31-36.
25. Wheelock JB, Rhoads RP, VanBaale MJ, Sanders SR, Baumgard LH, 2010, Effects of heat stress on energetic metabolism in lactating Holstein cows, J Dairy Sci, 93, 644–655.
26. Wheelock JB, Sanders SR, Shwartz G, Hernandez LL, Baker SH, McFadden JW, Odens LJ, Burgos R, Hartman SR, Johnson RM, Jones BE, Collier RJ, Rhoads RP, VanBaale MJ, and Baumgard LH, 2006, Effects of heat stress and rbST on production parameters and glucose homeostasis, J Dairy Sci, 89, (Suppl.1), 290–291, Abstract.
27. Wise M E, R E Rodriguez, D V Armstrong, J T Huber, F Wiersma, and R Hunter, 1988, Fertility and hormonal responses to temporary relief of heat stress in lactating dairy cows, Theriogenology, 29, 1027–1035.

ЕФЕКТ НА ТОПЛИНСКИОТ СТРЕС ВРЗ МЕТАБОЛИЧКИОТ И ЕНДОКРИНИОТ СТАТУС НА МЛЕЧНИ КРАВИ

Вујанац Иван[1], Хорват Јожеф[2], Шаманц Хореа[1], Кировски Данијела[3]

[1]*Катедра за внатрешни болести кај фармски животни, Факултет за ветеринарна медицина, Универзитет во Белград, Белград, Србија*
[2]*Ветеринарен Институт, Суботица, Суботица, Србија*
[3]*Катедра за физиологија и биохемија, Факултет за ветеринарна медицина, Универзитет во Белград, Белград, Србија*

АПСТРАКТ
Стресот е одговор на организот на промената на околината. Топлинскиот стрес е доста проучуван како резултат на климатските промени поради глобалното затоплување. Кај млечните крави стресот има негативно влијание врз производството на млеко, репродукцијата и здравјето. Тоа е од голема важност за физиолошките механизми кои настанале како резултат на стресот кај млечните крави и на тој начин се амортизира ударот од негативното влијание на топлинскиот стрес врз организмот на кравите. Метаболичкиот и ендоктиниот статус на кравите претрпува драстични промени во текот на топлински стрес. Концентацијата на тироидните хормони опаѓа, што е во согласност со механизмот на адаптација на зголемената амбиентална температура. Концентрацијата на кортизолот се зголемува, додека концентрацијата на инсулинот се намалува во случај на топлински стрес. Намалувањето на концентрацијата на инсулинот најверојатно настанува како резултат на намалено внесување на храна, во услови кога е зголемена амбиенталната температура. Концентрацијата на гликозата е намалена поради нејзино зголемено искористување во периферните ткива. Истовремено, намалена е оксидацијата на масните киселини, која веројатно е главен механизам на метаболичката адаптација на организмот на топлинскиот стрес. Имено, оксидацијата на гликозата ослободува помалку енергија, одколку оксидацијата на масните кислеини и затоа е повеќе фаворизиран метаболички пат во случај на зголемена амбиентална температура. Концентрацијата на уреата е зголемена при топлинскиот стес поради зголемен катаболизам на протеините кој процесот на гликонеогенеза го обезбедува со дополнително количество на аминокиселини.
Како заклучок, широко прифатена е тезата дека намалената продукција на млеко во текот на летните месеци е само последица на намалено внесување на храна во состојба на топлински стрес. Но, новите истражувања во оваа област, докажуваат дека намалувањето на млекопроизводството во состојба на топлински стрес не е во целост согласно со намаленото внесување на храна, затоа што е последица на метаболичка и ендокрина адптација на високата амбиентална температура. Знаејќи ги границите на овие механизми на адаптација, ризик факторите за здравјето на млечните крави дозволува да се дефинираат начините на кои може да се превенира негативното влијание на топлинскиот стрес на здравјето на млечните крави.
Клучни зборови: топлински стес, млечни крави

Plenary Lecture

UDC: 599.74-123:616-072.1

APPLICATION OF FLEXIBLE ENDOSCOPY IN CARNIVORES

Krstić Vanja

Small animal clinic, Faculty of Veterinary medicine, Belgrade, R. Serbia

ABSTRACT

Endoscopy is a diagnostic and therapeutic method which allows us to obtain important and realistic information about the organs being examined. Currently, two types of endoscopes are in use: the rigid and the flexible one.

Endoscope set contains flexible endoscope of various length and diameter, a camera, a light source, a video monitor and a suction and insufflation device.

Gastrointestinal endoscopy is a hugely significant method for making the correct diagnosis in gastrointestinal disorders. However, it cannot be considered as the sole or the basic method, but should be applied in conjunction with a detailed history of the disease, clinical examination, laboratory tests and X-rays.

Key words: endoscopy, small animals, gastrointestinal disorders

INTRODUCTION

Endoscopy is a diagnostic and therapeutic method which allows us to obtain very important and realistic information about the organs being examined. Currently, two types of endoscopes are in use: the rigid and the flexible one.

In its Small Animals Clinic, the Veterinary Medical Faculty of Belgrade uses ”STORZ” video endoscopes which contain a 140cm x 0.9cm flexible endoscope, a camera, a light source, a 14” monitor and a suction and insufflation device, while also in use is a human patient 50cm x 0.5cm bronchoscope with a working canal.

ENDOSCOPY EXAMINATION OF THE DIGESTIVE SYSTEM

Gastrointestinal endoscopy is a hugely significant method for making the correct diagnosis in gastrointestinal disorders. However, it cannot be considered as the sole or the basic method, but should be applied in conjunction with a detailed history of the disease, clinical examination, laboratory tests and X-rays.

● Esophagoscopy

Esophagoscopy is applied in animals with signs of disorders such as regurgitation, dysphagia and increased salivation and with suspected: esophagus inflammation, foreign object within the esophagus, narrowing of the esophagus, tumors within the esophagus and a persistent aorta arch.

● Gastroscopy

Gastroscopy reveals pathology of the gastric mucous, and also shows pathological disorders of the stomach caused by tumors or enlarged neighboring organs. An endoscopic biopsy enables a fast and reliable evaluation of numerous malformations. This diagnostic method is used in cases of suspected: acute or chronic gastric inflammation, gastric ulcer, gastric tumors, foreign objects within the stomach, its deformations, pylorus spasm.

● Enteroscopy

Endoscopy of the small intestine represents an important and the least invasive method for the examination of the small intestine and for taking biopsy specimens. Following the introduction of air via the endoscope, usually it is possible to see the lumen of the small intestine very clearly. The descending part of the duodenum can be examined in all animals in which the endoscope was passed through the pylorus and often, in cats and dogs, it is possible to reach the jejunum. In dogs over 5kg, it is possible to perform a retrograde ileoscopy following a full colonoscopy, as this provides a view of the upper and lower parts of the small intestine.

Enteroscopy is required when clinical signs show disorders in the morphology and functions of the small intestine, such as vomiting, diarrhea, hematemesis, melena, when palpation of the abdomen reveals thickening of the small intestine walls, changes in the appetite and weight loss. This method is applied in situations of suspected acute or chronic inflammation of the small intestine, foreign objects within the small intestine, duodenum ulcers or small intestine tumors.

● Colonoscopy

This special diagnostic procedure is carried out on animals with frequent bloody diarrhea, with a lot of mucous and tenesmus, chronic vomiting (especially in cats) and constipation. Endoscopy of the rectum and the colon is required in suspected: chronic inflammation of the colon, presence of parasites or foreign objects within the colon, tumors of the colon, narrowing of the large intestine or cecum inversion.

● Endoscopic Biopsy

The aim of endoscopic biopsy is to confirm the suspected nature of the lesions and to discount other diseases that present a similar endoscopic picture. Endoscopic biopsy samples can be taken from the mucous, solid organs, certain masses or intra articularly. The techniques for sample taking vary depending on the type of the sample. In veterinary practice the most frequent samples are taken from the mucous of the respiratory, gastrointestinal or urogential systems. The best way to obtain a good biopsy sample of the gastric mucous is to use forceps with

serrated edges or an instrument shaped like a „bayonet“. The optimum area to take biopsy samples is from the mucous of the gastric folds, with the required number of samples taken (4 – 8) in order to deterine whether the stomach is functioning normally. Compared to the gastric mucous, the small intestine mucous is not compact, therefore samples should be taken with a forceps with cups at the tips. In principle, two biopsy techniques are applied. One is to place the forceps on the visible areas of the small intestine, while the other is the blind biopsy technique with the sample taken from the furthest parts of the small intestine. Between 10 – 16 samples are required. It is recomanded that the biopsy of the mucous and submucous of the large inestine is carried out with a rigid biopsy instrument whose tip is at an angle between 30 – 40 degrees and that, if possible, two samples are taken.

REFERENCES

1. Brearley, M.J., Cooper., 1991, Color Atlas of Small Animal Endoscopy, Mosby, Year Book, Inc.
2. Krstić V., 2004, Endoskopija: tehničke karakteristike i dijagnostičke mogućnosti fiberoptičkih endoskopa, Zbornik radova šestog međunarodnog savetovanja iz kliničke patologije i terapije životinja, Budva, str.272.
3. Krstić N., Krstić V., 2007, Rendgenološka i endoskopska dijagnostika oboljenja digestivnog i respiratornog sistema pasa i mačaka, Beograd
4. Timothy C.M., 2005, Veterinary Endoscopy for the small animal practitioner, Elsevier Saunders.

ПРИМЕНА НА ФЛЕКСИБИЛНА ЕНДОСКОПИЈА КАЈ МЕСОЈАДИ

Крстиќ Вања

Клиника за миленици, Факултет за ветеринарна медицина, Белград, Р. Србија

АПСТРАКТ

Ендоскопија е дијагностичка и терапевтска метода која што ни овозможува да се добијат важни и реални информации за органите што се испитуваат. Во моментов, во употреба се два вида ендоскопи: крути и флексибилни.

Комплет (сет) на ендоскоп содржи флексибилен ендоскоп за различни должини и дијаметри, камера, извор на светлина, видео монитор и уред за вшмукување и вдувување.

Гастроинтестиналната ендоскопија е мошне значаен метод за изведување на правилна дијагноза при гастроинтестинални нарушувања. Сепак, таа не може да се смета како единствен или основен метод, но треба да се применува во комбинација со детална историја на болеста, клинички преглед, лабораториски тестови и употреба на рентгенски зраци.

Клучни зборови: ендоскопија, миленици, гастроинтестинални нарушувања.

UDC: 636.5.09:616.76-002-022.7(497.11)

INFECTIOUS BURSAL DISEASE VIRUS: THE CURRENT SITUATION IN SERBIA, CONTROL AND PREVENTION

Velhner Maja[1], Miljković Biljana[2], Dobrosavljević Ivan[3]

[1]*Scientific Veterinary Institute "Novi Sad", 21000 Novi Sad, Republic of Serbia,*
[2] *Veterinary Research Institute of Serbia, Belgrade, Republic of Serbia,*
[3]*Veterinary Institute Požarevac, Požarevac, Republic of Serbia*

ABSTRACT

Infectious bursal disease virus has not been eliminated from poultry in Serbia. The contagious nature of the virus contributes to the viral persistence. To cope and eliminate the disease from the poultry flocks, chickens must be vaccinated with attenuated vaccines at the proper time point. Maternal antibodies protect very young chickens but also interfere with the vaccine virus and restrict or post pone active immune response. We present results from the challenge experiment in broiler chickens and broiler breeders that were infected with the very virulent Infectious bursal disease virus at 10 and 14 days of age respectively. We conclude that the protection in chickens provided by maternal antibodies was not good and that 7 days post infection even in chickens with high antibody level, lesions in the bursa were severe. Because it is difficult to estimate the proper time for the vaccination, many farmers in Serbia have introduced intermediate plus vaccines in the poultry flocks. In attempt to maintain the good health, management on farms is slowly improving. Poultry producers are advised to control hygiene on farms, to perform disinfection of vehicles and pursue regular rodent control. The HACCP concept has been introduced in some farms and in coming years, at least some producers will possibly gains the benefit of the good management practice.
Key words: infectious bursal disease virus, chickens, vaccine, maternal antibodies

INTRODUCTION

The Infectious bursal disease virus (IBDV) is double stranded bisegmented none-enveloped RNA virus. The larger segment A encodes the polyprotein that is autocatalytically cleaved to obtain virus proteins VP2, VP3 and VP4. A small overlapping fragment encodes also the VP5 protein. Segment B encodes the viral polymerase and the protein is termed VP1 (Hudson et al., 1986, Mundt et al., 1995). The major structural protein is VP2. Between amino acids 206 to 350 there are neutralizing epitopes for antibodies recognition. Subsequently this hypervariable region is subjected to mutations (Azad et al., 1987). The sequences data from the variable part of a genome is necessary to determine the virulent potential of the virus. Serologically the virus is divided to serotype 1 and serotype 2. Pathogenic viruses belong to the serotype 1, while in serotype 2 group there are viruses found in turkeys and they appear to be none pathogenic for the chickens (rev by Lasher and Shane, 1994). Classical strains are found all around the globe, while variants are mostly detected in North America. Very virulent IBDV was found in America just recently (Stoute et al., 2009), but in other parts of the world it is quite common (Lasher and Shane 1994). IBDV is very contagious virus and it destroys an immunologically important organ in young chickens, the bursa of Fabricius. The destruction is due to the necrosis of B lymphocytes but also to apoptotic cell death. Apoptosis has been found in antigen positive cells but also in antigen negative cells. The mechanism of apoptosis in IBDV infection is still a subject of intensive research. It is evident that at one point it helps the virus to release from the infect cells, while on other hand it restrict virus spread by removing susceptible cells from the infected tissue (Jungmann et al., 2001). Because of several harmful effects of the virus, young chickens suffer from immunosuppression and sometimes are affected with other microorganism (Lukert and Saif, 1997).

The IBD is still causing problems in poultry industry in Serbia. One of the most important reasons is substantial amount of backyard chickens that are not always vaccinated and present the "reservoir" of the virus. The farmers that have just started to raise broilers are not knowledgeable enough in terms of vaccinations. Field veterinarians have more and more duties in solving health problems in poultry industry, but they do not understand always the concepts in protecting chickens against viral diseases. In such circumstances the best practice is to vaccinate chickens exposed to the risk of IBDV infection with intermediate plus vaccines. We describe here an experiment that was undertaken to determine the protective effect of the maternal antibodies in boiler breeders and broilers when the chickens are exposed to a very virulent virus.

MATERIAL AND METHODS

Fourteen day old broiler breeder chickens and broilers were purchased from the local hatchery and held in the experimental units of the Institute. Water and feed were provided *ad libitum*. The challenge virus CH/99 was isolated from the outbreak in a flock of pullets. During the outbreak this virus caused 20% of mortality in pullets previously vaccinated at three intervals, with the vaccine of intermediate virulence. The bursa homogenate was produced in chickens that were held in the experimental room of the Institute until 6 weeks of age and subsequently did not have antibodies to IBDV. The chickens were infected with the CH/99 virus and 3 days post infection (dpi) the bursal homogenate was prepared in physiological saline (PBS) pH 7.2-7.4 at the ration of 1:10 and used as inoculums in the experiment.

Two experiments were conducted. Fourteen broiler breeder chickens were infected at 14 days of age by oral inoculation of the CH/99 diluted tenfold in PBS. In second experiment fourteen broiler chickens were infected at 10 days of age in the same way. After challenge the chickens were sacrificed at 7 dpi, blood samples were taken and bursa was collected for pathohistological analysis. Control chickens were sacrificed at the end of experiment (7 dpi) and bursae were processed for pathohistology. Pathological lesions were determined according to the following formula: 20% follicular depletion was scored as 1, 20 to 50% depletion was scored 2, 50 to 70% depletion was scored 3 and 70 to 100% depletion was scored 4.

RESULTS

Maternally derived antibodies protected chickens against clinical IBDV during the experiment. Only in the group of broiler breeders, one chicken died. The bleedings on legs and pectoral muscle were evident on necropsy. In broiler breeders that have lower antibody titer, a sharp increase occurs 7 dpi. In one chicken with high antibody titer (2048) at the time of challenge, the titer increased to > 4096, (Table 1). The chicken had a severe depletion of lymphocytes in the bursa (bursal lesion score 4), (Fig 2).

Table 1. *Antibody titer for IBDV in broiler breeder chickens and bursa scores seven dpi*

Chick No	Ab titer at the time of infection	Bursa score	Antibody titer 7 dpi	Chick No	Ab titer at the time of infection	Bursa score	Antibody titer 7 dpi
1	128	4	4096	8	1024	4	1024
2	128	4	512	9	1024	4	4096
3	512	4	4096	10	2048	2	1024
4	512	died		11	2048	4	>4096
5	512	4	4096	12	4096	2	256
6	512	4	4096	13	4096	4	2048
7	1024	4	1024	14	4096	1	512

Table 2. *Antibody titer for IBDV in broiler chickens and bursa scores seven dpi*

Chick No	Ab titer at the time of infection	Bursa score	Antibody titer 7 dpi	Chick No	Ab titer at the time of infection	Bursa score	Antibody titer 7 dpi
1	128	4	4096	8	512	3	256
2	128	4	4096	9	512	4	1024
3	128	3	4096	10	1024	4	1024
4	128	4	1024	11	1024	4	128
5	128	4	512	12	2048	4	128
6	128	4	256	13	4096	4	1024
7	128	4	8192	14	8192	2	4096

In other chickens (breeders and broilers) the titer decreased at 7 dpi, in spite of the challenge. In most of the chickens the bursa lesion score was 4. In five chickens (broilers and breeders) the bursal score was 1 to 3 (Tables 1 and 2). This means that bursa of maternally immune chickens was protect to some level from damage caused by the virus. Significant depletion of lymphocytes was found and proliferation of connective tissue around the follicles. In medulla the necrosis was evident. In most of the samples in this experiment the complete destruction of bursal follicles was seen. The maternal antibodies were protecting the chickens against the lethal outcome of the disease but prevented quick antibody response during first 7 dpi. By pathohistology examination it was evident that the control bursas were intact (Fig 1).

CONCLUSION

Before the very virulent viruses have been found in poultry, it was believed that the maternally derived antibodies are one of the strongest weapons against the virus. In developed countries where very virulent viruses are not present any longer, this still holds the truth for broilers. The concept of "building up" the immunity against IBDV in breeders is therefore important. In this experiment the pathogenic virus destroyed the bursa of maternally immune chickens 7 dpi. The bursa was depleted from lymphocyte and the pathological changes were significant even in breeder chickens. In unfavorable situation in the filed the vaccine virus selected must be strong enough to overcome maternal antibodies and to provide immune response, before the field infection occurs. In Serbia the concept of multiple vaccinations with intermediate vaccines has been practiced for a long time. Several years back, poultry producers prefer to vaccinate with intermediate plus vaccines and the results are satisfactory. However, some small holdings that keep chickens for their own need do not vaccinate at all and this present a substation risk for the poultry industry.

The new concept of vaccination has been developed recently. Immune-complex vaccines are coated with antibodies and it has been thought that such antigen-antibody complex could provide immunity in young maternally immune chickens. However, in experimental conditions it was shown that the vaccine virus from immunocomplex formula replicates with a delay, thus providing an "empty window" as much as the field vaccination with classical attenuated vaccines (Iván et al., 2005).

The pathogenesis of IBDV has been studied extensively for many years back. The research relies on histology examination of target organs: the bursa, spleen, thymus and the bone marrow. It has been concluded that there are no significant differences in terms of amount of the virus or lesions in the bursa between one day old and 3 weeks old chickens if infected with classical strains. The influx of T lymphocytes also does not differ. The bursa is similarly destroyed after classical and very virulent virus infection, although there is evidence that the lesions in immunological organs appear faster and are more severe after very virulent virus attack. The important difference between these two viruses is that vvIBDV replicates to a higher extent in spleen and bone marrow comparing to classical types. Variant viruses do not cause inflammatory reaction in the bursa (rev by Velhner et al., 2008). In this experiment an interesting observation was made. In 3 out of 5 chickens with antibody titer of 2048 and 4096 (chickens number 13, Table 1 and chickens number 12 and 13, Table 2) the bursa was totally destroyed, but the antibody titer was not increased. This means that active immune response in this experiment was correlated with the level of maternally derived antibodies rather than with the bursa lesions, since all the chickens with antibody titer below 1024 responded serologically to the infection within 7 days. Indeed, it was noted that if vaccination occurs in maternally immune birds there could be a delay in an onset of antibody response. This is why the vaccination is complicated, especially in unfavorable circumstances in the field (Zorman Rojs et al., 2011).

Management on farms is as important as vaccination. In Serbia some improvements in management are obvious but presently they are still not at the satisfactory level. IBDV is more present in broiler flocks than layers although the latter are more susceptible. On multiple age broiler farms the virus may persist longer. The efficiency of a vaccination with intermediate vaccines is influenced with proper timing. This is probably why farmers readily vaccinate chickens against IBDV with the intermediate plus vaccines. The results are good, but this practice is not planned to last for substantial period of time.

Now we have a problem how to withdraw the vaccination with intermediate plus vaccines. Farmers who had problems in the past prefer to stick to such vaccination strategy rather than to have "an own experiment" in the field, since they are afraid of losses. Besides a possibility to place sentinel birds in vaccinated flocks, that could "pick" the virulent virus, it is not easy to monitor its presence. There is a possibility to detect the virus from a number of bursal homogenate by PCR and confirm its nature by sequencing. This would require large national project, sufficient funds and participation of many field veterinarians. To this end such possibility is not considered in Serbia. We keep in mind that vaccination of back yard chickens must be done and that it is an illusion to expect that chickens on farms will stay safe without safe surroundings.

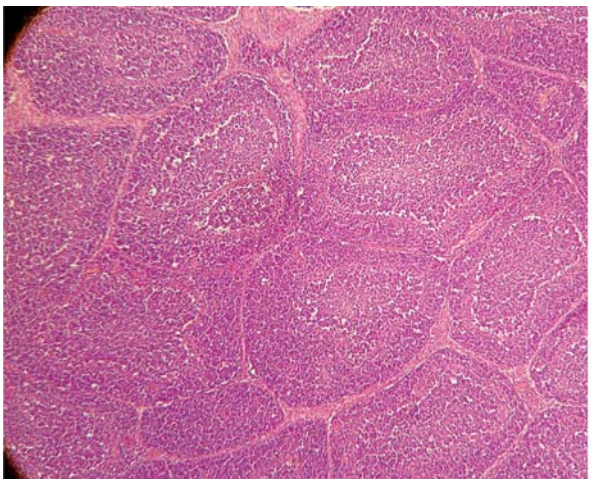


Figure 1 . Control bursa

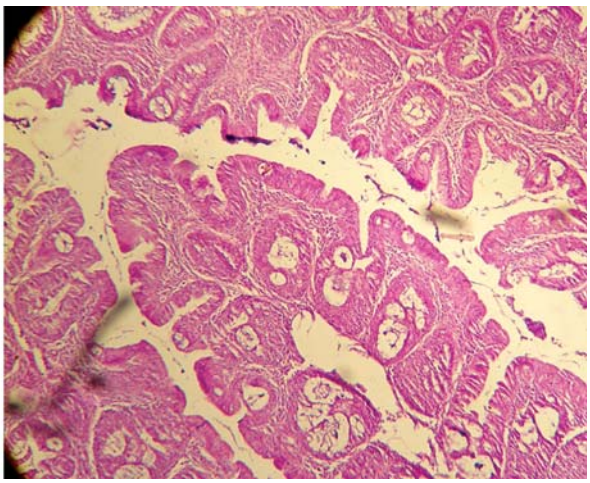


Figure 2. *Bursa from broiler chicken infected with very virulent IBDV at 10 day of age*

Vaccination of farm chickens even with intermediate plus vaccines will not solve the field problems related to IBDV. National or regional project is needed to perform monitoring to IBDV and to find out how safe chickens on farms are from the IBDV infection. Vaccination of backyard chicken is necessary not as a random event, but as a comprehensive and mass vaccination of all chickens raised for human consumption or for the chickens held as pets.

Acknowledgment: This work is supported by a grant from the Ministry of Education and Science, Republic of Serbia, Project Number TR 31071.

REFERENCES:

1. Hudson, P. J., McKern, N. M., Power, B. E. and Azad, A. A: Genomic structure of the large RNA segment of infectious bursal disease virus. Nucleic Acid Research, 14, 5001-5012, 1986.
2. Mundt E, Beyer J, Müller H: Identification of a novel viral protein in infectious bursal disease virus-infected cells. Journal of General Virology,76, 437-443, 1995
3. Azad AA, Jagadish MN, Brown MA, Hudson PJ: Deletion mapping and expression in *Escherichia coli* of the large genomic segment of birnavirus. Virology, 161, 145-152, 1987.
4. Lasher HN and Shane SM: Infectious bursal disease, Worlds Poultry Science Journal, 50, 133-166, 1994.
5. Stoute ST, Jackwood DJ, Somer-Wagner SE, Cooper GL, Anderson ML, Woolcock PR, Bickfor AA, Senties-Cué CG and Charlton BR: The diagnosis of very virulent infectious bursal disease in California pullets. Avian Diseases, 53. 321-326, 2009.
6. Jungmann, A., Nieper, H. and Müller H. 200. Apoptosis is induced by infectious bursal disease virus replcation in productively infected cells as well as in antigen-negative cells in their vicinity. Journal of General Virology, 82, 1107-1115.
7. Lukert PD and Saif YM: Infectious bursal disease in Diseases of poultry, Edited by Calnek BW, 10th edition Iowa State University Press, Ames, Iowa, p 721-738, 1997.
8. Iván J, Velhner M, Ursu K, Germán P, Mató T, Drén CN, Mészaros J: Delayed vaccine virus replication in chickens vaccinated subcutaneously with immune complex infectious bursal disease vaccine: quatification of vaccine virus by real-time polymerase chain reaction. Canadian Journal of Veterinary Research, 69, 135-142, 2005.
9. Velhner Maja: Infektivni burzitis pilića. Arhiv Vtereinraske Medicine, 1, 3-15, 2008.
10. Zorman-Rojs O, Krapež U, Slavec B, Juršić-Cizerl R, Poljanec T: Filed efficacy of different vaccines against infectious bursal disease in broiler flocks. Acta Veterinaria Hungarica, 59, 385-398, 2011.

ИНФЕКТИВНА БОЛЕСТ НА БУРЗАТА: СЕГАШНА СИТУАЦИЈА ВО СРБИЈА

Велхнер Маја[1], Миљковиќ Биљана[2], Добросављевиќ Иван[3]

[1]Научен ветеринарен институт "Нови Сад", Нови Сад, Србија,
[2]Научен ветеринарен институт на Србија, Белград, Република Србија,
[34] Ветеринарен институт Пожаревац, Србија

АПСТРАКТ

Вирусот на инфективната болест на бурзата не е елиминиран од популацијата на живина во Србија. Инфективната природа на вирусот придонесува за неговата перзистенција. За справување и елиминација на вирусот од живинските јата, пилињата мора да се вакцинирани со атенуирани во адекватно време. Матерналните антитела ги заштитуваат многу младите пилиња, но исто така интерферираат со вакциналниот вирус и го ограничуваат или одложуваат активниот имун одговор. Ги презентираме резултатите од експериментот со инокулација на силно вирулентен вирус на инфективната болест на бурзата кај бројлери и бројлески родители на старост од 10 и 14 дена, соодветно. Заклучуваме дека заштитата на пилињата од страна на матерналните антитела не е добра и 7 дена по инфекцијата дури и кај пилињата со високо ниво на антитела 7 дена по инфекцијата присутни се силни лезии на бурзата. Бидејќи е тешко да се пресмета превилното време на вакцинација, многу фармери во Србија вклучија инермедијарни плус вакцини во живинарските јата. Во обид за одржување на доброто здравје, менаџментот на фармите полека се подобрува. Одгледувачите на живина се советуваат да ја контролираат хигиената на фармата, да вршат дезинфекција на возилата и контрола на штетници. На некои фарми применет е HACCP концептот и во годините што доаѓаат некои произведувачи веројатно ќе добијат придобивки од добрите практитки на менаџмент.

Клучни зборови: вирус на инфективната болест на бурзата, пилиња, вакцина, матерални антитела

UDC: 637.4.065:579.842.14(497.11)

SERA AND YOLK HAETHCING EGGS` EXAMINATION ON SALMONELLA ENTERITIDIS ANTIBODIES

Miljković Biljana[1], Radojčić Marina[2], Velhner Maja[3], Ilic Živka[1], Dobrosavljević Ivan[4]

[1]*Scientific Veterinary Institute of Serbia, 11000 Belgrade, Republic of Serbia,*
[2] *Faculty of Veterinary Medicine, University of Belgrade, 11000 Belgrade*
[3] *Scientific Veterinary Institute "Novi Sad", 21000 Novi Sad,*
[4] *Veterinary Institute Požarevac, 12000, Požarevac*

ABSTRACT

This study has been carried out to determine the presence of the specific antibodies for *Salmonella Enteritidis*. A total of 1311 serum samples were analyzed. The 250 sera were collected from non-vaccinated broiler breeder chickens aged 40 weeks. 625 samples were from vaccinated (live vaccine was given on the 1st, then either on the 42-nd or the 56-th and either on the 112-th or 126-th day of life) broiler breeders aged 26-33 weeks. 228 samples were from 6 commercial non-vaccinated layer hens flocks (during the lay period) and 208 samples were taken from 30 to 40 day-old commercial chickens/broilers originating from non-vaccinated and vaccinated parents. A total number of 220 egg yolk samples from non-vaccinated broiler breeders were also examined. The ELISA *S.enteritidis g.m.* procedure was carried out according to the manufacturer's instructions for *S.enteritidis g.m.* (FlockChek Test Kit IDEXX, USA). Specific antiflagellar antibodies were found in broiler breeders that had been vaccinated previously but also in the non-vaccinated flocks. The commercial non-vaccinated layer hens and broilers tested negative. Yolks from eggs collected from non-vaccinated flocks tested positive in 66.37% of samples. It was concluded that good management practice and vaccination policy need to be implemented in poultry farms in Serbia.This shall in long term contribute to keep infections caused by microbes under control.

Key words: antibody, egg, yolk, serum, *Salmonella*, vaccination

INTRODUCTION

Monitoring and control of *Salmonella* infections is a very important part of food safety. *Salmonellae* usually do not cause disease in chickens but they are easily transmitted to humans. Infections with these microorganisms are of public health concern and much effort is needed to eliminate them from food. The sensitivity of the methods applied to monitor *Salmonella* in poultry industry is crucial. It was suggested that combining serological and bacteriological methods in determining health status of the poultry flocks is successful and has to be implemented with the top-down principle (1). In Serbia the vaccination of breeder flocks has begun and improvements of farm management are being observed. It is expected that vaccination will lower shedding but that management and high standards in biosecurity on the farms have to be implemented further. In terms of diagnostics, different methods that will provide sensitive and objective results and help to evaluate the infection/contamination levels are necessary.

Until now the methods most frequently used for *Salmonella* surveillance are bacteriology and serology. The plate agglutination test (mostly used for detection of antibodies to *S.Pullorum - Gallinarum*) is a quick and inexpensive test, important for initial screening and could be used for serological monitoring in the field and in laboratories because of its cross reactivity with *S. Enteritidis*. This test does not detect antibodies produced after vaccination with live SE oral vaccine but it detects antibodies if a field infection occurs. Thus, it could be used for serological monitoring in the field (2). Immunosorbent assay (ELISA) *S.enteritidis* test Kit (3) or *S.enteritidis g.m.* test Kit are also valuable methods for detection of *S. enteritidis* antibodies. Due to the *Salmonella* infection having rather intermittent course, sometimes it may be difficult to discover it by a bacteriological examination (4). In such circumstances a serological response of a flock could be indicative of a flock infection (6). Combining both methods is therefore beneficial and could provide the evidence about the spread of infection in the flock. In the previous work we showed that vaccinated poultry flocks responded poorly before the onset of lay and that the increase of antibody titer was seen most often during production phases (5).

The purpose of the present results is to monitor serological response in a limited number of poultry flocks in Serbia and to test the antibodies against *Salmonella* in the sera and egg yolks applying commercial ELISA. The goal is to expand and improve diagnostics of *Salmonella* infection on poultry farms in Serbia.

MATERIALS AND METHODS

The presence of *S.enteritidis g.m*. antibodies was studied in sera and egg yolk samples. The total number of 1311 serum samples was analyzed. The 250 sera were from non-vaccinated broiler breeders aged 40 weeks. 625 samples were from vaccinated broiler breeders aged 26-33 weeks. They were vaccinated with an oral live vaccine on the 1st, then either on the 42-nd or the 56-th and either on the 112-th or 126-th day of life . Also, 228 sera from 6 commercial non-vaccinated layers flocks (during the lay period) and 208 sera from 30 to 40-day-old commercial

chickens/broilers from (non-vaccinated and vaccinated parents) were analyzed. A total of 220 egg yolk samples originating from non-vaccinated broiler parents were also included in the study.

The ELISA *S.enteritidis g.m.* procedure was carried out according to the manufacturer's instructions *S. enteritidis g.m.* (FlockChek Test Kit IDEXX, USA), with Tecan Sunrise-Reader spectrophotometer (Austria). Preparation of serum samples was done in micro-dilution tubes or directly in the microtiter plate by adding an appropriate amount of serum to previously prepared sample diluents. If the field exposure is suspected the sera are to be diluted two-fold (1:2). Vaccinated flock serum samples are to be diluted 1:250; 1:500. Egg yolks are to be diluted two-fold (1:2) prior to being assayed and the samples must be mixed thoroughly before placing them into the microtiter well.

It is recommended that the absorbance should be at 650 nm. Samples with S/N ratio greater than or equal to 0.75 are considered negative within limits of the test. Samples with S/N ratio less than or equal to 0.59 are considered positive and should be confirmed by culture. If the S/N ratio is between 0.74 and 0.60 the sample should be retested (instructions from FlockChek Test Kit IDEXX, USA).

RESULTS

In the non-vaccinated parent flock of 40 weeks of age 250 sera were tested. High mean absorbance (S/N 0.56) was found and 30% of serum samples were positive to antibodies against *Salmonella*. In 5 vaccinated parent flocks 625 sera were tested when the flocks were between 26 and 33 weeks of age. *S.enteritidis g.m*. specific antibodies were found in 37.60% of serum samples and the obtained mean absorbance value was 0.58. Serum samples from commercial layers (No 228) and serum samples from commercial broilers (No 208), hatched from non- vaccinated and vaccinated parents were negative (Table 1).

At the same time we examined 220 incubated egg yolks from non-vaccinated flocks and the obtained results revealed presence of specific antibodies for *S.enteritidis* g.m. in 66.37% of samples. The mean absorbance value was 0.49.

Table 1. *S.enteritidis g.m. ELISA test mean absorbance values*

Flock type	Number of samples	Average of S/N ratio of number of samples (positive)	Average of S/N ratio of number of samples (negative)	% of positive sera	% of negative sera
1 Non-vaccinated 40 weeks broiler parent flock.	250	0.56 / 75	0.78 / 175	30.0	70.0
5 vaccinated broiler parent layer hens flocks 26-33 weeks.	625	0.58 / 235	0.76 / 390	37.60	62.40
6 commercial non-vaccinated egg laying hens.	228	-	0,76 / 228	-	100.0
Broilers from vaccinated and non-vaccinated parents.	208	-	0.77 / 208	-	100.0
Total No of sera samples	1311				
Incubated yolk eggs from non-vaccinated flock	220	0.49 / 74	0.75 / 146	66.37	33.63

CONCLUSION

A very important outcome of infections caused by *Salmonella* is the possibility of its vertical transmission. *Salmonella* found inside eggs means that such a transmission has happened. Egg shells may be contaminated on the surface and this poses a risk of further contamination. In such a case it spreads rapidly in the hatchery cabinet to other chickens and subsequently to the whole environment. Humans are most frequently infected after consuming the food prepared from undercooked table eggs, but also from the contaminated meat. Infections caused by *Salmonella* can be fatal to elderly and immunocompromised patients and also to children.

The producer of oral vaccine suggests that if low positivity occurs in 20-50% of the serum samples, about 2 weeks after the third vaccination and 8 weeks later, 10% is attributed to the vaccine response. If a sharp increase of the titer is evident, the field infection is suspected. This usually happens during production in breeders and layers as shown in this work. We could not find the antibodies to *S. Enteritidis* in broilers at the age of 30 to 40 days, and that is in correlation with the results of ELISA serology obtained by (7), who infected commercial broilers with low infectious doses, and found out that ELISA test was negative at 3-rd and 6-th week of age. Gast et al., (8) found that ELISA serology was well correlated with the fecal shedding in layer hens, infected with high doses of *S.* Enteritidis, but that the titer was not necessarily correlated with number of contaminated eggs. Combining both methods, the serology and the bacteriology is helpful in determining the *Salmonella* status in flocks. The high antibody titer and high percentage of positive sera in flocks may be

indicative for egg contamination.

The finding of *S. Enteritidis* specific antibodies in egg yolks is another reliable, fast and effective method in predicting *Salmonella* infection in poultry flocks. In this research 66.37% of egg yolk samples from flocks that had not been vaccinated were positive applying *S. Enteritidis g.m.* ELISA. In the research of (3) the number of positive yolks in eggs coming from non-vaccinated flocks was 23.07%. The differences are possibly due to the fact that in this research the flagella antigen from *S.enteritidis* was used, while in the work of (3) the antigen was LPS. Since *S.enteritidis* ELISA is a g,m flagellin-based assay, other *Salmonella* serotypes that share the epitopes of *g,m* flagella can potentially give positive results. The results of ELISA screening must always be confirmed by standard bacteriological methods.

To reduce *Salmonella* in poultry, a general recommendation is to improve biosecurity by good management practices and also to prevent environmental contamination. To obtain this goal it is important to estimate the level of infection and to start eliminating it as soon as possible and in the most acceptable way. In Serbia some improvements have been done in management and vaccination against *Salmonella* has started. In this work the serological testing showed that some flocks are still contaminated with *S. Enteritidis.* In the previous research we proved that *S. Enteriitidis* of the common genotype and phenotype could be subsequently found in food and humans (9).

ELISA test is used to determine the infectious and vaccination status of flocks, but this method is of a limited value. However, it could be used in monitoring of breeder flocks simultaneously with a bacteriological examination. ELISA focused on *S. Enteritidis* does not provide information about other serotypes that may be present in poultry flocks. Bacteriological examination of feces and environmental samples is therefore the leading strategy. Moreover, bacteriological examination gives us an opportunity to monitor resistance to antimicrobials in *Salmonella*. Surveillance of resistance to antimicrobial agents is another important issue and it has to be considered in each poultry health program. The rulebook on Animal Health Protection published in Official Gazette of RS 21/12, recommends that poultry in all housing systems must be tested for bacteria with antimicrobial resistance monitoring and serology.

To reduce the risk of infections caused by *Salmonella* is of a high priority. This includes screening of contaminated feed, bedding, water ,objects, animals, people, pests, rodents, vehicles, manure and mortalities from which *Salmonellae* can be introduced into flocks or houses. Vaccination of chickens is an additional control measure to decrease the shedding. Egg yolk antibodies were found to be more effective for predicting *S.enteritidis* in flocks than the ELISA antibodies in sera.

Acknowledgment: Part of this work was supported by the Ministry of Agriculture and Forestry and Water Management; Veterinary Directorate of the Republic of Serbia. The authors thank for the generous support by the Ministry of Education and Science, Republic of Serbia, Project number TR 31033.

REFERENCES

1. Wegener HC, Hald T, Lo Fo Wong D, Madsen M, Korsgaard H, Bager F, Gerner-Smidt P, Mølbak K 2008. Emerging Infectious Diseases, 9:774-780.
2. Miljkovic Biljana i Radojcic Marina, 2003: Imuni mehanizmi zastite kod primene oralne vaccine protiv S. Enetritidis. Zbornik referata i kratkih sadrzaja, Simpozijum V epizootiološki dani, Subotica**,** 277-279.
3. Radojčić Marina, Milic, N., Nišavic, J., Marković Maja 2011. Study of the presence of specific Salmonella enteritidis antibodies in chicken egg yolks competitive cELISA method. Acta veterinaria, 61: 205-214.
4. F**e**berwee,A., T.S.de Vries,.Elbers A.R.W and de Jong W.A. 2000. Results of *Salmonella* enteritidis vaccination field trial in broiler-breeder flocks in the Netherlands. Avian Dis. 44;249-255.
5. Potkonjak D, Velhner M, Orlić D, Kapetanov M, Živkov-Baloš M, Stojanović D 2007. Serological control on *Salmonella* in some poultry flocks in Vojvodina region. Lucrari Stintifice Medicina Veterinara, Timisoara, Vol XL, p 740-744.
6. Nicholas R A J. and Cullen GA 1991. Development and application of an ELISA for detecting antibodies to *Salmonella* enteritidis in chicken flocks. Vet Rec 128:74-76.
7. Velhner Maja, Stojanovc, I., Potkonjak Dubravka, Kapetanov M., Orlic, D., Rasic, Z. 2005. Salmonella enteritidis isolation from broiler chickens infected with low doses. Acta Veterinaria, 55:183-191.
8. Gast RK, Holt PS 2001. The relationship between the magnitude of the specific antibody response to experimental *Salmonella* enteritidis infection in laying hens and their production of contaminated eggs. Avian Diseases, 45: 425-431.
9. Kozoderović G, Velhner M, Jelesić Z, Stojaanov I, Petrović T, Stojanović D, Golić N 2011. Molecular typing and antimicrobial resistance of *Salmonella* Enteritidis isolated from poutrly, food and humans in Serbia. Folia Microbiologica 56: 66-71.

ИСПИТУВАЊЕ НА АНТИТЕЛА ПРОТИВ САЛМОНЕЛА ЕНТЕРИТИДИС ВО СЕРУМ И ЖОЛТОК НА ПРИПЛОДНИ ЈАЈЦА

Миљковиќ Биљана[1], Радојчиќ Марина[2], Велхнер Маја[3],
Илиќ Живка[1], Добросављевиќ Иван[4]

[1]Научен ветеринарен институт на Србија, Белград, Република Србија
[2]Факултет за ветеринарна медицина, Универзитет во Белград, Белград, Република Србија
[3]Научен ветеринарен институт "Нови Сад", Нови Сад,
[4] Ветеринарен институт Пожаревац

АБСТРАКТ
Оваа студија е направена за да се утврди присуството на специфични антитела за *Salmonella Enteritidis.* Анализирани се вкупно 1311 серуми. 250 серуми се земени од невакцинирани бројлерски родителски јата на возраст од 40 недели. 625 серуми се земени од вакцинирани бројлерски родителски јата (со жива вакцина 1-от ден и потоа на 42 или 56 ден и 112 или 126 ден) на возраст од 26 – 33 недели. 228 серуми се земени од 6 комерцијални јата на не вакцнинирани несилки (за време на период на несивост) и 208 примероци се земени од 30-от до 40-от ден кај комерцијални бројлери со потекло од вакцинирани и не вакцинирани родителски јата. Исто така испитани се и вкупно 220 примероци од жолтокот од не вакцинирани бројлерки родителски јата. Постапката на ЕЛИСА *S.enteritidis g.m.* е извршена согласно упатството на произведувачот (FlockChek Test Kit IDEXX, USA). Пронајдени се специфични антифлагеларни антитела кај вакцнинираните и не вакцнираните бројлерските родителски јата. Комерцијалните не вакцнирани несилки и бројелрите беа негативни. Жолтокот од јацата земени од не вакцинираните јата беше позитивен во 66,37% од случаите. Може да се заклучи дека е потребно да се имплементираат добри менаџмент практики и вакцинална политика во живинарските фарми во Србија. Ова на долг рок ќе придонесе инфекциите предизвикани од мироорганизми да бидат под контрола.
Клучни зборови: антитела, јајце, жолток, серум, *Salmonella,* вакцинација

UDC: 636.52/.58.09:616.24-002-022.7-085.371

ANTIBODY PATTERNS OF INFECTIOUS BRONCHITIS IN VACCINATED FLOCKS WITH CLINICAL SIGNS

Dodovski Aleksandar[1], Krstevski Kiril[1], Mitrov Dine[1], Naletoski Ivancho[2]

[1]Veterinary Institute, Faculty of Veterinary Medicine,
University "Ss. Cyril and Methodius" Skopje, Macedonia
[2]Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Wien, Austria

ABSTRACT
Infectious bronchitis (IB) is an acute, contagious and economically important viral disease of chickens caused by coronavirus infectious bronchitis virus (IBV). The highly transmissible nature of IB and the occurrence and emergence of multiple serotypes of the virus have complicated control by vaccination. In general, different serotypes of the virus do not cross-protect. Some strains of the virus are quite effective at inducing cross protection against other serotypes and are referred to as protectotypes. For surveillance purposes, ELISA is the method of choice, but field challenge can only be detected if flock antibody status is monitored continually. In our study 437 sera from 13 different farms were tested using indirect ELISA. Farms have undergone full vaccination schedule against IB. Here we report the antibody pattern of infectious bronchitis indicative of field infection in vaccinated flocks with clinical signs and pathomorphological lesions typical of IBV.
Key words: vaccinated flocks, infectious bronchitis, antibody pattern

INTRODUCTION

Avian (IB), an OIE-listed disease caused by coronavirus IBV, is an acute, contagious disease characterised primarily by respiratory signs in growing chickens; in broilers, production losses are due to poor weight gains, condemnation at processing and mortality, whilst in laying birds, losses are due to decreased egg production and poor egg quality. Infectious bronchitis has a significant economic impact and it is distributed globally (1). Some strains of the IBV are nephropathogenic and produce interstitial nephritis and mortality of up to 25%. Strains of the virus vary in the extent to which they cause pathology in nonrespiratory organs. Replication at enteric surfaces is considered to not normally result in clinical disease, although it does result in faecal excretion of the virus (2). Besides chickens, it was thought that pheasants are the only other species reported to be naturally affected but evidence is increasing that IBV has a wider host range – and not only in galliform birds e.g. the peafowl but also in non-galliform birds e.g. the teal (3).

There have been no reports of human infection with IBV. IBV has an enormous capacity to change both by spontaneous mutation and by genetic recombination (4). Although many countries share some common antigenic types, IBV strains within a geographic region are unique and distinct, examples are Europe, the United States of America and Australia (5). Commercially available IBV vaccines are live attenuated or inactivated. Many countries only permit live vaccines of the Massachusetts (Mass) type such as H120. Some countries may also have licensed vaccines to other live strains such as Connecticut, Arkansas, or Delaware 072 (USA) or the 4/91 (Europe). Vaccination-challenge tests have shown that the commercially available H120 vaccine did not provide sufficient protection against challenge with the QX-like isolates. The prevalence of variant Infectious Bronchitis (IB) serotypes must first be determined before contemplating the use of a vaccine containing these viruses. Local regulations should also be taken into account. Variant viruses may be present when "IB-like" problems are seen in flocks properly vaccinated with Massachusetts type vaccines (6). Studies have shown that vaccinating with two different types of IBV vaccine can provide broad protection against different IBV types (7). Serology as a diagnostic tool is complicated by continual use of live vaccines. For surveillance purposes, ELISA is the method of choice, regardless of the antigenic type of IBV involved. The assay is used to monitor the response to vaccination, but field challenge can only be detected if flock antibody status is monitored continually. The antigenic type of a challenge strain involved cannot be ascertained by ELISA (8). The basis of immunity to IBV is not well understood. Serum antibody levels do not correlate with protection, though local antibody is believed to play a role in protection of the respiratory tract (10). The profile of the serum antibody response depends on the method used to detect it. Following infection of chickens with a virulent strain of IBV, specific antibody was first detected by ELISA (plates coated with IBV), and later by VN and HI tests [28, 70–72].

The objective of the present work is to assess the antibody pattern of infectious bronchitis in vaccinated flocks with H-120 demonstrating "IB-like" problems as an indication of field infection.

MATERIALS AND METHODS

In order to determine the antibody level of infectious bronchitis in vaccinated flocks 437 sera were taken from 13 different farms consisting of layer, layer breeders and broiler breeders. All sera were taken after 1 - 2 weeks of reported onset of clinical signs and alteration of production parameters, except on index farm 10 where sera were taken twice after reported onset of clinical problems, 2 and 5 weeks respectively. All tested flocks have undergone typical vaccination protocol against IB, as follows: day 1 spray/aerosol, day 10 spray/aerosol,

day 32 – 37 in drinking water/spray, week 13 – 15 in drinking water/spray, week 16 – 17 intramuscular (killed vaccine). At the time of sampling the only used vaccine strain in all farms was H-120. All flocks at the time of sampling have undergone full vaccination schedule.

Detection of specific IBV antibodies in individual sera was performed using commercial IBV ELISA Kit. This test kit is designed in indirect ELISA format and measure IBV antibody bound to IBV antigen coated plates. Test procedure was as described in the manufacturer's protocol. Optical densities (OD) were measured on BDSL Immunoscan Plus spectrophotometer, using 405 nm filter. Positive and normal control sera were tested in duplicate in each test run and test results were considered as valid only if ODs of the controls were within the interval predetermined for quality assurance. Obtained IBV titers were representing comparison of the IBV antibody level within each tested serum and the IBV ELISA kit positive and normal control sera. The titers were demonstrated in geometric mean of titers (GMT). Descriptive statistics was performed an all obtained GMT titers on a flock basis.

RESULTS

Table 1. *Clinical signs and patomorphological lesions in observed farms*

Index farm	Respiratory signs	Decreased egg production	Nephropathogenic lesions	Ovary lesions
1	No	Yes	No	No
2	No	Yes	Yes	Yes
3	No	Yes	No	No
4	No	Yes	Yes	Yes
5	Yes	Yes	Yes	Yes
6	No	Yes	No	Yes
7	Yes	Yes	Yes	Yes
8	Yes	Yes	Yes	Yes
9	No	Yes	No	Yes
10	No	Yes	No	Yes
11	No	Yes	No	Yes
12	No	Yes	No	Yes
13	No	Yes	No	Yes

Table 2. *Antibody titers of IB in observed farms*

Index farm	Index barn (where applicable)	Number of samples	Minimum value of antibody titer	Maximum value of antibody titer	Coefficient of variation (CV%)	Geometric mean titer (GMT)
1	/	36	1544	30764	73,6	8084
2	/	37	10733	32461	26,1	23180
3	1	8	2635	23193	72,0	8756
	2	8	466	38288	148,2	3389
	3	8	7273	38529	58,0	17880
4	/	19	2927	25344	35,6	17307
5	/	20	350	30548	77,6	12194
6	1	20	11615	54992	31,2	40087
	2	20	314	55753	43,7	25817
	3	20	14916	53437	37,2	29175
	4	20	15538	58245	27,3	41047
7	/	19	296	35767	65,8	11424
8	1	16	21193	34333	15,7	28082
	2	21	3222	31215	53,0	12990
	3	22	1140	33281	48,9	13043
9	/	30	42	40911	100,1	5515
10	1	9	17675	27038	11,9	23746
	2	10	16770	26110	16,7	21104
	1	10	18484	41024	24,2	31544
	2	10	12608	27909	38,5	25776
11	/	20	2530	33764	47,1	15903
12	/	31	4727	29711	25,8	22186
13	1	11	173	14218	97,3	2800
	2	12	394	19072	97,9	3500

CONCLUSION

Despite having undergone full vaccination schedule against IB all flocks showed clinical signs and pathomorphological lesions typical of IBV. There are numerous data in the literature indicating field infections breaking through vaccine immunity (5). Variant viruses may be present when "IB-like" problems are seen in flocks properly vaccinated with Massachusetts type vaccines (6). On the other side, antibody patterns can be indicative of field infection breaking through vaccine immunity (9). From the results obtained it can be seen that antibody titers in tested flocks is highly variable and in the majority of flocks non-uniform reaching up to CV 148,2%. From the distribution plots it is evident that there are subpopulations within flocks which differ in terms of antibody level and uniformity. Antibody levels are well beyond protective levels for the IB. Vaccination alone should be improved and supervised by veterinarian. Therefore, we suggest that an IBV strain(s) other than vaccine strain is circulating in flocks in Macedonia. For full investigation of the field strain(s) molecular, phylogenetic and virus isolation techniques are necessary.

REFERENCES

1. Mardani K., Noormohammadi A.H., Ignjatovic J., Browning G.F., Typing infectious bronchitis virus strains using reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism analysis to compare the 3' 7.5 kb of their genomes, Avian Pathol. (2006) 35:63–69.
2. Dave Cavanagh, Coronavirus avian infectious bronchitis virus Vet. Res. 38 (2007) 281–297.
3. Liu S., Chen J., Kong X., Shao Y., Han Z., Feng L., Cai X., Gu S., Liu M., Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*), J. Gen. Virol. (2005) 86:719–725.
4. Cavanagh, D. & Gelb, J. (2008). Infectious bronchitis. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan & D.E. Swayne (Eds.). Diseases of Poultry 12th edn (pp. 117_135). Ames, Iowa, USA: Blackwell Publishing Professional.
5. J. J. (Sjaak) de Wit, Jane K. A. Cook and Harold M. J. F. van der Heijden, Infectious bronchitis virus variants: a review of the history, current situation and control measures Avian Pathology (Jun 2011) 40(3), 223_235
6. Worthington, K. J., Currie, R. J. W. & Jones, R.C. (2008). A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. *Avian Pathology, 37,* 274-257.
7. Cook J.K.A., Orbell S.J., Woods M. and Huggins M. (1999) Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes Avian Pathology 28, 477-485.
8. Ignjatović J, Sapats. Avian infectious bronchitis virus. S. Rev Sci Tech. 2000 Aug;19(2):493-508.
9. De Herdt P, Ducatelle AR, Uyttebroek AE, Sneep A, Torbeyns R. Infectious bronchitis serology in broilers and broiler breeders: correlations between antibody titers and performance in vaccinated flocks. Avian Dis. 2001 Jul-Sep;45(3):612-9.
10. Raggi L.G., Lee G.G., Lack of correlation between infectivity serological response and challenge results in immunization with an avian infectious bronchitis vaccine, J. Immunol. (1965) 94:538–543.

ПРИКАЗ НА АНТИТЕЛА НА ИНФЕКТИВНИОТ БРОНХИТИС КАЈ ВАКЦИНИРАНИ ЈАТА СО КЛИНИЧКИ ЗНАЦИ

Додовски Александар[1], Крстевски Кирил[1], Митров Дине[1], Налетоски Иванчо[2]

[1]Ветеринарен институт, Факултет за ветеринарна медицина Скопје,
Универзитет „Св. Кирил и Методиј" Скопје, Македонија,
[2]FAO/IAEA Оддел за нуклеарни техники во храна и земјоделие, Виена, Австрија

АПСТРАКТ

Инфектуивнио бронхитис (ИБ) е акутно, заразно и економски важно вирусно заболување на кокошките предизвикано од коронавирус на инфективниот бронхитис (ИБВ). ИБ мошне лесно се пренесува и појавата и избивањето на повеќе серотипови ја усложнуваат контролата со помош на вакцинација. Генерално, различните серотипови немаат вкрстена заштита. Некои соеви на вирусот се мошне ефикасни во предизвикување на вкрстена заштита кон други серотипови и се нарекуваат протектотипови. За мониторинг, ЕЛИСАТА е метода на избор, но теренските соеви можат да се откријат само ако статусот на антитела на јатата се следи постојано. Во нашата студија испитани се 437 серуми од 13 различни фарми користејќи индиректна ЕЛИСА. Сите фарми беа подложени на целосен вакцинален протокол за ИБ. Во овој труд известуваме за шаблонот на антитела на инфективниот бронхитис кај вакцинирани јата со манифестни клиничи знаци и патоморфолошки лезии типични за ИБВ. Истиот укажува на теренска инфекција со ИБВ.

Клучни зборови: вакцнирани јата, инфективен бронхитис, шаблон на антитела

UDC: 636.4.09:[616.98:578.833.3}:615.371(497.7)

EVALUATION OF VACCINATION COVERAGE AGAINST CLASSICAL SWINE FEVER IN FARM PIGS IN MACEDONIA AND POSSIBLE REASONS FOR THE POOR RESULTS

Djadjovski Igor[1], Krstevski Kiril[1], Mitrov Dine[1], Mrenoshki Slavcho[1], Acevski Sinisha[1], Cvetkovikj Iskra[1], Celeska Irena[1], Kirandjiski Toni[2], Naletoski Ivanco[3]

[1]Veterinary Institute, Faculty of Veterinary Medicine - Skopje, University Ss. Cyril and Methodius - Skopje, Macedonia;
[2]Food and Veterinary Agency, Skopje, Macedonia;
[3]Animal Production and Health, IAEA, Vienna, Austria.

ABSTRACT
Although eradicated from most European Union (EU) member states, Classical Swine Fever (CSF) continues to cause serious problems in different parts of the world, including South-Eastern European countries. According to the annual order, issued every year by the Food and Veterinary Agency (FVA), vaccination against CSF is mandatory on all territory of Macedonia. For monitoring of vaccination coverage, 29 serum samples per farm were calculated to be sufficient, to be able to detect 1 positive (vaccinated) animal for assumed prevalence of 10%, with 95% confidence level. From 2002 tested serum samples only 1001(50.00%), gave positive result, 67 (3.35%), were ambiguous and 934 (46.65%), were negative. Reasons for this situation can be various and all should be carefully examined and proper actions should be taken in order to correct certain gaps in the current vaccination practice.
Key words: Pigs, CSF, vaccination coverage, ELISA.

INTRODUCTION

Classical swine fever (CSF), also known as hogcholera, is a serious disease of pigs caused by the CSF virus (CSFV) of the family Flaviviridae, genus Pestivirus (Wengler et al., 1995). Although eradicated from most European Union (EU) member states, CSF continues to cause serious problems in different parts of the world, including South-Eastern European countries (Edwards et al., 2000; Vargas et al., 2004). According to the annual order, issued every year by the Food and Veterinary Agency (FVA), vaccination against CSF is mandatory on all territory of the country. Adult pigs have to be vaccinated once, and piglets two times per year, according to the instructions of the manufacturer of the vaccine. Only immune pigs can be put on market, or pigs vaccinated at least seven days prior to commerce. For evaluation of general situation with vaccination coverage, in the framework of the ongoing regional IPA project for Rabies and CSF (Capacity building of the veterinary service for implementation of EU Acquis), it was decided to carry out a monitoring for presence of neutralizing antibodies against CSFV in pigs from commercial farms, slaughtered in registered abattoirs.

MATERIALS AND METHODS

29 serum samples per farm were calculated to be sufficient, to be able to detect 1 positive (vaccinated) animal for assumed prevalence of 10%, with 95% confidence level. From September 2011 until January 2012, a total of 2002 blood samples from pigs originating from 75 pig farms were collected. All 2002 serum samples were tested using the ELISA method (CHEKIT CSF-Sero Ab, IDEXX), according to the manufacturer's protocol. Optical densities (OD) of the samples and controls were measured at 450 nm using BDSL Immunoscan spectrophotometer. Results were considered valid only if required criteria for validity based on obtained ODs for positive and negative controls were fulfilled.

RESULTS

From 2002 tested serum samples only 1001(50.00%), gave positive result, 67 (3.35%), were ambiguous and 934 (46.65%), were negative.

Table 1. *Monitoring for presence of neutralizing antibodies against CSFV in farm pigs in 2011.*

CHEKIT CSF-Sero Ab, IDEXX	**Number of samples**	**Percent of Positivity (%)**
POS	1001	50.00%
NEG	934	46.65%
AMB	67	3.35%
TOTAL	**2002**	

DISCUSSION

Taking into account that the samples originated from farm pigs, where high level of vaccination coverage was expected, the percentage of positivity was assumed to be around 80%. On the other hand, numbers in table 1reveal different situation. These results give a solid ground to believe that the situation with vaccination coverage in backyard pigs is even worse. Good vaccination coverage is essential for control of CSF. Afterwards investigation showed that on some farms vaccination was not carried out at all, whereas on some other farms despite the clear evidence of regular purchasing of an adequate number of vaccines in respect to number of pigs and proper vaccination, results were also unsatisfactory. Reasons for this situation can be various and all should be carefully examined. Probably the most important factor is the economical factor. This factor play major role in the backyard sector, but do not explain in total the situation in the commercial farm sector.

Other reason for this situation could be presence of some low virulent virus strains that might be circulating among the pig population in Macedonia, causing mild, atypical symptoms in pigs that do not arouse suspicion on CSF among veterinarians and owners. This form can be characterized with low reproductive performances, higher mortality in the litter and late onset of the disease especially in piglets 3 to 4 months of age, which ends up dead and unreported.

Quality of vaccines is also factor that should be taken in to account when discussing bad vaccination results. Vaccination in RM is performed with live attenuated vaccines. Currently four different vaccines are in use in RM, all containing attenuated China strain (Mrenoshki et al., 2011). So far no vaccine has been subjected to testing for determination of the virus titre, upon arrival in Macedonia.

Handling of vaccine (storage, maintenance of the cold chain, time from opening until spending), is the next thing that should be checked in order to resolve this situation.

Important information that is missing, when interpreting the results from the monitoring, is the age of the pigs that were sampled at the slaughterhouses, and time of their vaccination. This information is very important and because of this, slaughterhouse is may be not the best place for collecting samples.

However if we want to minimize the damage of possible CSF outbreaks that might happen in transition from vaccination to non - vaccination (stamping out), policy, some actions towards determination and corrections of the gaps in the current vaccination practice must be taken.

REFERENCES

1. Edwards, S., Fukusho, A., Lefevre, P., Lipowski, A., Pejsak, Z., Roehe, P., Westergaard, J., 2000. Classical swine fever: the global situation. Vet. Microbiol. 73, 103–119.
2. Mrenoshki, **S.,** Naletoski, I., Mitrov, D., Cvetkovik, I., Krstevski, K., Djadjovski, I., Markic, Z. Classical Swine Fever in Macedonia - Situation and Chalenges. Book of abstracts, 9th symposium - Health protection, Selection and Reproduction of Pigs. Silver Lake, Serbia, 26-28.05.2011.
3. Vargas, T.M., Calcagno, F.N., Lubroth, J., 2004. Situation of classical swine fever and the epidemiologic and ecologic aspects affecting its distribution in the American continent. Ann. NY Acad. Sci. 1026, 54–64.
4. Wengler,G., Bradley,D.W., Collett,M.S., Heinz,F.X., Schlesinger,R.W., Strauss,J.H.,1995. Flaviviridae. In: Murphy,F.A., Fauquet,C.M., Bishop,D.H.L., Ghabrial,S.A., Jarvis,A.W., Martelli,G.P., Mayo,M.A., Summers, M.D. (Eds.), Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. Springer Verlag, NewYork, pp. 415–427.

ПРОЦЕНКА НА УСПЕШНОСТА НА ВАКЦИНАЦИЈАТА ПРОТИВ КЛАСИЧНА СВИНСКА ЧУМА КАЈ ФАРМСКИ СВИЊИ ВО МАКЕДОНИЈА И МОЖНИ ПРИЧИНИ ЗА СЛАБИТЕ РЕЗУЛТАТИ

Цаџовски Игор[1], Крстевски Кирил[1], Митров Дине[1], Мреношки Славчо[1], Ацевски Синиша[1], Цветковиќ Искра[1], Целеска Ирена[1], Киранџиски Тони[2], Налетоски Иванчо[3]

[1]Ветеринарен Институт, Факултет за ветеринарна медицина - Скопје, Универзитет Св. Кирил и Методиј - Скопје, Македонија;
[2]Агенција за храна и ветеринарство, Скопје, Македонија;
[3] Катедра за Здравствена заштита и продукција кај животните, МААЕ, Виена, Австрија.

АПСТРАКТ

Иако искоренета од најголемиот број на земји членки на Европската унија (ЕУ), класичната чума кај свињите (КЧС), продолжува да претставува сериозен проблем во различни делови на светот, вклучувајќи ги и земјите од југо-источна Европа. Според годишната наредба, која се издава секоја година од страна на Агенцијата за храна и ветеринарство (АХВ), вакцинацијата против КЧС е задолжителна на целата територија на Македонија. За мониторинг на вакцинацијата, пресметано е дека 29 серуми по свињарска фарма се доволни за да се открие едно позитивно (вакцинирано), животно за претпоставена застапеност од 10% и со 95% веродостојност. Од 2002 крвни серуми кои беа тестирани, само 1001 (50,00%) дадоа позитивен резултат, 67 (3,35%) беа сомнителни, додека 934 (46,65%) беа негативни. Причините за ваквата состојба можат да бидат различни и сите тие би требало внимателно да се разгледаат и да се превземат соодветни мерки за коригирање на одредените пропусти во моменталната вакцинална практика.

Клучни зборови: Свињи, КЧС, вакцинација, ЕЛИСА.

UDC: 639.113.09:[616.98:578.824}:615.371(497.7)

MONITORING BAIT UPTAKE THROUGH TETRACYCLINE PRESENCE AND AGE STRUCTURE OF FOXES IN ORAL VACCINATION AGAINST RABIES CAMPAIGNS IN R. MACEDONIA

Cvetkovikj Aleksandar[1], Radeski Miroslav[1], Mrenoshki Slavcho[1], Kirandjiski Toni[2], Krstevski Kiril[1], Dzhadzhovski Igor[1], Gjurovski Ivica[1], Branko Angjelovski[1], Cvetkovikj Iskra[1], Florence Cliquet[3]

[1]*Veterinary Institute, Faculty of veterinary medicine, Skopje, Macedonia*
[2]*Animal health and welfare sector, Food and veterinary agency, Skopje, Macedonia*
[3]*Anses, Laboratoire de la rage et de la faune sauvage de Nancy, Malzéville Cedex, France*

ABSTRACT
Key objectives of this paper were to present the prevalence of tetracycline presence in fox's teeth and jaws and their age structure after two performed campaigns for oral rabies vaccination in 2011 in Macedonia and to evaluate the successfulness of the bait uptake. The method used for detection of tetracycline was based on fluorescent microscopy of fine sections of teeth and mandibular bones of foxes in ultra-violet light. Age determination in the canine sections was based on presence/absence and number of annual growing lines in the cementum. The total number of examined samples was 141 (n=141) out of which 86 (60,9%) were tetracycline positive. The Southeastern region had the most positive samples [26 (68,4 %)]. Ninetyeight samples (69,5 %) originated from foxes older than three years, although there was high prevalence of tetracycline positive samples in all age classes. The presented results showed relatively high percentage of baits uptake by foxes for 2011 in Macedonia, but more thorough sampling on national level is needed, especially in Northeastern, Polog, Skopje and Vardar region.
Key words: rabies, monitoring of vaccination, tetracycline, fox, age determination

INTRODUCTION

Rabies as a zoonotic viral disease is of a great concern in the European countries. The main reasons are the ability of the virus for easy transmition through direct contact with saliva from the infected animals and the fatal results for human and other animals after the development of the disease's symptoms. Although rabies is widely known as a disease of dogs and cats, the analyses show that the wild animals are the main reservoirs. From the mid of the twentieth century the disease virtually disappeared from dogs, established itself in the fox population in Eastern Europe and unavoidably spread on south and west. Following the high co-adaptation of the current rabies virus strain to the fox, and due to the fox's ecology, no other species play a significant role in maintaining the disease in the infected areas. Therefore red foxes (*Vulpes vulpes*) are considered as the main reservoir as well as the main vector of the virus [1].

Countries across Europe have created vaccination strategies of foxes in order to monitor and control this disease. As a result of implemented oral rabies vaccination campaigns there was a great improvement of rabies situation across the Europe, especially in western European countries [1]. Conducted researches concluded that oral application of inactivated rabies vaccines cannot confirm satisfactory level of immunization in red foxes [2]. Therefore, in vaccine strategies for foxes attenuated vaccines are used.

Fox vaccination monitoring is crucial for the successfulness of implemented vaccination strategy. The ability of tetracycline molecule to incorporate into bones and teeth and to serve as a long term post-mortem tissue marker is widely used in oral rabies vaccines for monitoring the bait uptake by wildlife. Therefore, WHO expert committee on Rabies suggested that the testing for the occurrence of a biomarker (tetracycline) – incorporated into the bait is one of the three methods that could be used for the evaluation of vaccination [3]. Tetracycline, as a marker of bait uptake, provides a life-long marking of bones and teeth that is easily detected on post-mortem. At the same time, during the tetracycline determination, the age of the animal can be determined, which provides additional data of the conducted vaccination strategy on one region.

At the Balkan region, including Macedonia, the main vector and reservoir of the disease, also, is the red fox (*Vulpes vulpes*), which constitutes 88% of the rabies cases [4]. Therefore, part of the European Instrument for Pre-Accession Assistance (IPA) project "Capacity building of the veterinary services for implementation of EU Acquis" has a component for control and eradication of rabies in Macedonia. The project has started on 16 August 2010 and in order to create a multiannual strategy for control (oral vaccination of foxes), improve passive surveillance and reporting, as well as to enhance public awareness for the risks of this disease and ways of its prevention.

In 2011, as part of this project, two oral vaccination campaigns were completed. The first one was initiated in spring 2011 (May, 19 – June, 9) and the second campaign was undertaken in autumn (October, 11 – 31). The aerial distribution of vaccine Fuchsoral® (SAD B19 strain) was performed using an automatic dropping system. A total of 500 000 baits per campaign were dropped, covering the whole territory of the country excluding water

surfaces and dense urban settlements. The vaccine bait density was 21.6 baits/km^2, the distance between dropping lines was 500 – 600 meters [4].

The main objectives of this paper were to present the presence of tetracycline in fox's teeth after two performed campaigns for oral vaccination in 2011 in Macedonia on national and regional level; to determine the age structure profile of collected samples of foxes on national and regional level; to demonstrate the predominant age category of foxes in bait uptake and to evaluate the successfulness of the bait uptake campaign for oral vaccination of foxes for 2011.

MATERIALS AND METHODS

The method used for detection of tetracycline in canine and premolar teeth and mandibular bone of red foxes is based upon the protocol of ANSES, Nancy Laboratory for rabies and wildlife [5]. The detection method uses the fluorescence of tetracycline in ultra – violet light. First, sections of teeth and jaw were made, according to the protocol for "section of teeth and bone of foxes to monitor age and bait uptake" by ANSES, Nancy Laboratory for rabies and wildlife [6]. The canine is fixed by its point and a transversal section is done. The cut is done between 2 and 3 mm from the end of the root. For very young animals, the mandible is sectioned at right angle at the beginning of the first premolar. The result is acceptable when the section is 0.2 to 0.6 mm thick and when different elements may be identified such as the end of the root, pulp cavity, dentine, cementum and some pieces of bone [6]. The created section is placed on the microscope slide on a drop of buffered glycerol. Tetracycline lines will appear as more or less intense yellow lines on the bluish background. These lines could be found at the premolar teeth (characteristic for the young foxes), dentine and cementum of the canine, as well as into the mandibular bone. Depending of the number of uptake baits, the number of campaigns that animals were subjected and the age of the animal, the tetracycline lines will vary in thickness, number and location, respectively (Figure 1).

Regarding the age determination of foxes, the procedure "Age determination on sections of teeth" by ANSES, Nancy Laboratory for rabies and wildlife was used [7]. Buffered glycerol was dropped on the microscope slide. The transversal fine section of fox's canine was submerged into the glycerol. Using classical transmitted light microscope the annual growing lines were searched in the tooth cementum. The main parameters for the age determination are: the size (diameter) of pulp cavity, presence/absence of cementum and the number of dark lines in cementum that correspond to the number of winter passed by the animal. These parameters are summarized and expressed as fox's age through the following age classes: 0-1 year; 1-2 years; 2-3 years and over 3 years.

All of the results (positive/negative samples and age of foxes) were processed statistically and geographically analyzed using the LABIS® (Laboratory Information System) software and MS Excel 2010.

RESULTS

In 2011 the total number of examined samples for detection of tetracycline was 141 (n=141) of which 86 (60,99%) samples were tetracycline positive. Predominantly tetracycline was found in mandibular bone, canine dentine, canine cementum and in the dentine of the first premolar respectively. The Southeastern region had the most positive samples on tetracycline presence, 26 (68,42 %) out of 38 collected samples, while at the Northeastern region there was no positive sample with the lowest rate of collected samples (Table 1).

CONCLUSIONS

The presented results showed relatively high percentage of baits uptake by foxes for 2011 in Macedonia. In the regions with high number of collected samples the percentage of positive samples was high, suggesting successfulness of the implemented vaccination campaigns. However, the number of collected samples from Northeastern, Polog, Skopje and Vardar region is very low, so the level of bait uptake could not be determined. Therefore, more frequent sampling from the mentioned regions is necessary.

Regarding the age structure of collected samples, most of them were older than 3 years. Although there is lower percentage of occurence of other age classes, a high affinity of bait uptake was noted in all age classes. This led us to a conclusion that the implemented vaccination campaigns are equally influencing all age categories of foxes.

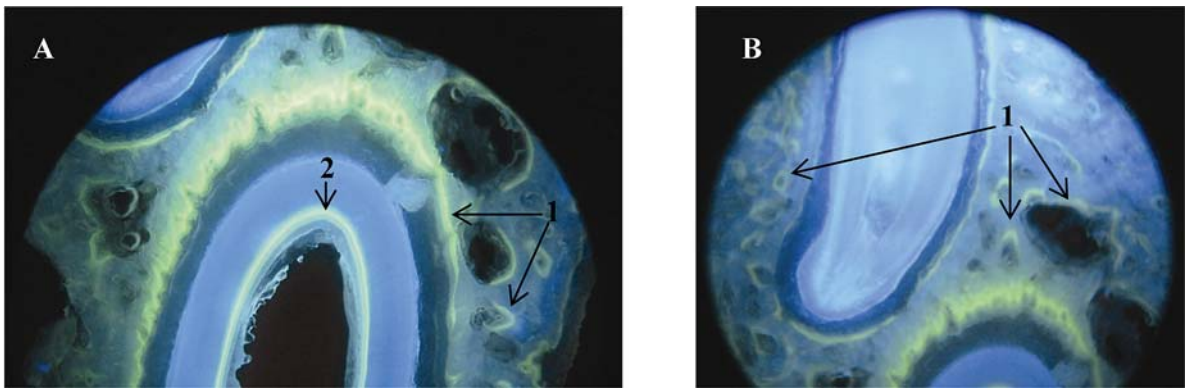


Figure 1. *Fluorescence of tetracycline in positive tetracycline samples seen from the transversal fine section of fox's (A) canine and (B) premolar. 1 - Tetracycline presence in mandibular bone; 2 – Tetracycline lines presence in the dentin of the canine tooth.*

Table 1. *Tetracycline presence distribution in the examined samples on national and regional level*

Statistical Region	No. Examined	No. Positive	No. Negative	Positive samples %
Eastern	**26**	**18**	**8**	**69,23**
Northeastern	**1**	**0**	**1**	**0,00**
Pelagonia	**34**	**21**	**13**	**61,76**
Polog	**4**	**2**	**2**	**50,00**
Skopje	**5**	**2**	**3**	**40,00**
Southeastern	**38**	**26**	**12**	**68,42**
Southwestern	**26**	**15**	**11**	**57,69**
Vardar	**7**	**2**	**5**	**28,57**
TOTAL:	**141**	**86**	**55**	**60,99**

Ninetyeight samples (69,5 %) originated from foxes older than three years, and the rest 43 samples (30.49%) were within the other age classes (2-3, 1-2 and 0-1). The distribution of positive tetracycline samples according the age class showed that there was high percentage of positive samples in all age classes, especially in age classes 0-1 and over 3 years. (Figure 2).

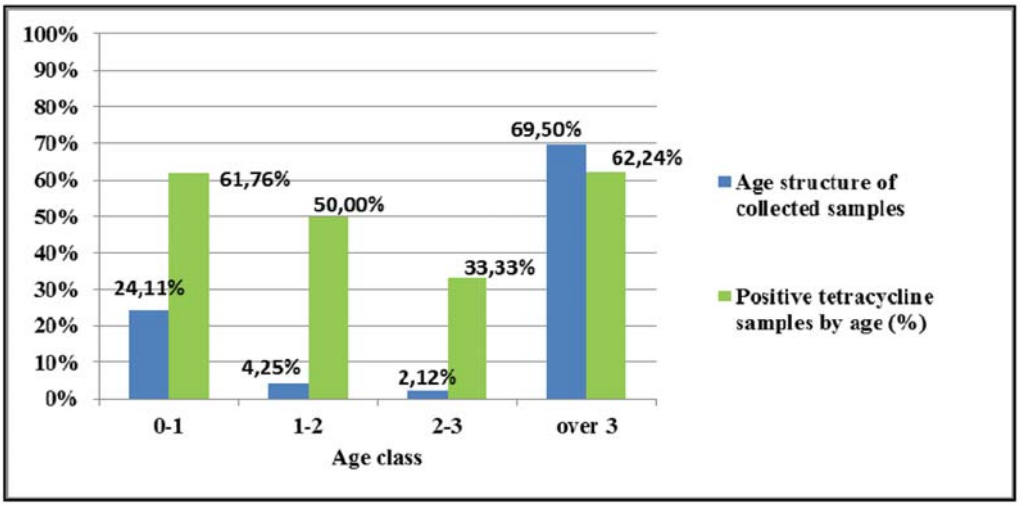


Figure 2. *Age structure and distribution of positive tetracycline samples. The age structure is presented as a percentage from the total number of samples. The category "positive tetracycline samples" is shown as a percentage of positive samples in the appropriate age class.*

REFERENCES

1. European commission, (2002).*The oral vaccination of foxes against rabies*. Report of the Scientific Committee on Animal Health and Animal Welfare. Health & consumer protection directorate-general, Directorate C - Scientific Opinions
2. Atanasiu, P., Metianu, T., and Bolanos, A., (1982). *Evaluation d'une vaccination rabique expérimentale par la voie orale et intestinale avec des vaccins tués concenters et non concentrés*. Comp. Immun. Microbiol. Infect. Dis. 5: 1-3, 187-191
3. WHO, (1992). *8th Report of WHO Expert Committee on rabies*, Geneva
4. Davcheva K., Cliquet F. (2012). *Epidemiological situation of rabies in Macedonia in 2011*. ANSES, Nancy Laboratory for Rabies and Wildlife
5. *Detection of tetracycline in teeth and bone specimens by fluorescence*. ANSES, Nancy Laboratory for Rabies and Wildlife, 3
6. *Section of teeth and bone of foxes to monitor age and bait uptake*. ANSES, Nancy Laboratory for Rabies and Wildlife, 2
7. *Age determination on sections of teeth*. ANSES, Nancy Laboratory for Rabies and Wildlife, 2

МОНИТОРИНГ НА ВНЕСОТ НА ВАКЦИНАЛНИ МАМКИ ПРЕКУ ДЕТЕКЦИЈА НА ТЕТРАЦИКЛИН И ВОЗРАСНА СТРУКТУРА НА ЛИСИЦИ ВО КАМПАЊИТЕ ЗА ОРАЛНА ВАКЦИНАЦИЈА ПРОТИВ БЕСНИЛО ВО Р. МАКЕДОНИЈА

Цветковиќ Александар[1], Радески Мирослав[1], Мреношки Славчо[1], Киранџиски Тони[2], Џаџовски Игор[1], Крстевски Кирил[1], Ѓуровски Ивица[1], Бранко Анѓеловски[1], Цветковиќ Искра[1], Флоренс Клике[3]

[1]*Ветеринарен институт, Факултет за ветеринарна медицина, Скопје, Македонија*
[2]*Сектор за здравствена заштита и благосостојба на животните, Агенција за храна и ветеринарство, Скопје, Македонија*
[3]*Лабораторија за беснило и диви животни во Нанси, Малзевил Цедекс, Франција*

АПСТРАКТ

Главни цели на овој труд беа да се прикажат преваленцата на присуството на тетрациклин во забите и вилиците од лисици, нивната возрасна структура по две спроведени кампањи за орална вакцинација против беснило во 2011 година и евалуација на успешноста на внесот на вакциналните мамки. Детекцијата на тетрациклин базираше на флуоресцентна микроскопија на фини секции од забите и долните вилици на ултра-виолетова светлина. Возрасната структура во секциите од забите беше одредена на база на присуството/отсуството и бројот на годишни линии на раст во цементот. Вкупно беа испитани 141 (n=141) вилица, од кои 86 (60,9%) беа тетрациклин позитивни. Најмногу позитивни примероци [26 (68,4 %)] имаше во Југоисточниот регион. Деведесет и осум (69,5 %) испитани примероци потекнуваа од лисици постари од три години и имаше висока преваленца на тетрациклин позитивни примероци кај сите возрасни класи. Резултатите покажаа висок процент на внес на вакцинални мамки кај лисиците во текот на двете кампањи во 2011 година, иако на национално ниво, потребно е потемелно мострирање особено во Северноисточниот, Полошкиот, Скопскиот и Вардарскиот регион.

Клучни зборови: беснило, мониторинг на вакцинација, тетрациклин, лисица, одредување на возраст

UDC: 636.7.09:616.995.41

MOST IMPORTANT FOOD-BORNE DISEASES OF DOGS CAUSED BY TICKS AND ITS CONTROL

Pavlovic Ivan[1], Petkovic Dragana[2], Kukovska Valentina[3], Stamenkovic Vojislav[3], Jovcevski Srdjan[4], Pavlovic Miloš[5], Jovcevski Stefan[4], Elezovic Milica[5]

[1]Institute of Veterinary Medicine of Serbia, Belgrade, Serbia,
[2] Veterinary Ambulance "Petwellness", New Belgrade, Serbia
[3] Royal Vet Belgrade, Serbia, [4] Veterinarna Clinic "INO-VET" Kumanovo, R.Macedonia,
[5] Faculty of Veterinary Medicine, Belgrade, Serbia,

ABSTRACT
From veterinary aspect ticks caused food-born disease most important are babesiosis, ehrlichiosis, and lyme boreliosis.
Dog babesiosis is caused by *Babesia canis*. The principal vector is *R. sanguineus, D. marginatus* and *D.reticulatus* which has been specially demonstrated as a vector in Europe. Common clinical manifestation of disease is anemia, haemoglobinuria, fever, pale of mucous membranes and etc.
Canine ehrlichiosis is a disease in dogs caused by *E. canis*. It is now known that canine ehrlichiosis can be caused by multiple etiologic agents with different host cell tropisms including monocytes, granulocytes, and platelets. Clinically, *E. canis* infections are characterized by acute, subclinical, and chronic stages of infection.
Lyme borreliosis, caused by the *Borrelia burgdorferi* is the most prevalent arthropod-transmitted zoonosis in Europe. *Ixodes ricinus* is a source, vector and reservoir in the epizootic process of *Borrelia burgdorferi*. This species is one of the most widely distributed in all balcan countries.
Tick control is most important measures for prevention of this diseases. With aim to protect against ticks as one of the best measures are use of spot on drugs which is applied to the skin like fipronile (FRONTLINE, FRONTLINE PLUS) or other drugs. Drop on methods is used like a small dollop of the solution is squeezed from a tiny tube and gently rubbed into the pet's coat at the base of the neck, right where it connects to the shoulders at the back of the head. Through a process called translocation, the ointment works its way through the pet's coat. The oil in the coat slowly dispenses through the hair over 30 days. The initial translocation normally completes in about 24 hours.
Key words: ticks, babesiosis, ehrlichiosis, lyme boreliosis, control

INTRODUCTION

Food-borne diseases of dogs transmitted by ticks presented great problem worldwide. As the incidence of tick-borne illnesses increases and the geographic areas in which they are found expand, it becomes increasingly important that health professionals be able to distinguish the diverse, and often overlapping, clinical presentations of these diseases. From veterinary aspect most important was babesiosis, ehrlichiosis, and lyme boreliosis. Because ticks can harbor more than one disease-causing agent, dogs can be infected with more than one pathogen at the same time, compounding the difficulty in diagnosis and treatment.

This seasonal characteristic is present especially in arthropod-borne parasitoses, primarily because population densities of vectors or intermediate hosts vary throughout the year. Ticks tend to be more active during warmer months, though this varies by geographic region and climate. Areas with woods, bushes, high grass, or leaf litter are likely to have more ticks. The change of seasons may have an influence on disease prevalence and may result in a periodical occurrence.

From these reason, except the therapy of diseases animals, we must take care to prevention of ticks bite.

Ticks

Ticks are small arachnids in the order Ixodida. Along with mites, they constitute the subclass Acarina. Ticks are ectoparasites (external parasites), living by hematophagy on the blood of mammals, birds, and sometimes reptiles and amphibians.

In research on ticks as reservoirs and vectors of the causative agents of disease, it is of great importance to ascertain elements of zoogeographic distribution, particularly the possible presence of species which might be indicators of a disease. For an ecosystem to support ticks, it must satisfy two requirements: there must be a high enough population density of host species in the area, and there must be high enough humidity for ticks to remain hydrated.

Ixodids ticks have three distinct life stages. Larvae which emerge from the egg have six legs. After obtaining a blood meal from a vertebrate host, they molt to the nymphal stage and acquire eight legs. Nymphs feed and molt to the next and final stage - the adult, which also has eight legs. After feeding once more, the adult female hard ticks lay one batch of thousands of eggs and then die. Only one blood meal is taken during each of the three life stages. The time to completion of the entire life cycle may vary from less than a year in tropical regions to over three years in cold climates, where certain stages may enter diapause until hosts are again available. Many hard ticks can go for several months without feeding if not unduly duressed by environmental conditions.

Ticks seek hosts by an interesting behaviour called "questing." Questing ticks crawl up the stems of grass or perch on the edges of leaves on the ground in a typical posture with the front legs extended, especially in response to a host passing by. Certain biochemical such as carbon dioxide as well as heat and movement serve as stimuli for questing behaviour. Subsequently, these ticks climb on to a potential host which brushes against their extended front legs.

Ixodid ticks feed for extended periods of time on their hosts, varying from several days to weeks, depending on such factors as life stage, host type, and species of tick. The outside surface, or cuticle, of hard ticks actually grows to accommodate the large volume of blood ingested, which, in adult ticks, may be anywhere from 200-600 times their unfed body weight

From epidemiological aspects *Rhipicephalus, Dermacentor* and *Ixodes* was the most important tick genus. The population dynamics of recorded tick species are known for their two maxima a year - in spring (April-May) and in autumn (September-October). The considerable interchange between spring and autumn tick populations can be attributed mainly to environmental conditions.

Babesiosis

Dog babesiosis is caused by haemoprotozoan parasites of the genus *Babesia (Piroplasmidae: Apicomplexa)*. Two species of genus *Babesia* - *B.canis* and *B. gibsoni* are of great importance owing to wide range of infected animals in *Canidae* family. *Babesia canis* is endemic in Europe, Southern Africa, Asia and the Americas. This is a large piroplasm, pyriform in shape, 4-5 micrometer in length, pointed at one and round at other. Frequently there is a vacuole in the cytoplasm. The pyriform forms may lie at an angle to another, but pleomorphism of shape may be seen, organism varies from amoeboid to ring forms. *Babesia gibsoni* organisms are smaller (1-2.5 μm in diameter) and appear as round to oval or ring-shaped organisms, usually single, in red blood cells.. *Babesia gibsoni* is found in Southern Asia, the Middle East and Northern Africa. Recent reports indicate that *Babesia gibsoni* is an emerging vector-borne disease among dogs in Europe and the United States, as well.

There are three subtypes of *Babesia canis: B. canis canis, B. canis vogeli* and *B. canis rossi*. These strains differ in virulence, geographic location and tick vector, but are identical in appearance. In the United States, the most common strain is *B. canis vogeli,* which is the least pathogenic form. *Babesia canis canis* infections occur world-wide, but most often in Europe. *Babesia canis canis* infections occur world-wide, but most often in Europe.

Dog babesiosis is (like other babesiosis) a tick-borne disease. The principal vector of *B. canis* is *Rhipicephalus sanguineus* which occurs throughout the world; it has been specially demonstrated as a vector in Europe, south and central Africa, both America and south Asia. The species of the genus *Dermacentor- D. marginatus* and *D. reticulatus* - have been incriminated in Europe including Russia as vectors of dog babesiosis. At West Balkan area, *R. sanguineus* and *D. reticulatus*, rarely *D. marginatus*, are impointed as vectors of dog babesiosis. The change of seasons may have an influence on disease prevalence and may result in a periodical occurrence. This seasonal characteristic is present especially in arthropod-borne parasitoses, primarily because population densities of vectors or intermediate hosts vary throughout the year.

The Babesia parasite will infect the red blood cells of the dog, and this will cause the dog's immune system to kill the infected red blood cells. This will kill the Babesia parasite, but if a great number of red blood cells are killed in the process it will give your dog anaemia. The immune system can also run amok and start killing uninfected red blood cells too.

Infected dogs may exhibit either peracute, acute or subclinical signs of disease. Pathogenicity is increased in young dogs, immunosuppressed dogs, heavily parasitized dogs, and when there is exposure to a virulent strain or concurrent infection with other tick-borne pathogens (ehrlichiosis, hepatozoonosis, leishmaniasis).

Peracute signs include acute onset of hypotensive shock, vasculitis, extensive tissue damage, hypoxia and death. Signs of acute disease include weakness, fever, lethargy, hemolytic anemia, thrombocytopenia, splenomegaly, lymphadenopathy, pale of mucous membranes, a yellow colouring of the eyes (and skin), and urine that looks red or orange (haemoglobinuria).

Less common signs include ascites, peripheral edema, ulcerations, stomatitis, gastroenteritis, acute renal failure and rhabdomyolysis. If parasites infest the central nervous system, a dog with Babesiosis can display neurological problems, as well as local inflammation. The lungs can also become damaged by Babesiosis, and some dogs will also suffer from liver problems.

Acute infections of virulent strains of Babesia canis have been associated with induction of the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) secondary to massive immunostimulation and cytokine release. Signs of MODS can include coagulopathies (DIC), adult respiratory distress syndrome (ARDS), cerebral dysfunction and acute renal failure.

Ehrlichiosis

Canine ehrlichiosis is a general term that originally described a tick-transmitted disease in dogs caused by *E. canis*. It is now known that canine ehrlichiosis can be caused by multiple etiologic agents (*E. canis, E. chaffeensis, E. ewingii, A. phagocytophila,* and *A. platys*) with different host cell tropisms including monocytes, granulocytes, and platelets. Serosurveys conducted in 35 states indicate that the overall prevalence rate of canine ehrlichiosis is approximately 1.5%, with southwestern states having the highest prevalence, 2-6%.

Clinically, *E. canis* infections are characterized by acute, subclinical, and chronic stages of infection. In the acute stage of the disease, dogs may resolve the disease, but develop subclinical persistent infections, and thus, become asymptomatic carriers of the infection. A severe chronic form of the disease sometimes occurs, in which the response to antibiotic therapy is poor and dogs often die from massive hemorrhage, severe debilitation or secondary infection. Ehrlichiae have a complex life cycle involving a tick vector and a mammalian host and have developed strategies to establish persistent infections in the natural hosts to insure their survival and transmission in nature.

E. canis is transmitted between dogs by the brown dog tick *Rhipicephalus sanguineus* and is maintained in nature by persistent infection of both wild and domestic dogs. Nymphal or larval ticks typically are infected with

E. canis by feeding on a persistently infected dog, and maintain the infection transtadially as the tick molts from stage to stage. The mechanism of persistent ehrlichial infection has not yet been determined, but recent studies have identified important immunoreactive proteins and potential pathogenic mechanisms of ehrlichiae.

The acute stage of disease is due largely to a vasculitis. The organism replicates in circulating monocytes, and subsequently in mononuclear phagocytic cells throughout the body. The infected monocytes bind to vascular endothelial cells and initiate a vasculitis and subendothelial cell infection. (Because of the vasculitis, dogs with ehrlichiosis may also demonstrate edema, but this is less characteristic than in RMSF.)

The chronic stage of disease reflects bone marrow suppression.

The thrombocytopenia in ehrlichiosis may be due to consumption of platelets, sequestration of platelets in the spleen, immune-mediated destruction of platelets, decreased bone marrow production of platelets, or some combination of these mechanisms. Overall, however, the basis for ehrlichial thrombocytopenia remains unclear.

Ehrlichia canis infection in dogs is divided into 3 clinicopathologic stages:

Acute phase of disease characterized fever, anorexia, lethargy, lymphadenopathy and thrombocytopenia. This phase begins 1-3 weeks after exposure. Most dogs recover at this point, but others progress to the subacute and chronic phases

Subacute phase of disease characterized hypergammaglobulinemia (polyclonal or sometimes monoclonal gammopathy), thrombocytopenia and anemia. Usually subclinical, but can last months to years

Chronic phase of disease characterized lethargy and weight loss.

Lyme borreliosis

Lyme borreliosis, caused by the spirochaete *Borrelia burgdorferi* s. l., is the most prevalent arthropod-transmitted zoonosis in Europe. Concerning domestic animals, Lyme borreliosis has been reported in dogs, cats, horses, cattle andsheep Research carried out on Lyme disease in Serbia so far pointed to the species *Ixodes ricinus* L. (*Acari:Ixodidae*) as a source, vector and reservoir in the epizootic process of *Borrelia burgdorferi*. This species is one of the most widely distributed in all Balcan countries.

The majority of dogs that are infected with Lyme disease show no symptoms at all. Most of these dogs are identified through routine yearly testing at veterinary hospitals. We do not know why some dogs are affected with symptoms and others are not. In some animal species, the age at which they are infected has a lot to do with the development of symptoms - younger animals were affected more severely than more mature ones.

However, a small portion of infected dogs do develop sore, painful joints weeks or months after infection. Some of these dogs run low-grade fevers. The signs you read about in humans with Lyme almost never occur in pets.

Simple arthritis is usually constant in the joints it affects. Many dogs with Lyme disease have recurrent lameness of the limbs due to inflammation of the joints. Others, meanwhile, may develop acute lameness, which lasts for only three to four days but recurs days to weeks later, with lameness in the same leg, or in other legs. Better known as "shifting-leg lameness," this condition is characterized by lameness in one leg, with a return to normal function, and another leg is then involved; one or more joints may be swollen and warm; a pain response is elicited by feeling the joint; responds well to antibiotic treatment.

Some dogs may also develop kidney problems. If left untreated, it may lead to glomerulonephritis, which causes inflammation and accompanying dysfunction of the kidney's glomeruli (essentially, a blood filter). Eventually, total kidney failure sets in and the dog begins to exhibit such signs as vomiting, diarrhea, lack of appetite, weight loss, increased urination and thirst, fluid buildup in the abdomen and fluid buildup in the tissues, especially the legs and under the skin.

Neurological disease (behavioral changes, seizures) and heart complications, which are sometimes seen in humans, are rare in dogs.

Prevention and Control

Preventing exposure to the ticks that carry all tick-borne diseases is the best means of preventing diseases. This is especially important in peak tick season or if dog spends time in the woods or tall grass (consider avoiding these areas in tick season). Once a host is found, the tick climbs on and attaches its mouthparts into the skin, beginning the blood meal. Once locked in place, the tick will not detach until its meal is complete. It may continue to feed for several hours to days, depending on the type of tick. On dogs, ticks often attach themselves in crevices and/or areas with little to no hair – typically in and around the ears, the areas where the insides of the legs meet the body, between the toes, and within skin folds.

Owners should inspect their dogs daily for ticks. Prompt removal of ticks within 24 hours should prevent disease transmission, because it has been reported that the tick must be attached for two to three days to transmit the organism. In kennels where puppies are being lost to disease, aggressive tick-control measures should be instituted including spraying the environment as well as treating animals. Although it has not been proven, it is likely that any blood-sucking insect that moves from dog to dog could directly transmit the organism through blood contamination. Therefore, an insecticide that prevents biting flies, fleas, and mosquitoes would have the best chance of preventing spread of this organism in a kennel situation.

In aim to protection against ticks as one of the best measures were use of spot on drugs which is applied to the skin like fipronile (FrontLine, frontline plus) or other drugs. Drop on methods is used like a small dollop of the solution is squeezed from a tiny tube and gently rubbed into the pet's coat at the base of the neck, right where it connects to the shoulders at the back of the head. Through a process called translocation, the ointment works its way through the pet's coat. The oil in the coat slowly dispenses through the hair over 30 days. The initial translocation normally completes in about 24 hours.

REFERENCES

1. Milutinović, M., Aleksić-Bakrač, N., Pavlović, I, (1998/7) Faunistic and ecological notes on ticks (Acari: Ixodidae, Argasidae) in the extended area of Belgrade.Magy.Allator.Lapja 120: 434-436.
2. Pavlović, I., Pavlović, I., Terzin V.,Petković

Dragana,Terzin Dragana (2002) The first occurence of Erlichia canis at dogs in Belgrade area 12th FAVA Congress – Globalization – Challanges to the Veterinary Profession, Kuala Lumpur, Malaysia, 28
3. Pavlović, I., Milutinović M.,Terzin V.,Petković Dragna,Terzin Dragana (2002) Tick fauna of dogs from Belgrade area and its role in epizootiology of dog babesiosis 12th FAVA Congress – Globalization – Challanges to the Veterinary Profession, Kuala Lumpur, Malaysia, 26
4. Pavlović, I., Milutinović Marija, Radulović Ž.,Petković Dragana, Terzin V., Terzin Dragana, (2003) Epizootiological research of canine babesiosis in the Belgrade district. Lucrari Stiintifice Medicina Veterinara 46 (5), 365-370
5. Pavlović, I., Terzin V.,PetkovićD.,Ćurčin Lj.,StankovićB.,TerzinD.,ĆurčinK.(2006):Erlihioza pasa na području Beograda, Zbornik radova i kratkih sadržaja 18.savetovanja veterinara Srbije, Zlatibor, 97-98
6. Pavlović, I., Terzin V., Petković Dragana, Ćurčin Klara, Terzin Dragana,Ćurčin Lj., Stanković B. (2007): Protozoarne infekcije pasa prenosive krpeljimaZbornik radova i kratkih sadržaja IX simpozijuma epizootiološki dani sa međunarodnim učešćem, Srebrno jezero, 69-70
7. Pavlović, I., Jovičić Dubravka, Terzin V., Savatić Isidora, Marković Slavica (2011) Influence of ecological factors to spread of dog babesiosis at Belgrade area Book of Abstracts International Scientific Conference on Sustainable Development in the Function of Environment Protection, Belgrade, Serbia, 221
8. Pavlović, I., Terzin V., Petković Dragana, Terzin Dragana, Vojinović Dragica (2011):Higijenskih mera u preventivi i suzbijanju erlihioze pasaZbornik radova XXII Savetovanja dezinfekcija, dezinsekcija i deratizacija u zaštiti zdravlja životinja i ljudi sa međunarodnim učešćem, Kaštel-Ečka, Lovački dvorac, 225-228
9. Riek, P.F. 1968. Babesiosis:220-268. In: Infectious Blood Diseases of man and Animals, vol.2, Waineman D.& Ristić M. (ed), Academic Press, New York-London.
10. Zahler, M., Gothe, R. 1977. Endemic risk of Babesia canis by Dermacentor reticulatus epidemiological study. Tieraztl. Prax. Ausg. Klantiere Heimtiere 25, 6: 666-670.

НАЈВАЖНИ БОЛЕСТИ ОД ХРАНА КАЈ КУЧИЊА ПРЕДИЗВИКАНИ ОД КРЛЕЖИ И НИВНА КОНТРОЛА

Павловиќ Иван[1] ,Петковиќ Драгана[2], Куковска Валентина[3], Стаменковиќ Војислав[3], Јовчевски Срѓан[4], Павловиќ Милош[5], Јовчевски Стефан[4], Елезовиќ Милица[5]

[1]Институт за ветеринарна медицина во Србија, Белград, Србија, [2]Ветеринарна амбуланта "Петвелнес", Нов Белград, Србија, [3]Ројал Вет Белград, Србија,[4]Ветеринарна клиника " ИНО-ВЕТ"Куманово, Р. Македонија, [5]Факултет за Ветеринарна Медицина, Белград, Србија,

АПСТРАКТ

Од ветеринарен аспект крлежите кои предизвикуваат болести кои се пренесуваат преку храна најважни се бабезиоза, ерлихиоза и лајмска борелиоза.

Кучешката бабезиоза ја предизвикува Babesia canis. Главниот вектор е *R. sanguineus, D. marginatus u D.reticulatus.* кои особено се демонстрирани како вектор во Европа. Вообичаена клиничка манифестација на болеста е анемија, хемоглобинурија, треска, бледило на мукозните мембрани итн.

Кучешката ерлихиоза е болест кај кучињата предизвикана од E canis. Денеска е познато дека кучешката ерлихиоза може да биде предизвикана од различни етиолошки причинители со различен домаќински клеточен тропизам, вклучувајќи ги моноцитите, гранулоцитите и тромбоцитите. Клиничкати E canis инфекциите се карактеризираат со акутна, субклиничка и хронична форма на инфекција.

Лајмската борелиоза предизвикана од *Borrelia burgdorferi* е најчесто застапена артроподно пренесена зооноза во Европа. *Ixodes ricinus* е изворот, векторот и резервоарот во епизоотскиот процес на *Borrelia burgdorferi.* Овој вид е еден од најшироко распространетите во сите балкански држави.

Контролата на крлежите е најважната мерка за превенција на овие болести. Како заштита против крлежите како една од најдобрите мерки се користат топикални медикаменти кои се аплицираат на кожата како фипронил (ФРОНТЛАЈН, ФРОНТЛАЈН ПЛУС) или други медикаменти. Методот на капнување се употребува кога мала количина на солуција се истисне од тенкото цевче и нежно се протрие во крзното на миленичето во основата на вратот веднаш каде се спојува со рамото на задниот дел од главата. Низ процес наречен транслокација, маста делува поминувајќи низ крзното на миленичето. Маста во крзното полека се диспензира низ влакната за 30 дена. Иницијалната транслокација нормално се комплетира за 24 часа.

Клучни зборови: бабезиоза, ерлихиоза, лајмска борелиоза, контрола

UDC: 636.7.09:[616.98:579.881.3.083.3(497.7)

SEROLOGICAL EVIDENCE OF EHRLICHIA CANIS AND LEISHMANIA INFANTUM CO-INFECTION IN NATURALLY EXPOSED DOGS ON THE TERRITORY OF REPUBLIC OF MACEDONIA

Stefanovska Jovana[1], Kochevski Zoran[1], Iskra Cvetkovikj[2], Farkas Robert[3], Ivancho Naletoski[4], Mrenoshki Slavcho[2],

[1] *Department of parasitology and parasitic diseases Faculty of Veterinary Medicine in Skopje,*
[2] *Department of microbiology and immunology Faculty of Veterinary Medicine in Skopje,*
[3]*Faculty of Veterinary Science, Szent Istvan University, Budapest, Hungary*
[4]*International Atomic Energy Agency, Animal Production and Health Section, Joint FAO/IAEA Division, Vienna, Austria*

ABSTRACT
Ehrlichia canis and *Leishmania infantum* are vector born diseases which also persist on the territory of Republic of Macedonia. Clinical and haematological signs of these diseases are very similar and establishing a differential diagnosis sometimes can be problematic, since antibodies against *E. canis* are cross-reactive to *Leishmania* sp. antigen present in common serological tests used to detect dogs infected by *Leishmania* sp. The aim of this study was to define the rate of co-infection among two diseases and presence of specific antibodies in dog samples in R. of Macedonia. A total of 144 serum samples were tested for the detection of *E. canis* antibodies, using a commercial ELISA kit (SNAP® 4Dx®; IDEXX Laboratories, Inc. U.S.A.), and for detection of anti-*Leishmania* antibodies, with indirect fluorescent antibody test (IFAT, in-house method). The total number of positive samples on presence of antibodies on both diseases were 79, and the overlapping (coexistence of antibodies to *E. canis* and antibodies to *L. infantum*) was detected in 13 of them (16,4%). This finding shows that when the suspicion of ehrlichiosis or leishmaniasis in dogs is present, it will be very beneficial to use simultaneous serological tests which will provide correct diagnosis of the infection.
Key words: Dog, Ehrlichiosis, Leishmaniosis, Co-infection

INTRODUCTION

Ehrlichia canis and *Leishmania infantum* are vector born diseases which also persist on the territory of R. Macedonia. The causative agents of these diseases are intracellular pathogens present in monocyte/macrophage cells. *E. canis* is transmitted by ticks and *L. infantum* by phlebotomine sand flies, but both vectors have similar activity and transmission period. Moreover, clinical and haematological signs of these diseases are very similar. Establishing a differential diagnosis sometimes can be problematic, since antibodies against *E. canis* are cross-reactive to *Leishmania* sp. antigen present in serological tests used to detect dogs infected with *Leishmania* sp. [1]. The aim of the study was to detect the presence, distribution and frequency of the co-infection with these two diseases in dogs in R. of Macedonia.

MATERIAL AND METHODS

A total of 144 dogs from different races and ages, randomly chosen from 8 regions in the country, different clinics and animal asylum houses were studied. According to the gender, 80 (55,5%) were males and 64 (45,5%) females, and according to the origin 87 (60,4%) were from urban and 57 (39,5%) from rural areas. From each animal, blood samples were taken in order to determine the seroprevalence of *E. canis* and *L. Infantum* antibodies.

Serum samples were investigate for *E. canis* antibodies using a commercial ELISA kit (SNAP® 4Dx®; IDEXX Laboratories, Inc. U.S.A.), according to the manufacturer's manual. The commercially available in-clinic ELISA detects antibodies against peptides from p30 and p30-1 outer membrane immunodominant proteins of *E. canis* [2]. Reported sensitivities/specificities of the SNAP® 4Dx® for *E. canis* is 98.8%/100% [3].

L. ifantum antibodies were detected with indirect fluorescent antibody test (IFAT), in-house method. Cultured promastigotes from *L. infantum* zymodem MON-1 9MCAN/HR/2003/LLM-1282 were used to prepare antigen and were washed and fixed with cold acetone on multiwell slides [4]. Promastigotes were exposed to serial dilutions of serum in phosphate buffered saline (PBS) pH 7,2 for 30 min. at 37^{0}C, washed thoroughly and than incubate with goat-anti-dog FITC IgG (Southern-Biotech). Serial dilutions of each dog serum staring from the threshold titer of 1:40 were examined, until a visual end-point was reached. A cut off titer ≥1:80 was taken as a positive result.

RESULTS

As it is shown in a Table 1, the co-infection with both diseases is found in 6 regions in the country, in both genders, in different breeds and in relatively large range of age (2 - 9 years).

While 29 (18,7%) dogs showed positive result for the presence of *E. canis* antibodies, a total of 50 (34,7%) dogs showed positive result on leishmania infection above the cut-off point. Co-infection with both agents was found in 13 animals (16,4%) although the chronic canine ehrlichiosis can coexists with other diseases such as leishmaniosis in significantly higher rates (49%) [5]. The titer of positive dogs for *L. infantum* varied from 1:160 to 1:20480 (Table 1). There was no significant difference among titers of positive dogs with leishmaniasis only and the co-infected dogs with ehrlichia.

Table 1. *Distribution of co-infected dogs according to country region, gender, breed and age*

Epizootiological region	*E. canis* and *L.infantum* positive co-infected dogs			
	Gender	*Breed*	*Age (years)*	*Titer of L. infantum positive dogs*
Gevgelija	male	mix breed	3	2560
Skopje	male	mix breed	7	640
Strumica	male	three colored dogs	2,5	640
Strumica	female	mix breed	2	160
Tetovo	female	mix breed	3	20480
Tetovo	female	Schnauzer	3	20480
Veles	female	Samoyed	9	320
Veles	female	German shepherd	8	320
Veles	male	Posavian hound	5	10240
Veles	male	mix breed	5	5120
Probistip	male	mix breed	2	640
Probistip	male	mix breed	3	160
Probistip	male	Posavian hound	2	160

CONCLUSIONS

The coexistence of the ehrlichiosis and leishmaniasis in dogs in R. Macedonia has been detected across the all of the territory of R. of Macedonia.

The dogs infected were of genders, different breeds and all age categories.

Although the percentage of co-infected dogs was not very high (16,4%) still it demonstrate that when the clinician have a suspicion for ehrlichiosis or leishmaniasis in dogs, it can be very beneficial to use simultaneously serological tests which will clearly distinguish antibodies from both pathogens and help in establishment of final and accurate diagnosis of disease(s).

REFERENCES

1. Mancianti, F., Pedonese, F. and Poli, A. Evaluation of dot enzyme-linked immunosorbent assay (dot-ELISA) for the serodiagnosis of canine leishmaniosis as compared with indirect immunofluorescence assay. Veterinary Parasitology, v. 65, n. 1, p.1-9, 1996.
2. O'Connor, T.P., Hanscom, J.L., Hegarty, B.C., Groat, R.G. and Breitschwerdt, E.B. Comparison of an indirect immunofluorescence assay, western blot analysis, and a commercially available ELISA for detection of Ehrlichia canis antibodies in canine sera. Am J Vet Res 2006, 67:206-210.
3. Chandrashekar, R., Mainville, C.A., Beall, M.J., O'Connor, T., Eberts, M.D., Alleman, A.R., Gaunt, S.D. and Breitschwerdt, E.B. Performance of a commercially available in-clinic ELISA for the detection of antibodies against Anaplasma phagocytophilum, Ehrlichia canis, and Borrelia burgdorferi and Dirofilaria immitis antigen in dogs. Am J Vet Res 2010, 71:1443-1450.
4. Mancianti, F. and Maciani, N., 1988. Specific serodiagnosis of canine leishmaniasis by indirect immunofluorescence, indirect hemmaglutination and counterimmunoelectrophoresis. Am. J.Vet. Res., 49: 1409-1411.
5. Sanchez Visconti, G. and Tesouro Diez, M. (Eds.), 2001. Ehrlichiosis. Canis et Felis. San Román, Madrid, pp. 49–56.

СЕРОЛОШКО ДОКАЖУВАЊЕ НА КОИНФЕКЦИЈА ПОМЕЃУ EHRLICHIA CANIS И LEISHMANIA INFANTUM КАЈ ПРИРОДНО ИЗЛОЖЕНИТЕ КУЧИЊА НА ТЕРИТОРИЈАТА НА РЕПУБЛИКА МАЕКДОНИЈА

Стефановска Јована[1], Кочевкси Зоран[1], Искра Цветковиќ[2], Фаркаш Роберт[2], Иванчо Налетоски[4], Мреношки Славчо[2]

[1] Одделение за паразитологија и паразитарни заболувања, Факултет за ветеринарна медицина во Скопје,
[2] Оддделнеие за микробиологија и иммунологија, Факултет за ветеринарна медицина во Скопје,
[3]Факултет за ветеринарни науки Сент Иштван универзитет, Будимпешта, Унгарија
[4]Интернационална агенција за атомска енергија, Животниско производство и одделние за здравје,со единета ФАО/ ИАЕА Дивизија Виена, Австрија

АПСТРАКТ

Ehrlichia canis и *Leishmania infantum* се векторски преносливи болести кои се присутни и на територијат на Република Македонија. Клиничките и хематолошките знаци на овие болест се слични и воспоставувањето на диференцијална дијагноза понекогаш може да биде проблематично, со оглед на тоа што антителата против *E. canis* вкрстено-реагираат со антигенот на *Leishmania sp*. присутен во рутинските серолошки тестови кои се користат за откривање на кучињата инфицирани со *Leishmania sp*. Целта на оваа студија беше да се дефинира бројот на ко-инфекции на овие две болести преку присуството на специфични антитела кај кучињата во Р. Македонија. Тестирани се вкупно 114 кучешки серуми на присуство на антитела против *E. canis*, со користење на комерцијален *ELISA* кит *(SNAP® 4Dx®; IDEXX Laboratories, Inc. U.S.A.)*, и на присуство на антитела против *Leishmania*, со употреба на индиректна имунофлуоресценција (*in-house* метод). Вкупниот број на позитивни мостри серум беше 79, а преклопување (присуство на антителата против *E. canis* и оние против *L. infantum*) е детектирано кај 13 од нив (16,4%). Овие наоди упатуваат на тоа дека кога постои сомнеж на присуство на ерлихиоза или лајшманијаза кај кучињата, од голема корист може да биде симултаната апликација на серолошките тестови кои овозможуваат коректна дијагноза на инфекцијата.

Клучни зборови: Куче, ерлихиоза, лајшманијаза, ко-инфекција.

UDC: 636.7.09:616-089.5

COMPARISON OF THE ANESTHETIC EFFECTS OF XYLAZINE/KETAMINE, PROPOFOL AND ZOLETIL IN DOGS

Ilievska Ksenija[1], Trenkoska-Spasovska Pandorce[2], Trojacanec Plamen[1]

[1] Department of Surgery, orthopedics and ophthalmology, Faculty of Veterinary medicine, Skopje, Macedonia
[2] Veterinary Clinic, Dr. Naletoski, Skopje, Macedonia

ABSTRACT
The objective of this study was to evaluate the anesthetic effect of ketamine/xylazine combination, propofol and zoletil in 15 dogs of similar age and body weight. The dogs from the first and second group were premedicated with acepromazine (0.2 mg/kg, i/m) and xylazine (2 mg/kg) respectively, followed by single intravenous injection of combination of xylazine (1.0 mg/kg) and ketamine (10 mg/kg) (group 1) and intermittent bolus of propofol (5 mg/kg) (group 2) for induction and maintenance of general anesthesia. Induction and maintenance of anesthesia in the group 3 was achieved by intravenous injection of zoletil (7 mg/kg) 15 minutes after subcutaneous application of atropine (0,05 mg/kg). Time of induction of anesthesia, respiratory and heart rate, rectal temperature as well as oxygene saturation, were recorded at the time of induction and during the maintenance of anesthesia. Duration of the induction period in the first group had a mean value of 5,5 minutes while the dogs from the second group, induced with propofol had significantly shorter time of induction (1,5 minutes), whereas mean value of the induction time in group 3 was 3 minutes. The duration of anesthetic period had mean values of 37, 8 minutes; 8,0 minutes and 23,5 minutes in group 1, group 2 and group 3, respectively. Satisfactory levels of analgesia and muscular relaxation were achieved in all dogs. Recovery period in the group 1 was longer compared to groups 2 and 3. The results demonstrate that these anesthetic combinations despite the similar anesthetic effect during the anesthetic period had significant differences in induction and anesthetic period.
Key words: anesthesia, propofol, ketamine, xylazine, zoletil

INTRODUCTION

Anesthesia is defined as a reversible depression of CNS that provides immobilization, loss of sensation, analgesia and muscle relaxation. According to the type of the drug and the route of drug administration, general anesthesia is mainly classified as injectable (intramuscular, subcutaneous or intravenous injection) or inhalation anesthesia (Waelbers et al. 2009). There is no ideal anesthetic drug that alone provides all positive effects of general anesthesia. In order to provide adequate anesthesia, combination of two or more anesthetic drugs, defined as balanced anesthesia, are widely used in small animal practice (Demirkan et al. 2002). Premedication drugs aim to: induce sedation, provide adequate analgesia, decrease salivation and airway secretion, decrease potential dangerous effects of the anesthetic drugs, prevents vomiting and regurgitation and to provide adequate induction and smooth recovery from general anesthesia (Thurmon, 1996). Combinations of tranquilizers, sedatives and dissociative anesthetics are commonly used in small animal practice due to low equipment costs and availability. Tranquilizers are used for relief from anxiety, decrease of anesthetic drug dose, preventing vomits and secure smoother recovery (Thurmon 1996; Seymor and Gleed, 1999).

Acepromazine maleate is one of the most commonly used phenothiazine tranquilizer in small animal practice. Tranquilization, antiemetic and antihistaminic properties, sedation, decreased adrenaline-induced ventricular arrhythmias are some of the desirable effects of acepromazine (Seymor and Gleed, 1999). However, acepromazine has no analgesic effect, induces hypotension, hypothermia, bradicardia and respiratory depression (especially in brachiocephalic breeds). Recommended doses for acepromazine is 0,03-0,2 mg/kg for i/m, i/v and s/c injection. Xylazine is an α_2 adrenoreceptor agonist that produces analgesia, muscle relaxation, sedation and anxiolysis (Seymor and Gleed, 1999). As premedication drug, α_2 adrenoreceptor antagonists can reduce the dose of the injectable or inhalation anesthetic drug up to 50 %. Recommended dose for xylazine is 0,25-1,0 mg/kg i/m or i/v injection with onset of action between 10-15 minutes after intramuscular injection and 3-5 minutes after intravenous injection (Thurmon, 1996; Seymor and Gleed, 1999). After application, analgesia is provided for 15-30 minutes whereas sedation is prolonged for 1-2 hours (Demirkane et al., 2002). Xylazine as other α_2 agonists can provide adequate analgesia, muscle relaxation and chemical restraint, but also may cause bradicardia, hypothermia, vomiting, decreased cardiac output and other haemodynamic disturbances (Demirkane et al. 2002). Xylazine should not be used during pregnancy since it can increase uterine tonus. Xylazine is compatible for use in combination with buprenorphine, butorphanol and ketamine.

Ketamine hydrochloride is a dissociative anesthetic drug that interrupts ascending transmission from the unconscious to conscious parts of the brain (Thurmon, 1996; Waelbers et al., 2009). The anesthesia produced by ketamine is characterized by cataleptic state in which

the eyes remain open. Ketamine is fast acting anesthetic drug that provides good analgesia, normal pharyngeal-laryngeal reflexes, absence of muscle relaxation, restless recovery, convulsion in dogs, abnormal behavior in post-anesthetic period, dose related cardiovascular effects and minimal respiratory depression (Hellebrekers, 1996; Waelbers et al., 2009; Sindak et al., 2010). Initial decreasing of respiratory and minute volume are noticed after single bolus of ketamin, but both parameters returns to baseline values within 15 minutes (Haskins et al., 1985). Respiratory depression with so called breath-holding or "apneustic" breathing is also one of the characteristic of ketamine (Seymor and Gleed, 1999). In order to reduce or eliminate the side effects, combinations of ketamine and tranquilizers or sedative drugs are commonly used in small animal practice. The combination with α_2 adrenergic agonist produces good general anesthesia, improves muscle relaxation and eliminates convulsion during recovery and it has also been used for immobilization of wild and captive carnivores, (Hall et al. 2001; Sindak et al. 2010). Single bolus of ketamine/xylazine combination at 2-10 mg/kg and 0,7-1,0 mg/kg, respectively, by i/m or i/v injection provides 20-40 minutes of surgical anesthesia (Thurmon, 1996).

Zoletil is a combination of dissociative anesthetic - Tiletamine hydrochloride and benzodiazepine tranquilizer - Zolazepam hydrochloride. Tiletamine has greater analgesic effect and longer duration of action and is more potent than ketamine whereas zolazepam plays role as an anticonvulsant and muscle relaxation drug. Combination of tiletamine/zolazepam (Zoletil or Telazol) induces tachycardia dose related respiratory depression and irregular breathing (Seymor and Gleed, 1999). Low, but clinical effective doses have minimal effect on respiration (Thurmon, 1996). Salivation is controlled with previous application of anticholinergic drugs (atropine 0,05 mg/kg s/c or glycopirolate 0,01 mg/kg i/m or s/c). Temporary hypothermia is one of the additional side effects of Zoletil. Desirable effects of zoletil are fast onset of surgical anesthesia (30-60 seconds following intravenous injection and 5-8 minutes following intramuscular injection), no hepatic or renal toxicity, good analgesia, muscle relaxation and smooth recovery (Thurmon, 1996). Recommended doses varies between 2,0-8,0 mg/kg i/m or i/v injection. Combination of tiletamine-zolazepam-ketamine-xylazine has been used in feral cats for elective procedures (Williams et al., 2002).

Propofol is injectable, short acting, fast metabolizing anesthetic drug, chemically unrelated to barbiturates or other anesthetic drugs (Tsai et al., 2008). Rapid onset and short duration of action is due to rapid uptake to CNS and redistribution from the brain to other tissues (Thurmon, 1996). Propofol is sedative-hypnotic drug with minimal analgesic effect used for induction and maintenance of general anesthesia by single bolus or continuous infusion (Grimm et al., 2001). Abscence of excitation during induction and recovery, rapid onset of anesthesia, respiratory and cardiovascular depression, rapid and smooth recovery are some of the desirable effects induced by propofol (Seymor and Gleed, 1999). Apnea and bradicardia are usually related to the speed of injection of the drug – the slower the injection, the lower the incident of apnea and bradycardia. Induction dose in unpremedicated dogs ranges between 4-6 mg/kg i/v whereas the dose in premedicated patient is reduced to 3-4 mg/kg (Thurmon, 1996; Seymor and Gleed, 1999).

The objective of this study was to compare the anesthetic effects of ketamin/xylazine combination, propofol and zoletil for elective surgical procedures in small animal practice.

MATERIAL AND METHODS

The study was carried out on 15 dogs, at similar age and body weight undergone elective surgical procedure (OVH). All patients were in good health condition. The dogs were fasted for 12 hours, without water restriction. According to their physical status the patients were categorized as ASA 1. After the clinical examination, dogs were divided in three groups (5 dogs in each group). Patients were premedicated by i/m injection of 0,2 mg/kg Acepromazine maleate (Castran, Interchemie, Holland) in group 1 (Ket./Xyl.) and 2 mg/kg Xylazine (Xyla 2%, Interchemie, Holland) in group 2 (Prop.). Subcutaneous injection of atropine sulfate 0,05 mg/kg was administered 15 minutes prior induction in the group 3 (Til./Zol.). An intravenous 22G catheter was placed into the cephalic vein in all dogs for administration of the anesthetic drug. Combination of 1 mg/kg xylazine and 10 mg/kg ketamine for induction and maintenance of anesthesia was applied by i/v injection in dogs from the group 1. In the group 2, 5mg/kg Propofol (Braun, Melsungen) was administered for induction, while maintenance of general anesthesia was achieved by intermittent boluses of 1/2 of induction dose of propofol. Induction and maintenance of anesthesia in the group 3 was carried out by 8 mg/kg Tiletamine/zolazepam (Zoletil 50, Virbac) i/m, approximately 15 minutes after administration of atropine and continues with 4 mg/kg by i/v injection. All patients were intubated after induction of surgical anesthesia but no oxygen was administered in order to compare the influence of different anesthetic procedures on oxygen saturation.

Evaluation of the clinical effects of all combinations was graded from + - ++++ according to Atalan et al. (2002) whereas +- refers to the dogs with preserved reflexes and ++++ - refers to the dogs unresponsive to all reflexes, absents of occulopalpebral reflex and good muscle relaxation.

Time of induction (period from application to clinical effect), anesthetic period and recovery period (period from extubation to sternal recumbence) were recorded. Also, heart rate, respiratory rate, oxygen saturation and pupillary reflex were monitored before, at time of induction and every 15 minutes until extubation. Oxygen saturation (pO_2) was monitored by lingual probe connected to patient monitor (Mindray MEC-1200Vet, China) from induction until resumption of lingual movement. In order to provide satisfacory analgesia in group 2, 1 mg/kg i/m ketoprofen (Ketonal, Lek) was administrated. Analgesia in all three groups was assessed by classic needle prick in the paws. Absence of limb withdrawal or any limb movement was considered as "yes" analgesia.

RESULTS AND DISCUSSION

Times of induction and duration of anesthesia as well as other clinical parameters are shown in Tables 1 and 2.

Table 1. *Mean values of duration of induction, anesthetic period and analgesia (minutes)*

Group	***Induction***	***Anesthetic period***	***Analgesia with needle prick***
Ket./Xyl.	5,5±1,0	37,8±7,5	45±3,2
Prop.	1,5±0,5	8,0±4,0	No analgesia
Til./Zol.	3,0±0,8	23,5±3,2	33±2,6

Table 2. *Mean values of heart rate, respiratory rate and recovery time (minutes)*

Group	***Heart rate***	***Respiratory rate***	***Recovery time***	***Clinical effect of anesthesia***
Ket./Xyl.	142	22	140±12,5	++++
Prop.	108	25	42±5.5	+++
Til./Zol.	98	20	110±6,2	++++

Beginning of sedation period varies between the groups. Rapid sedation was achieved in the group Prop. after i/m administration of 2 mg/kg xylazine unlike the group Ket./Xyl., where light sedation with acepromazine was achieved. During the sedation period, vomiting reflex was observed in two dogs from the group Prop.

All the anesthetic combination enabled rapid and smooth induction of anesthesia, however significant differences in the induction time were detected between the Ket./Xyl. group versus Prop. and Til./Zol groups. Increased muscle tone and spontaneous movement were noticed in one dog from the group Ket./Xyl during induction.

Mean duration of the anesthetic period in the Ket./Xyl. group (37,8 ±7,5) was longer than the Prop. group and Til./Zol group. (8,0±4,0 and 23,5±3,2, respectively). Significant differences in recovery period were also recorded. The most rapid recovery was detected in Prop. group (42±5.5), while longer recovery was recorded in the Ket./Xyl. group (140±12,5). Muscle twitches, slightly increased muscle tone and rolling with the tongue were recorded in the group Til./Zol., during the recovery. Dogs from the Prop. group, had more attempts from lateral to sternal recumbency than other groups. Duration of analgesia or lack of response to needle prick was shorter in the Til./Zol. (33±2,6) compared to the Ket./Xyl group (45±3,2). Mild reaction to needle prick was noticed in Prop group.

Respiratory depression, apnea and bradycardia were recorded in 3 dogs from the Prop. group, while as slightly increased heart rate and arrhythmia were noticed in all dogs from the Ket./Xyl. Group. Rectal temperature decreased at 15-20 min after the induction for 0,2-0,5^{0}C. (mean measured temperature was 36,5^{0}C in all patients). Initial respiratory depression (apnea) occurs in all dogs from the Ket./Xyl group after bolus injection. Atropin was administered only in the Til./Zol. group to prevent bradycardia. The eyes remained open in all dogs from the Ket./Xyl group.

Mean values of pO2 varied between 75-82% during the surgery in all groups. The lowest pO2 was recorded in Ket./Xyl group (68%) in the first 15 minutes after induction with tendency for stabilization to ~80% in the following period. The patients in the other groups had constant pO2 during the whole anesthetic period (78 ± 3.5%), however, still lower than the desirable level for normal tissue oxygenation (>95%).

The clinical anesthetic effect was satisfactory in all three groups with minor drop in the Prop. group.

In order to avoid undesirable effect of ketamin (convulsions, increased muscle tone, excitation during recovery and movements) combination of α_2 agonist such as xylazine HCL has been wildly used for immobilization of wild and domestic animals. Despite the findings of Sindak et al. (2010) that ketamine-xylazine combinations induces arrhythmia and bradicardia, none of the dogs from the Ket./Xyl group revealed bradycardia, however several occasions of dysrhytmia were noticed. Xylazine/ketamine combination induced profound anesthesia (37,8 min.) similar to Atalan et al. (2002). Recovery time during xylazine/ketamine combination from extubation to walking reported by Atalan et al. (2002) was shorter than that measured in Ket./Xyl. group.

Adequate analgesia was achieved in Ket./Xyl and Til./Zol. groups, tested byb a needle prick. When propofol was used as a single drug for induction and maintenance of anesthesia during painful procedures, administration of analgetic drug was required. Additional analgesia was provided in the group Prop. by i/m application of NSAID. Despite the recommended dose of 2-4 mg/kg in sedated animals, in our study 5 mg/kg propofol was administered in previously premedicated dogs without any undesirable effects.

CONCLUSIONS

Intravenous anesthesia is an excellent alternative for inhalation anesthesia and it has been wildly used in small animal practice. The results reported in the study indicate that all three anesthetic combinations provide rapid induction and smooth recovery from anesthesia. Acepromazine as a premedication drug induces relatively poor sedation compared to xylazine. Reliable analgesia was achieved in group Ket./Xyl and group Til./Zol., but due to the lack of analgesic properties of propofol additional application of ketoprofen was needed in the group Prop. for acceptable level of analgesia. The longest period of surgical anesthesia after single bolus dose was resorded when ketamin/xylazine combination was used. Despite the short duration of surgical procedures (30-45 min), the shortest recovery time was observed when propofol was used. All animals, subjected to any of the proposed anesthetic procedures, must be continuously

oxygenated via intubation or face mask, in order to attain adequate tissue oxygen saturation during surgery.

REFERENCES

1. Atalan G., Deemirkan I., Günes V., Cihan M., Celebi F., Citil M. (2002): Comparison of Xylazine+Ketamine HCL Anesthetic agnets wit Acepromazine +Butorphanol+Ketamine Combinations for their Clinical and Cardiorespiratory Effects in dogs, Veteriner Cerrahi Dergisi 8 (3-4), 35-40;
2. Demirkan I., Atalan G., Gokce H.I., Ozaydin I., Celebi F. (2002): Comparative study of Butorphanol-Ketamin and Xylazine-Ketamin HCL Combinations for Their Clinical and Cardiovascular/Respiratory Effects in Healthy Dogs, Turkish Journal of Veterinary Animal Science, 26, 1073-1079;
3. Grimm K.A., Thurmon T.S., Tranquilli W.J., Benson G.J., Greene S.A. (2001): Anesthetic and Cardiopulmonary Effects of Propofol in Dogs Premedicated with Atropine, Butorphanol, and Medetomidine, Veterinary Therapeutics, vol. 2 (1), 1-9;
4. Hall L.W., Clarke K.W., Trim C.M. (2001): Veterinary Anesthesia, 10 edition, Saunders
5. Hellebrekers L.J. (1996): Injection anesthesia in dogs and cats, Veterinary Quarterly, 18 sup.1, 62-63;
6. Seymor C. and Gleed R. (1999): Manual of Small animal Anesthesia and Analgesia, BSAVA;
7. Sindak N., Camkerten I., Ceylan C. (2010): Clinical Evaluation of Ketamine-Xylazine Anesthesia in Bozova Greyhounds, Journal of Animal and Veterinary Advances, 9 (15), 2025-2029;
8. Thurmon J.C., Tranquilli W.J., Benson G.J. (1996): Lumb & Jones Veterinary Anesthesia, Williams and Wilkins, third edition;
9. Tsai Y.C., Wang L.Y., Yeh L.S. (2008): A Clinical Comparative Study of Propofol Total Intravenous Anesthesia in Dogs, Journal of Veterinary clinical Science, vol. 1(2), 52-60;
10. Waelbers T., Vermoere P., Polis I. (2009): Total intravenous anesthesia in dogs, Vlaams Diergeneeskunding Tijdschrift, vol. 78, 160-169;
11. Wiliams L.S., Levy J.K., Robertson S.A., Cistola A.M., Centonze L.A. (2002): Use of the anesthetic combination of tiletamine, zolazepam, ketamine and xylazine for neutering feral cats, JAVMA, vol. 220, 1491-1495.

СПОРЕДБА НА АНЕСТЕТСКИТЕ ЕФЕКТИ НА КСИЛАЗИН/КЕТАМИН, ПРОПОФОЛ И ЗОЛЕТИЛ КАЈ КУЧИЊА

Илиевска Ксенија[1], Тренкоска-Спасовска Пандорче[2], Тројачанец Пламен[1]

[1]Катедра по Хирургија, ортопедија и офталмологија, Факултет за ветеринарна медицина, Скопје, Македонија
[2] Ветеринарна амбуланта Др. Налетоски, Скопје, Македонија

АПСТРАКТ

Целта на овој труд беше да се процени анестетскиот ефект на ксилазин/кетамин, пропофол и золетил кај 15 кучиња, на слична возраст и телесна тежина. Кучињата од првата и втората група беа премедицирани со ацепромазин (0,2 мг/кг и/м) односно ксилазин (2 мг/кг), проследена со единечна интравенска апликација на комбинација на ксилазин (1 мг/кг) и кетамин (10 мг/кг) (група 1) и интермитентен болус на пропофол (5 мг/кг) (група 2) за индукција и одржување на општата анестезија. Индукцијата и одржување на анестезијата во група 3 беше постигната со интравенска апликација на золетил (7 мг/кг), 15 минути по субкутаната апликација на атропин (0,05 мг/кг). Времето на индукција на анестезија, респирации и пулс, телесната температура како и сатурацијата со кислород беа мерени од почетокот на индукцијата и во текот на општата анестезија. Времетраењето на периодот на индукција во група 1 имаше средна вредност од 5,5 минути додека кучињата од втората група кај кои индукцијата беше изведена со пропофол имаа значително пократко време на индукција (1,5 минути), а пак во група 3 средната вредност на периодот на индукција изнесуваше 3 минути. Времетраењето на анестетскиот период имаше средна вредност од 37,8 минути; 8,0 минути и 23,5 минути во група 1, група 2 и група3. Кај сите кучиња беше постигнато задоволително ниво на аналгезија и мускулна релаксација. Периодот на будење во група 1 беше подолг во споредба со групите 2 и 3. Добиените резултати укажуваат дека овие анестетски комбинации и покрај сличниот ефект постигнат во тек на анестетскиот период имаа значителни разлики во тек на идукцијата и анестетскиот период.

Клучни зборови: анестезија, пропофол, кетамин, ксилазин, золетил

UDC: 636.7.09:616.153.1:[616-022.7:579.861.2

CREATINE KINASE ACTIVITY IN DOGS WITH EXPERIMENTALLY INDUCED STAPHYLOCOCCUS AUREUS INFECTION

Zapryanova Dimitrinka[1], Mircheva Teodora[1], Lalev Damyan[2]

[1]*Department of Pharmacology, Animal Physiology and Physiological Chemistry, Trakia University-Faculty of Veterinary Medicine, Stara Zagora, Bulgaria*
[2]*Research Institute of Mountain Stockbreeding and Agriculture, Troyan, Bulgaria*

ABSTRACT
In the dog, creatine kinase (CK) is mostly present in the skeletal muscles, myocardium, brain and intestine. CK release from the cells and reaches the plasma mostly via the lymphatic route and then remains in the plasma compartment. CK is an enzyme found predominantly in skeletal muscle and significantly elevated serum activity is largely associated with muscle damage. Plasma increases in dogs are associated with cell membrane leakage and will therefore be seen in any condition associated with muscular inflammation. The study was induced in 15 mongrel male dogs (n=9 in experimental group and n=6 in control group) at the age of two years and body weight 12-15 kg. The infection was reproduced by inoculation of 5 ml 24h broad culture of *Staphylococcus aureus* strain with density of $3,1x10^9$ cfu/ml. The plasma activity of creatine kinase was evaluated at 0, 6, 24, 48, 72 hours and on days 7, 14 and 21 by a kit from Hospitex Diagnostics. The aim of our study was to determine whether staphylococcal infection may cause enhancement of CK-activity in dogs. In the experimental group the plasma concentration of the CK-activity were significantly ($p<0,01$) higher at the 72 hour and on day 7 (101,6±5,92 U/L; 100,7±3,61 U/L) compared to the control group. At the end of the study (21 days) the values had returned to the initial levels – 85,5±2,65 U/L. The results of this study suggest that the evaluation of CK in an infected animals is of limited value.
Key words: creatine kinase, *Staphylococcus aureus*, dogs

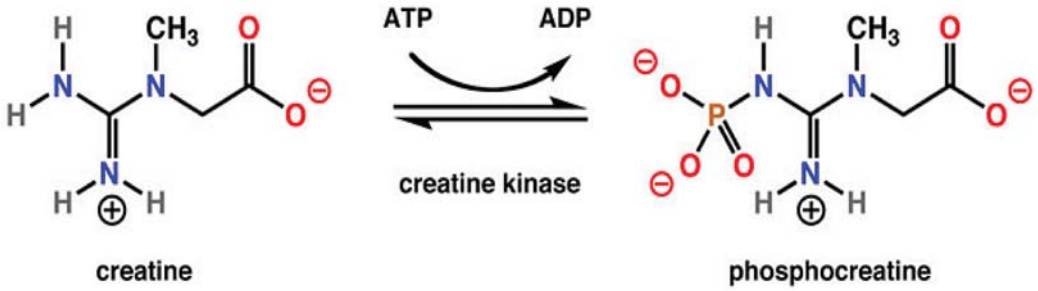
INTRODUCTION

Creatine kinase (CK) (EC 2.7.3.2) catalyses the reversible exchange of high-energy phosphate bonds between phosphocreatine (PCr) and ADP (adenosine diphosphate), regenerating ATP (adenosine triphosphate) from ADP produced during muscle contractions. The main function of the cytosolic CK is replenishment of ATP at sites of high-energy demand (Genet et al., 2000). The reaction catalyzed by CK allows energy storage as phosphocreatine when demand is low, and when energy demand increases CK enables rapid restoration of the intracellular pool of ATP necessary for muscle contraction. Three cytosolic isoenzymes have been describes: MM-muscle type; MB-heart and other tissues, and BB-brain type (Genet et al., 2000). Creatine kinase is an enzyme found predominantly in skeletal muscle and significantly elevated serum activity is largely associated with muscle damage. It is a sensitive indicator of muscle damage, but is not specific as to cause. Plasma increases in dogs are associated with cell membrane leakage and will therefore be seen in any condition associated with muscular inflammation, necrosis or degeneration (Nevill et al.,2010).

Staphylococcus aureus (*S. aureus*) is oft associated with suppurative infections and is recognized as an inherent member of the microflora of the skin of humans and dogs (Hoekstra and Paulton, 2002). Toxin-mediated diseases caused by *S. aureus* include range from cutaneous infections to infections of wounds, osteomyelitis, endocarditis, bacteremia with metastatic complications, toxic shock syndrome. The bacterial components and secreted products that affect the pathogenesis of *S. aureus* infections are numerous and include surface-associated adhesins, exoenzymes, exotoxins. According to some authors De Kimpe (1995) and Thiemermann (2002) the key elements in *S. aureus* are peptidoglycan (PepG) and lipoteichoic acid (LTA), which are a component of cell wall, synergize to cause shock and organ dysfunction. In this study were observed the clinical signs and creatine kinase activity in dogs. Inflammation, infection or trauma in dogs can induce secondary muscle involvement, which may be indicated by increased activities of the CK.

MATERIAL AND METHODS

The experiment was approved by the Ethic Com-



mittee at the Faculty of Veterinary Medicine. The experimental animals were provided by the municipality of Stara Zagora. The study was performed on 9 mongrel dogs (experimental group) and 6 mongrel dogs (control group) at the age of 2 years and body weight 12-15 kg. The dogs were housed in metal cages. They were exposed to a 12h light-dark cycle at room temperature (20-22^{0}C). They were fed a commercially available diet of dog pellet twice daily and had free access to water. Prior to the experiment the animals were vaccinated with vaccine Nobivac® DHPPiLR, Intervet International B.V and treated per oral against internal parasites with Caniverm®, Bioveta, A. S. Czech Republic, 1 tablet/10 kg B.W., and external parasites with Bolfo® Puder, Bayer, Germany. The infection was reproduced by inoculation of 5 ml 24h broth culture of *S. aureus* strain with density of 3,1x10^9 c.f.u./ml and same quantity saline in control dogs. Blood samples were collected into heparinized tubes before inoculation (hour 0) then at hours 6, 24, 48, 72 and on days 7, 14, 21. At the same time was taking blood and from controls. Heparinised blood was centrifuged (1500g, 10 minutes) within 30 min after collection. Plasma was immediately separated and stored at -20°C until analysis. Total CK-activity was determined by a kit from Hospitex Diagnostics.

The statistical analysis of the data was performed using one way analysis of variance (ANOVA). The results were processed with software Statistica v.6.1 (StatSoft Inc., 2002). All results are presented as mean and standard error of the mean (Mean ± Err). The statistical significance of parameters was determined in the LSD test at $p < 0.05$.

RESULTS

The changes in the CK concentration during the staphylococcal infection in our study are shown in fig.1 and table 1. In the experimental and control groups, total creatine kinase activities were followed during a period of 21 days. Blood creatine kinase activity were slightly influenced by staphylococcal infection. In the experimental group, initial levels (before inoculation) were 85,48±6,54 U/L and 24 hours after this, CK levels began to rise (92,97±5,48 U/L) and remained high until the 14th day (93,2±1,86 U/L) of the study compared to control values. From the 24th hour, the CK concentration showed consistent upward trend and at 48th h the mean values were 100,17±5,98 U/L. This study indicated significant differences (**p<0,01) in dogs with staphylococcal infection in comparison to the control group at 72th h and on day 7. At the 72th hour, CK levels reached significant peak elevation – 101,6±5,92 U/L in compared to the controls – 77,08±5,27 U/L. On the 7th day, creatine kinase activities remained stilll higher – 100,72±3,61 U/L to controls – 71,98±4,99 U/L.

In the dog, CK release from the cells and reaches the plasma mostly via the lymphatic route and then remains in the plasma compartment. It is rapidly cleared with a half-life of about 2 hours. Muscle disorders are the main source of plasma CK elevations, moreover sex has no influence on the plasma CK activity, which is higher in young dog than in adults (Aktas et al., 1993).

Inflammation or trauma can induce secondary muscle involvement, which may be indicated by increased activities of the CK. Other causes of cell injury are bacterial toxins, which can lead to loss of cell integrity. The mechanisms of this injury are not fully understood, but raised protein catabolism in the muscle cell is suspected (Neumann, 2005). This author demonstrates that dogs and cats with metabolic diseases have increased CK-activity. Bacterial infections involving muscle are relatively uncommon. Myositis may result from contiguous sites of infection, penetrating trauma, vascular insufficiency, or by hematogenous dissemination. The infecting organism is related to the mechanism of the infection. For instance, an acute bacterial infection of skeletal muscle that is the result of hematogenous spread is most commonly due to *Staphylococcus aureus* (Crum-Cianflone, 2008). In most cases involve only a single muscle group. The first stage occurs with local swelling, mild pain, local erythema and variable fevers are common findings in soft tissue infections. During this "invasive phase," bacteria have begun to infect the muscle.

Infection accompanied by local and general systematic signs-enhanced fever, increase heart and respiratory rates at 24th h, which are indicators for non-specific response and signs of inflammation. At the 6th h after *S. aureus* implication observed painfulness and oedema of the soft tissue. We watched at the 24th h enlargement of inguinal lymphatic nodes in the limp which was injected and reduced appetite and impaired motor activity in dogs. These clinical symptoms are observed by other authors at the 24th h after inoculation of bacteria (Georgieva et al., 2010). At this time CK activity showed upward tendency and reached levels 92,97±5,48 U/L. One of the experimental dogs had oedema on the scrotum. After 48th h, some of the dogs were purulent conjunctivitis eye infection and at this time the CK concentrations were higher (100,17±5,98 U/L) than initial levels (85,48±6,54 U/L). Giese et al. (2008) reported that in experimentally induced infection in dogs with avian influenza virus (H5N1) also occurs with conjunctivitis eye infection and elevated creatine kinase values within 24-48 hours post infection. According to these authors, the cause of the increased CK levels could have been nonspecific muscle injury. In area of the inoculation of bacteria, hair loss and appeared erosions on tissues. Some authors showed that *Staphylococcus aureus* induced infection (Georgieva et al., 2010) cause elevation in CK-activity in rabbits. They reported that the maximum levels observed at the 48th h after inoculation. In our study, at this time we observed higher values, too but CK reached a peak concentrations at the 72th h. The normal range of the total-CK activity in dog is higher than that in man (up to 84 U./l in dog, up to 50 U./l in man). On 7th day the inspection revealed the lack of oedema and pain, recovered appetite and dogs walked normally again. No clinical symptoms associated with infection were noticed although the blood CK levels remained higher (100,72 U/L).

CONCLUSION

In conclusion we think that our results show that infection caused by *Staphylococcus aureus* may induce secondary elevation of creatine kinase activity in dogs and is an indicator for muscle demage at the site of injection. In the experimental group the plasma CK concentration was significantly (p<0,01) higher than in the control group since 72 h up to day 7. These changes may be due to progression of infection and this elevation of CK-activity could be use as a marker for detection of staphylococcal infection in dogs.

REFERENCES

1. Genet S., R. K. Kale, N. Z. Baquer. Effects of free radicals on cytosolic creatine kinase activities andprotection by antioxidant enzymes and sulfhydryl compounds. *Molecular and Cellular Biochemistry* 210: 23-28, 2000.
2. Nevill B., A. Leisewitz, A. Goddard, P. Thompson. An evaluation of changes over time in serum creatine kinase activity and C-reactive protein concentration in dogs undergoing hemilaminectomy or ovariohysterectomy. *Journal of the South Africak Veterinary Assiciation* (Tydskr. S. Afr. vet. Ver.) 81(1): 22-26, 2010.
3. Hoekstra K. A. and R. J. L. Paulton. Clinical prevalence and antibicrobial succeptibility of *Staphylococcus aureus* and *Staphylococcus intermedius* in dogs. *Journal of Applied Microbiology*, 93: 406-413, 2002.
4. De Kimpe S. J., Kengatharan M., Thiemermann C., Vane J. R.: The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. Proceedings of the National Academy of Sciences USA,92; 10359-10363, 1995.
5. Thiemermann C.: Interactions between lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*: a structural and functional analysis. *Microbess and Infection* 4; 927-935, 2002.
6. Georgieva T., Petrov V., . Zapryanova D, Marutsov P., Nikiforov I., Rusenova N., Zarkov I., Penchev Georgiev I., Dinkova V. Creatin kinase activity in rabbits with *Staphylococcus aureus* infection. Book of Proceedings pp. 126-127, 14th International conference on production diseases in farm animals (ICPD), 20-24 June 2010, Ghent, Belgium.
7. Giese M., T. C. Harder, J. P. Teifke, R. Klopfleisch, A. Breithaupt, T. C. Mettenleiter, T. W. Vahlenkamp. Experimental infection and natural contact exposure of dogs with avian influenza virus (H5N1). *Emerging Infectious Diseases*, vol. 14, №2, 308-310, 2008.
8. Aktas M., D. Auguste, H. P. Lefebvre, P. L. Toutain, J. P. Braun. Creatine kinase in the dog: a review. *Veterinary Research Communications*, 17: 353-369, 1993.
9. Neumann S. Serum creatine kinase activity in dogs and cats with metabolic diseases. *Deutsche Tierarztliche Wochenschrift*, 112(9): 343-347, 2005.
10. Crum-Cianflone N. F. Bacterial, fungal, parasitic, and viral myositis. *Clinical Microbiology Reviews*, 21(3): 473-494, 2008.

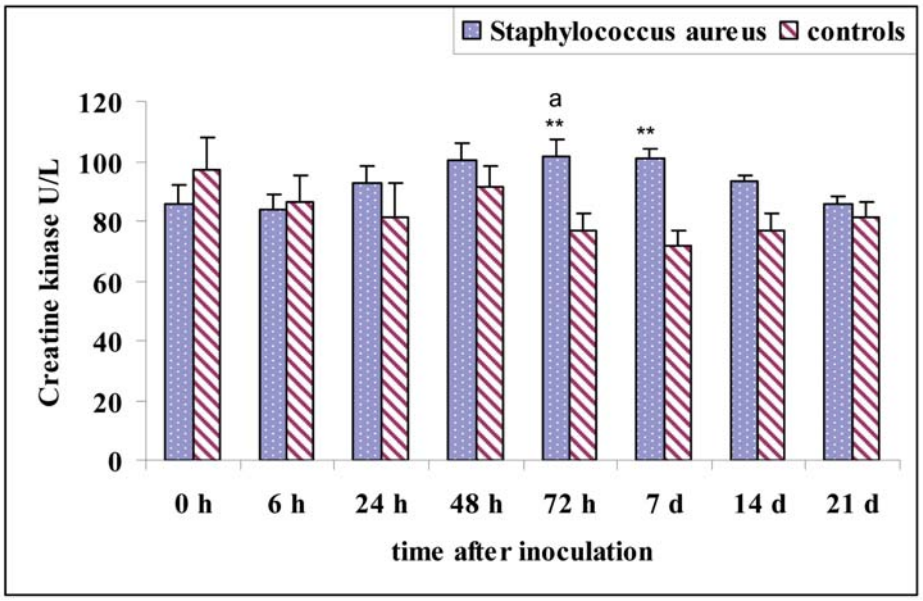


Figure 1. *Blood creatine kinase activities (U/L) in dogs with experimental infection induced by Staphylococcus aureus (3,1x10^9 cfu/ml) (mean±SE)*

*** - compared with the control group p<0,01; a – from baseline (0 hour)*

Table 1. *Total CK-activity in experimental and control dogs*

Time after inoculation	Infected dogs (n=9) means±SE	Non-infected dogs (n=6) means±SE
0 h	85,48 ± 6,54	97,26 ± 10,8
6 h	83,57 ± 5,11	86,36 ± 9,00
24 h	92,97 ± 5,48	81,48 ± 11,2
48 h	100,17 ± 5,98	91,46 ± 7,05
72 h	101,60 ± 5,92**;a	77,08 ± 5,27
Day 7	100,72 ± 3,61**	71,98 ± 4,99
Day 14	93,2 ± 1,86	77,11 ± 5,1
Day 21	85,55 ± 2,65	81,45 ± 4,74

*** - compared with the control group p<0,01; a – from baseline (0 hour)*

АКТИВНОСТА НА КРЕАТИН КИНАЗАТА КАЈ КУЧИЊА СО ЕКСПЕРИМЕНТАЛНО ИНДУЦИРАНА ИНФЕКЦИЈА СО STAPHYLOCOCCUS AUREUS

Заприанова Димитринка[1], Мирчева Теодора[1], Лалев Дамиан[2]

[1]Катедра за Фармакологија, Анимална физиологија и Физиолошка хемија, Тракиски Универзитет, Факултет за ветеринарна медицина, Стара Загора, Бугарија
[2]Истражувачки институт за планинско сточарење и земјоделие, Тројан, Бугарија

АПСТРАКТ
Кај кучињата креатин киназата (CK) е најмногу застапена во скелетните мускули, миокардот, мозокот и цревото. CK се ослободува од клетките и достигнува во плазмата најчесто преку лимфните патишта и останува во плазминиот оддел. CK преставува ензим кој претежно се наоѓа во скелетмата мускулатура и сигнификантното покачување во серумот значително е поврзано со мускулните оштетувања. Зголемувањето на нивото во плазмата кај кучиња е поврзано со губење на клеточната мембрана и поради тоа би била присутна при секоја состојба каде е присутна мускулна инфламација. Истражувањето беше спроведено кај 15 кучиња мелези (n=9 во експерименталната група и n-6 во контролната група)на возраст од 2 години и телесна тежина од12-15кг. Инфекцијата беше предизвикана со инокулација на 5 мл 24 ч широка култура na *Staphylococcus aureus* coj со густина од 3,1x10^9 cfu/ml. Плазматската активност на креатин киназата беше проценувана на0, 6, 24, 48, 72 часа и на 7, 14 и 21 ден со кит на Hospiteks Dijagnostik. Целта на ова истражување беше да се детерминира дали стафилококната инфекција може да предизвика зголемување на CK-активноста кај кучињата. Во експерименталната група плазма концентрацијата на CK-активноста беше сигнификантно (p<0,01) повисока на 72 часа и на 7 ден (101,6±5,92 U/L; 100,7±3,61 U/L) споредбено со контролната група. На крајот од истражувањето (21 ден) вредностите се вратија на почетните нивоа-85,5±2,65 U/L. Резултатите од ова истражување сугестираат дека проценката на CK кај инфицираните животни е со ограничена вредност.

Клучни зборови: креатин киназата, *Staphylococcus aureus,* кучиња

UDC: 636.2.034.09:616.36-008.847.9

EFFECT OF NEGATIVE ENERGY BALANCE ON IGF SYSTEM IN DAIRY COWS.

Kirovski Danijela[1], Šamanc Horea[2], Vujanac Ivan[2], Prodanović Radiša[2], Đurić Miloje[3], Sladojević Željko[4]

[1]*Department of Physiology and Biochemistry, Faculty of Veterinary Medicine University of Belgrade, Belgrade, Serbia*
[2]*Department of Farm Animal Disease, Faculty of Veterinary Medicine University of Belgrade, Belgrade, Serbia*
[3]*Department of reproduction, fertility and artificial insemination, Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia*
[4]*Veterinary Station „Veterina system Sladojević", Gradište, Bosna and Herzegovina, Republic of Srpska*

ABSTRACT
The objective of this study was to examine the effect of negative energy balance on IGF system in dairy cows. Sixteen puerperal cows (few days after calving) were chosen from the commercial dairy herd and placed in the study. On day 12 after calving, liver percutaneous biopsies were obtained. Lipid content in liver tissue was determined. Cows were divided into two groups of equal size based on the degree of lipid accumulation in the liver: cows with mild fatty liver (less than 20% fat) and cows with moderate to severe fatty liver (more than 20% fat). Three blood samples were taken from each animal: the first 12 days after calving, second 30 days after calving and third 60 days after calving. IGF-I, IGFBP-2, IGFBP-3 and IGFBP-4 in the blood serum were measured. IGF-I concentration was significantly lower in cows with mild compared to cows with moderate to severe fatty liver at all three examined periods. Relative abundance of IGFBP-2 was significantly higher in cows with moderate to severe compared to cows with mild fat liver at day 12 after calving. Thereafter there was no difference in abundance of IGFBP-2 among two groups of cows. Relative abundance of IGFBP-3 was significantly lower in cows with moderate to severe fatty liver compared to cows with mild fatty liver at all three examined periods. Relative abundance of IGFBP-4 was lower in cows with moderate to severe compared to cows with mild fat liver during all examined period, but the difference was significant only at day 60 after calving. In conclusion, cows that suffer from moderate to severe fatty liver have more pronounced negative energy balance which, according to our results, affects hepatic synthesis of IGF-I, and other components known to modulate the bioavailability and stability of circulating IGF-I.
Key words: fatty liver, energy balance, IGF system

INTRODUCTION

High-yielding dairy cows cannot consume sufficient nutrients in early lactation to support the level of milk yield. Peak milk production, at about 8 to 10 weeks postpartum, occurs earlier than maximum energy intake, causing cows to be in negative energy balance (NEB). To compensate for the NEB, the dairy cow mobilizes body fat reserves in the form of non-esterified fatty acids (NEFA). Mobilized NEFA are taken up mainly by the liver and are either oxidized in the mitochondria to produce energy or exported in the form of TAG-rich very low density lipoproteins (VLDL). When the uptake of NEFA by the liver exceeds their disposal through oxidation or export as VLDL, fatty liver syndrome develops to different extend (Grummer, 1993). Fatty liver usually provokes other metabolic diseases and reproductive problems in lactating cows that are initially derived from a state of NEB during early lactation period.

During the post partum period of NEB, substantial changes in endocrine system also occur, characterized by an increase in growth hormone and a decrease in insulin, thyroid hormones and insulin like growth factor-I (IGF-I). Blood IGF-I is bound to specific binding proteins (IGFBPs) which affect the transport and bioactivity of IGF-I and its half life in blood plasma (McGuire et al., 1992). Use of Western ligand blot analyses has shown that IGFBPs migrate with apparent molecular masses of 25, 28, 35 and 45 – 53 kDa for IGFBPs 4, 1, 2 and 3 respectively (Cohick et al. 1992; Kirovski et al., 2008). Studies have demonstrated that IGFBP-3 binds most of the immunoreactive IGF-I (Baxter and Martin, 1989) and is regulated by growth hormone and IGF-I itself (Zapf et al. 1989). Simmons et al (1994) reported an increase in IGFBP-2 levels just after parturition while IGFBP-3 remains lower. These variations in hormones and their binding proteins at late dry and early lactation periods in high yielding dairy cows reflect the variation in energy balance. There are evidence of the link of NEB and decline in fertility as well as reduced concentrations of IGF-I (Taylor et al., 2005).

The objective of this study was to examine the influence of negative energy balance on the IGF system as one of the main indicators of energy status of early lactation dairy cows.

MATERIALS AND METHODS

Sixteen puerperal cows (few days after calving) were chosen from the commercial dairy herd and included in the study. Cows were kept in tie-stall barn for the duration of experiment. During the examined period all cows were fed a standard ration. On day 12 after calving, liver percutaneous biopsies were obtained using a biopsy instrument following the method described in details by Šamanc et al (2010). For pathohistological determination of lipids, sections were made using a freezing microtome and stained with Sudan III. Lipid content in the hepato-

cytes was determined through computer image analysis (Software Q Win). Cows were divided into two groups of equal size based on the degree of lipid accumulation in the liver: cows with mild fatty liver (< 20% fat, $n = 8$) and cows with moderate to severe fatty liver syndrome (> 20 % fat, $n = 8$). Three blood samples were taken by jugular venipuncture from each animal: the first 12 days after calving (early puerperal period), the second 30 days after calving and third 60 days after calving. Samples obtained using a sterilized needle were placed into tubes and allowed to clot spontaneously at room temperature. The serum was decanted, centrifuged at 3000 × *g*, portioned into aliquots of 1.5 mL, and stored in polypropylene microtubes at −20°C until analysis. To compare blood metabolite concentrations without influence of daily rhythms, samples were taken 4 to 6 hours after morning feeding. Concentrations of IGF-I in sera were measured by radioimmunoassay (RIA; INEP-Zemun, Serbia). Intra-assay coefficients of variation (CV) ranged from 3.1% to 7.2%. For the IGF-I RIA, binding proteins were removed by acid-ethanol treatment followed by cryoprecipitation. Electrophoresis and immunoblotting of IGFBPs were performed as described by Hossenlopp et al (1986). Serum samples were diluted 1:20 (for the analysis of IGFBP-2 and -4) or 1:40 (for the analysis of IGFBP-3), in 0.05 M sodium phosphate buffer, 0.15 M NaCl, pH 7.5 (PBS), mixed with an equal volume of reducing sample buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue; pH 6.8), boiled for 5 min and loaded onto the gels (30 µL). Samples were subjected to SDS-PAGE under non-reducing conditions (using a 10 % gel). Molecular mass markers (Bio-Rad Laboratories, Hercules, CA, USA) were run in parallel. Electrophoresis was performed in a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories) at a constant voltage (150 V) for 1.5 h. Proteins were transferred to a nitrocellulose membrane (Protran, Whatman, PerkinElmer, Boston, USA) in a Mini-PROTEAN 3 Cell at a constant voltage of 25 V for 1 h. Nonspecific binding on membranes was prevented by immersing membranes in 0.01 M Tris-HCl buffer, 0.15 M NaCl, 0.1% (v/v) Tween-20, pH 7.4 (TBST) containing 5% nonfat dry milk, for 1 h. Membranes were left overnight at 4 °C in TBST containing 1% nonfat dried milk and primary antibody: goat polyclonal anti-IGFBP-2 (sc-6002), anti-IGFBP-3 (sc-6004) or anti-IGFBP-4 (sc-6005) produced by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) at a final dilution of 1:1000. Membranes were further incubated with swine anti-goat IgG antibody coupled to horseradish peroxidase (Biosource, Camarillo, USA) diluted 1:10000 in TBST at room temperature for 1 h. Immunoreactive proteins were visualised by autoradiography using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK) containing luminol as a supstrate. The X-ray films and developing reagents were purchased from KODAK (Paris, France). Films were scanned and analysed by densitometry using the ImageMaster TotalLab software, version 2.0 (Amersham, UK). The intensity of the protein bands was expressed in arbitrary units. All results are expressed as means ± SD. Student t test was administered to identify differences between groups. The differences were considered significant at $P < 0.05$.

RESULTS

The mean IGF-I concentrations were significantly lower in cows with moderate to severe fatty liver than in cows with mild fatty liver at day 12 (11.30 ± 1.08 nmol/L versus 16.80 ± 3.02 nmol/L; $p < 0.01$), day 30 (16.80 ± 3.02 nmol/L versus 21.61 ± 5.01 nmol/L; $p < 0.05$) and day 60 after calving (17.70 ± 3.76 nmol/L versus 29.79 ± 5.99 nmol/L; $p < 0.001$). In group with moderate to severe fatty liver, IGF-I concentrations increased significantly from day 7 after calving until day 30. There was no significant difference in IGF-I concentrations between day 30 and day 60 after parturition. In cows with mild fatty liver, IGF-I significantly increased from day 7 after calving and until day 60 of lactation.

At day 12 after calving the relative abundance of IGFBP-2 bands was significantly higher in cows with moderate to severe compared to cows with mild fatty liver (14.08 ± 2.11 ADU/ 10 µL versus 11.28 ± 2.11 ADU/10 µL, $p < 0.05$) while there was no difference in IGFBP-2 abundance at days 30 and 60 between groups (14.90 ± 1.88 ADU/ 10 µL versus 14.26 ± 1.71 ADU/ 10 µL and 16.65 ± 1.93 ADU/ 10 µL versus 14.86 ± 2.17 ADU/ 10 µL, respectively).

At days 12, 30 and 60 after calving the relative abundance of IGFBP-3 bands was significantly lower in cows with moderate to severe compared to cows with mild fatty liver (5.84 ± 2.02 ADU/ 10 µL versus 8.13 ± 1.59 ADU/ 10 µL at day 12, $p < 0.05$; 8.61 ± 1.25 ADU/ 10 µL versus 11.33 ± 1.85 ADU/ 10 µL at day 30, $p < 0.01$; 13.50 ± 2.06 ADU/ 10 µL versus 17.05 ± 2.03 ADU/ 10 µL at day 60, $p < 0.01$)

The relative abundance of IGFBP-4 was lower in cows with moderate to severe fatty liver compared to cows with mild fatty liver and the difference was significant at day 60 after calving (15.02 ± 2.56 ADU/ 10 µL versus 17.48 ± 2.27 ADU/ 10 µL at day 12; 17.57 ± 2.49 ADU/ 10 µL versus 20.95 ± 3.25 ADU/ 10 µL at day 30; 18.13 ± 2.25 ADU/ 10 µL versus 21.29 ± 3.16 ADU/ 10 µL at day 60 after calving, $p < 0.05$).

DISSCUSION

The objective of this study was to examine the effect of negative energy balance on IGF system that is considered to be main signals of a shift in energy balance around parturition. Our results showed that cows with mild fatty liver and therefore mild negative energy balance succeeded to avoid severe postpartum blood IGF-I depression. Namely, after parturition high yielding dairy cows are exposed to NEB (Grummer et al., 2010). NEB in early lactation is usually associated with low serum IGF-I concentrations resulting from depressed synthesis of IGF-I by the liver (Sharma et al., 1994). As expected, average IGF-I concentration was significantly higher in cows with mild compared to cows with moderate to severe fatty liver at day 12 after calving meaning that NEB is associate with IGF concentration in blood. Cows with mild fatty liver also had much earlier rise in IGF-I postpartum which is, probably, related to improved energy status of these cows. It is known that IGF-I circulates in the blood bound to several specific binding proteins. Immunoblotting results obtained in this study are in excellent agreement with those published by Cochick et al. (1992). The major IGFBP species is IGFBP-3, which in most physiological conditions binds more than 75% of the circulating IGF-I. Association with IGFBPs increases IGF-I half-life, as compared with free IGF-I, and IGFBPs are thought to regulate bioavailability of IGF in target tissues (Kostecka and Blahovec, 2002). In accordance with findings of Sharma et al (1994) and Formigoni et

al (1996), our results have shown that serum IGFBP-3 levels, like those of IGF-I, were the lowest just after parturition, whereas IGFBP-2 level was highest at the same time. There was earlier increase in serum IGFBP-3 concentrations and to a greater extent, similarly to the effect it had on IGF-I in cows with mild fatty liver. This result is in accordance with literature data (Formigoni et al., 1996). IGFBP-2 is negatively correlated with plasma IGF-I levels (Sharma et al., 1994). Lower IGFBP-2 is usually consistent with more positive energy balance in dairy cows.

CONCLUSION

Cows that suffer from moderate to severe fatty liver have more pronounced negative energy balance which, according to our results, affects hepatic synthesis of IGF-I, and other components known to modulate the bioavailability and stability of circulating IGF-I.

ACKNOWLEDGEMENTS:

This work was supported by Ministry of Science and Technology, Republic of Serbia, Project Grant No 46002 and Ministry of Science and Technology, Republic of Srpska Government.

REFERENCES

1. Baxter RC, Martin JL, 1989, Binding proteins for the insulin-like growth factors: structure, regulation and function, Prog Growth Factor Res, 1, 49-68.
2. Cohick WS, McGuire MA, Clemmons DR, Bauman DE, 1992, Regulation of insulin-like growth factor-binding proteins in serum and lymph of lactating cows by somatotropin, Endocrinology, 130, 1508-14.
3. Formigoni A, Cornil MC, Prandi A, Mordentu A, Rossi A, Portetelle d, Ranaville R, 1996, Effect of propylene glycol supplementation around parturition on milk yield, reproduction performance and some hormonal and metabolic characteristics in dairy cows, J Dairy Res, 63, 11-24.
4. Grummer RR, 1993, Etiology of lipid-related metabolic disorders in periparturient dairy cows, J Dairy Sci, 76, 3882-96.
5. Grummer RR, Wiltbank MC, Fricke PM, Watters RD, Silva-Del-Rio N, 2010, Management of dry and transition cows to improve energy balance and reproduction, J Reprod Dev, 56, S22-8.
6. Hossenlopp O, Seurin D, Sergovia-Quinson B, Hardouin S, Binoux M, 1986, Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competetive binding studies. Anal. Biochem, 154, 138-143.
7. Kirovski D, Lazarević M, Baričević-Jones I, Nedić O, Masnikosa R, Nikolić JA, 2008, Effects of peroral insulin and glucose on circulating insulin-like growth factor-I, its binding proteins and thyroid hormones in neonatal calves, Can J Vet Res, 72, 253-8.
8. Kostecka Z, Blahovec J, 2002, Animal insulin-like growth factor binding proteins and their biological functions, Vet Med-Czech, 47, 75-84.
9. McGuire MA, Vicini JL, Bauman DE, Veenhuizen JJ, 1992, Insulin-like growth factors and binding proteins in ruminants and their nutritional regulation, J Anim Sci, 70, 2091-10.
10. Šamanc H, Stojić V, Kirovski D, Jovanović M, Cernescu H, Vujanac I, 2010, Thyroid hormones concentrations during the mid-dry period: an early indicator of fatty liver in Holstein – Friesian dairy cows, J Thyroid Res, 2010, ID 897602.
11. Sharma BK, Vandehaar MJ, Ames NK, 1994, Expression of insulin-like growth factor-I in cows at different stages of lactation and in late lactation cows treated with somatotropin, J Dairy Sci, 77, 2232-41.
12. Taylor VJ, Cheng Z, Pushpakumura PG, Beever DE, Wathes DC, 2004, relationship between the plasma concentrations of insulin-like growth factor I in dairy cows and their fertility and milk yield, Vet Rec, 155, 583-588.
13. Zapf J, Martin JL, Häsler H, Binz K, Guler HP, Schmid C, Froesch ER, 1989, Recombinant insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats, Proc Natl Acad Sci, 86, 3813-

ЕФЕКТ НА НЕГАТИВНИОТ ЕНЕРГЕТСКИ БИЛАНС НА IGF СИСТЕМОТ КАЈ МЛЕЧНИТЕ КРАВИ

Кировски Данијела[1], Шаманц Хореа[2], Вујанац Иван[2], Продановиќ Радиша[2], Ѓуриќ Милоје[3], Сладојевиќ Жељко[4]

[1]Катедра за физиологија и биохемија, Факултет за ветеринарна медицина, Универзитет во Белград, Белград, Србија
[2]Катедра за внатрешни болести кај фармски животни, Факултет за ветеринарна медицина, Универзитет во Белград, Белград, Србија
[3]Катедра за репродукција, фертилитет и вештачко осеменување, Факултет за ветеринарна медицина, Универзитет во Белград, Белград, Србија
[4]Ветеринарна станица „Ветерина Систем Сладојевиќ“, Градиште, Босна и Херцеговина, Република Српска

АПСТРАКТ

Предмет на ова истражување е да се утврди ефектот на негативниот енергетски биланс на IGF системот врз млечните крави. Шеснаесет постпартални крави (неколку дена после телење) беа избрани од комерцијално млечно стадо и се обработени во оваа студија. На 12 ден по телењето беа земени примероци од црниот дроб со перкутана биопсија. Беше одредена содржината на масти во црниот дроб. Кравите беа поделени во две групи, врз база на степенот на акумулација на мастите во црниот дроб: крави со умерено замастување на црниот дроб, (помалку од 20% масти) и крави со значително до тешко замастување на црниот дроб (повеќе од 20% масти). По три примероци крв беа земени од секое животно: првиот 12 дена по телењето, вториот 30 дена по телењето и третиот 60 дена по телењето. Концентрациите на IGF-I, IGFBP-2, IGFBP-3 и IGFBP-4 беа измерени во крвниот серум. Концентрацијата на IGF-I беше сигнификантно пониска кај кравите

со умерено во споредба кај кравите со значително или тешко замастување на црниот дроб во сите три испитувани периода. Релативното зголемување на IGFBP-2 беше сигнификантно кај кравите со значително до тешко во споредба со кравите со умерено замастување на црниот дроб 12 дена по телењето. Во останатите периода не се јави разлика во количината на IGFBP-2 помеѓу двте групи на крави. Релативната коцентрација на IGFBP-3 беше сигнификантно пониска кај кравите со значително до тешко замастување на црниот дроб, во сите три испитувани периода. Релативната концентрација на IGFBP-4 беше пониска кај кравите со значително до тешко во споредба кај кравите со умерено замастување на црниот дроб во тек на целиот испитуван период, но разликата беше сигнификантна само 60 ден по телењето. Како заклучок произлегува дека кравите кои страдаат од умерено до тешко замастување на црниот дроб, имаат поизразен негативен енергетски биланс, кој, според нашите резултати влијаат врз хепаталната синтеза на IGF-I, и другите познати компоненти кои ја модулираат биоспособноста и стабилноста на циркулирачкиот IGF-I.

Клучни зборови: замастување на црниот дроб, енергетски биланс, IGF систем

UDC: 636.7.09:618.19-006.6-085.277.065

APPLICATION OF IRON CHELATOR DESFERIOXAMINE IN DOGS WITH MALIGNANT MAMMARY GLAND TUMORS TREATED WITH EPIRUBICIN

Todorova Irina[1]

[1]*Department of Veterinary Surgery; Faculty of Veterinary Medicine; Trakia University, Stara Zagora, Bulgaria*

e-mail: irkatodorova@abv.bg

ABSTRACT

Anthracyclines are anticancer drugs, most commonly used for therapy of canine malignant mammary tumours. It is known that anthracyclines cause cardiotoxicity, partly due to their interaction with iron. Iron overload potentiates the cardiotoxicity of the antracyclines. Desferioxamine (Desferal) is a clinically approved iron chelator used to treat iron overload. The aim of this study was to investigate the effect of iron chelators to reduce anthracycline-induced cardiotoxicity in dogs with spontaneous mammary gland tumors treated with chemotherapy with epirubicin and cyclophosphamide. For this purpose, we examined the serum iron, total iron binding capacity (TIBC), transferrin saturation (Fe sat), latent iron binding capacity (LIBC) and atrial natriuretic peptide (proANP) as a biomarker for anthracycline-induced cardiotoxicity. The investigation was carried out in 7 bitches aged 7-13 years and weighing 7-32 kg, patients of the Small Animal Clinic of the Faculty of Veterinary Medicine, Stara Zagora with histopathologically confirmed malignant mammary gland tumors. The therapy consisted in surgical removal of the tumour and chemotherapy with epirubicin and cyclophosphamide associated with iron chelator desferal. It was shown that implementation of the iron chelator Desferioxamine during chemotherapy was associated with lower levels of serum iron and transferrin saturation (Fe sat). The level of natriuretic peptide proANP decreased significantly during complex therapy including iron chelator Desferioxamine, in dogs with malignant mammary gland tumors. Implementation of the iron chelator Desferioxamine during chemotherapy prevents the heart from anthracycline-induced cardiotoxicity, as evidenced by a decrease in natriuretic peptide proANP.

Key words: dogs, mammary tumours, chemotherapy, Desferioxamine, pro ANP

INTRODUCTION

Anthracyclines are known to possess a dose-related cardiotoxicity whose mechanisms are not fully understood (Hrdina et al., 2000). According to Hershko et al. (1993) and Schimmel et al. (2004) anthracycline cardiotoxicity is mediated by formation of free radicals, leading to oxidative stress which could result in serious complications (Schimmel et al., 2004).

It is known that anthracyclines cause cardiotoxicity, partly due to their interaction with iron. It is well known that anthracyclines bind iron strongly, forming metal ion complexes (Gianni & Myers, 1992). Iron plays an important role in anthracycline toxicity helping to convert of superoxide radicals to highly toxic hydroxyl radicals by Haber-Weiss reaction (Hershko et al., 1993). Iron overload potentiates the cardiotoxicity of the doxorubicin (Hershko et al., 1993). Doxorubicin binds iron from ferritin, transferrin and microsomal membranes (Gianni & Myers, 1992). In a study of cell cultures of rat heart was found that iron overload worsens anthracycline toxicity and that interaction can be prevented by prior treatment with iron chelators (Hershko et al., 1993). Desferal is a clinically approved iron chelator used to treat iron overload. Treating with iron chelators such as deferoxamine or deferipron eliminate the harmful effects of iron overload and as a result inhibits doxorubicin cardiotoxicity (Link et al., 1996).

According to Hoke et al. (2005) the oxidant-generating activity of doxorubicin is thought to be responsible for the cardiotoxic side effects of the drug, but it is unclear whether it is also required for its anti-tumour activity. To test whether an iron-chelating antioxidant would interfere with the tumor-killing activity of doxorubicin, nude mice were transplanted with xenografts of human breast cancer MDA-MB 231 cells and then treated with doxorubicin and/or desferal. Not only desferal did not interfere with the anti-tumor activity of doxorubicin, it inhibited tumor growth on its own. In vitro studies confirmed that desferal inhibits breast tumor growth. In contrast to its effect on tumor cells, desferal did not inhibit growth of normal breast epithelial cells. The data indicate that the anti-tumor activity of doxorubicin is not dependent on iron-mediated ROS production. Furthermore, desferal may have utility as an adjunctive chemotherapy due to its ability to inhibit breast tumor growth and cardiotoxic side effects without compromising the tumor-killing activity of an anthracycline chemotherapy drug (Hoke et al., 2005)

The clinical use of the chelator desferrioxamine (DFO) reduces the toxic effects of doxorubicin in vitro (Hershko et al., 1993) and in vivo (Saad et al., 2001).

Early detection of cardiac injury is important for the prevention and control of anthracycline cardiotoxicity. The methods used for its early identification include ECG, biochemical markers and functional tests and morphological examinations (Hrdina et al., 2000).

As biomarkers for early detection it can be used natriuretic peptides (atrial natriuretic peptide-proANP; B-type natriuretic peptide-BNP; N-terminal pro-BNP (NT-pro-BNP), and cardiac troponin T (cTnT), and cardiac

troponin I (cTnI) (Hayakawa et al., 2001; Mavinkurve-Groothuis et al., 2008). Plasma levels of these peptides (proANP, BNP) are increased in proportion to the severity of heart disease (Hayakawa et al., 2001). They significantly correlated with cardiac systolic function, but not with diastolic function (Hayakawa et al., 2001). In dogs, the study of natriuretic peptides are also used in the diagnosis of heart disease (Tarnow et al., 2009).

In recent years, our scientific and clinical interest focused on the treatment of patients with spontaneous mammary gland tumors by combining surgery with chemotherapy. It was found a significant increase of iron in the blood of treated patients. Given the hypotheses on the mechanisms of anthracycline cardiotoxicity consider it reasonable to deepen and expand our research patients through the application of iron chelators. This study is a continuation of our previous studies on chemotherapy in dogs with malignant mammary tumors.

MATERIAL AND METHODS

● Patients

The investigation was carried out in 7 bitches aged 7-13 years and weighing 7-32 kg, patients of the Small Animal Clinic of the Faculty of Veterinary Medicine, Stara Zagora with histopathologically confirmed malignant mammary gland tumors.

The therapy consisted in surgical removal of the tumour and chemotherapy associated with iron chelator therapy.

The surgical intervention consisted of removal of tumour masses by partial (regional and unilateral) or total (bilateral) mastectomy with or without removal of inguinal lymph nodes as required by oncosurgery principles. The choice of surgical technique depended on tumour size and the number of affected mammary glands. The anaesthetic protocol was routinely performed. After catheterization (22G or 24G, according to dog's size) of the antebrachial cephalic vein, dogs were premedicated with 0.02 mg/kg 0.1% atropine sulfate (Sopharma, Bulgaria) subcutaneously. The induction of anaesthesia was done by slow intravenous injection of 0.5 mg/kg diazepam (Diazepam 0.5%, Sopharma, Bulgaria) and 10 mg/kg ketamine (Ketaminol, Intervet, Netherlands) 10 min after atropine administration. After endotracheal intubation, general anaesthesia was maintained with 1–1.5 vol% halothane (Narcotan, Leciva, Czech Republic) and oxygen flow at 2–3 L/min.

The histological diagnosis was done according to the WHO classification of canine mammary gland tumours (Misdorp, 1999). Histological diagnosis of dogs was as followed: 4 dogs with simple carcinoma, and each dog of the rest with complex carcinoma, carcinosarcoma and myxosarcoma.

The distribution of dogs according to the TNM staging system (TNM stands for tumour, nodes, and metastases) was as followed: 2 dogs (29 %) in the third stage, 4 dogs (57 %) in the fourth stage and 1 dog (14 %) in the fifth stage.

The chemotherapy in all dogs consisted in administration of the cytostatics epirubucin (Farmorubicin, Pharmacia & Upjohn, Italy) and cyclophosphamide (Endoxan, Asta Medica, Frankfurt) as followed: 1) intravenous injection of epirubicin at a dose of 20 (dogs weighing <10 kg) or 30 mg/m2 (dogs > 10 kg) once weekly for 3 consecutive weeks; 2) intravenous injection of cyclophosphamide at 100 mg/m2, once weekly, 3 days after epirubicin injection for 3 consecutive weeks.

Prior to each injection of epirubicin, iron chelator desferrioxamine (DFO, Desferal ®, fl. Á 500 mg, Novartis Pharma, Germany) was administered at 5 mg / kg intramuscularly.

● Blood analyses

Blood samples were obtained from the jugular vein at the following intervals: prior to the surgery (period 1); 10 days after the surgery prior to first epirubicin chemotherapy (period 2); 17 days after the surgery prior to second epirubicin injection (period 3); 24 days after the surgery prior to the third epirubicin injection (period 4) and 39 days after the surgery (period 5).

● Biochemical assays

Serum iron (mmol / l) was assayed by the method of Garcic (1979) including the formation of blue colored tertiary complex after reaction with hromazurol B and cetyltrimethylammonium bromide. The intensity of staining is proportional to the concentration of iron in serum.

Total iron binding capacity (TIBC, mmol/l). Iron binding protein transferrin in serum is saturated with an excess of ferric ions. Unbound (excess) iron is absorbed on aluminum oxide and precipitation. Then, defining the iron associated with transferrin (TIBC) in the supernatant.

Transferrin saturation (Fe sat, Transferrin Saturation - TFS,%) is the most useful indicator of iron status of the organism. Determined as the ratio of serum iron and total iron binding capacity in percentage by the formula (http://www.fpnotebook.com/Hemeonc/Lab/IrnStrtn.htm):

Fe sat, % (TFS) = 100 x Serum Iron (ug/dl) / TIBC (ug/dl)

The normal value in dogs is approximately 33%.

Latent iron binding capacity (LIBC, mmol / l). A measure of free transferrin in plasma, determined as the difference between TIBC and serum iron is as follows:

LIBS (mmol/l)=TIBS (mmol/l) – Serum iron (mmol/l)

Atrial natriuretic peptide (proANP, fmol/ml) (Canine Cardioscreen - proANP 31- 67 ELISA, Guildhay limited, England) – ELISA

The statistical analysis of data was performed by the non-parametric Friedman's test for two-way repeated measures analysis. In case of significant P-values ($P<0.05$), the non-parametric Tukey HSD test was then applied.

RESULTS

The results from blood biochemical assays are presented in Table 1. There were statistically significant differences in serum iron and transferrin saturation (Fe sat). Serum iron was significantly decreased in the third (23 (17 - 46), µmol/l; $p<0,05$) and fourth period (24 (18 - 35), µmol/l; $p<0,05$), compared to the first period (38 (32 – 45), µmol/l). Transferrin saturation were statistically significantly decreased also in the third (30 (17-46), %; $p<0,05$) and fourth period (27 (21-39), %; $p<0,05$), compared to the first period (40 (36-56)%).

Table 1. *Parameters related to metabolism of iron in the blood of dogs with malignant tumors of the mammary gland during different periods of treatment including surgical removal of the tumour and chemotherapy associated with iron chelator Desferal - prior to the surgery (period 1); 10 days after the surgery prior to first epirubicin chemotherapy (period 2); 17 days after the surgery prior to second epirubicin injection (period 3); 24 days after the surgery prior to the third epirubicin injection (period 4) and 39 days after the surgery (period 5). Data are presented as median (range). * P≤0.05; ** P≤0.01 for each period within a group vs baseline (period 1).*

Parameters	Period 1	Period 2	Period 3	Period 4	Period 5
Serum iron (μmol/l)	38(32-45)	40(18-48)	23(10-31) *	24(18-35) *	26(19-37)
TIBC (μmol/l)	91(81-94)	75(58-105)	77(48-107)	91(66-100)	89(67-101)
Fe sat (%)	40(36-56)	44(31-66)	30(17-46) *	27(21-39) *	37(20-51)
LIBC (μmol/l)	50(36-58)	40(21-65)	51(36-87)	58(41-76)	64(33-75)

Atrial natriuretic peptide (proANP) in the blood of dogs with malignant mammary gland tumors in different periods of combined therapy are presented in Figure 1. It was found statistically significant decrease in the value of third (756.53 (631.66-1994.4), fmol/ml; p< 0,01) and fourth period (799.21(496.50-799.21), fmol/ml; p< 0,01), compared to the first period (2323.4 (1870.3-3195.8, fmol/ml).

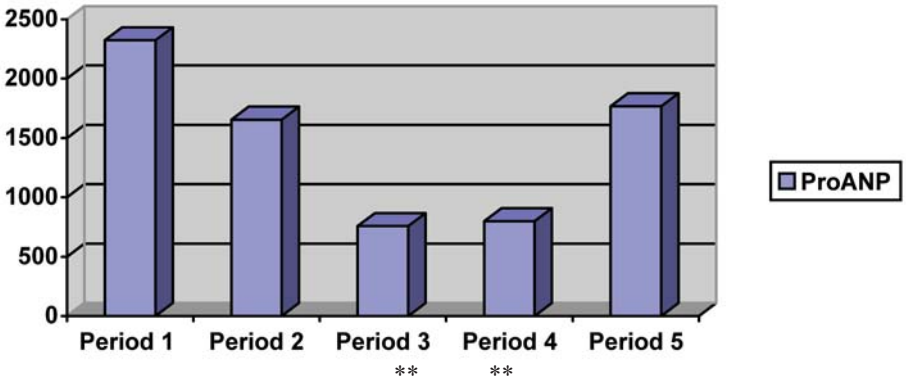


Figure 1. *Value of natriuretic peptide ProANP (fmol / ml) in blood of dogs with malignant tumors of the mammary gland during different periods of treatment including surgical removal of the tumour and chemotherapy associated with iron chelator Desferal - prior to the surgery (period 1); 10 days after the surgery prior to first epirubicin chemotherapy (period 2); 17 days after the surgery prior to second epirubicin injection (period 3); 24 days after the surgery prior to the third epirubicin injection (period 4) and 39 days after the surgery (period 5). Data are presented as median (range). * P≤0.05; ** P≤0.01 for each period within a group vs baseline (period 1).*

CONCLUSION

- Implementation of the iron chelator Desferioxamine during chemotherapy was associated with lower levels of serum iron and transferrin saturation (Fe sat).
- The level of natriuretic peptide proANP decreased significantly during complex therapy including iron chelator Desferioxamine, in dogs with malignant mammary gland tumors.
- Implementation of the iron chelator Desferioxamine during chemotherapy prevents the heart from anthracycline-induced cardiotoxicity, as evidenced by a decrease in natriuretic peptide proANP.

REFERENCES

1. Hrdina, R., V. Gersl, I. Klimtova, T. Simunek, J. Machackova & M. Adamcova, 2000. Anthracycline-induced cardiotoxicity. Acta Medica (Hradec Kralove), 43, 75−82.
2. Hershko, C., G. Link, M. Tzahor, J. P. Kaltwasser, P. Athias, A. Grynberg & A. Pinson, 1993. Anthracycline toxicity is potentiated by iron and inhibited by deferoxamine: Studies in rat heart cells in culture. Journal of Laboratory Clinical Medicine, 122, 245−251.
3. Schimmel, K. J., D. J. Richel, R. B. van den Brink & H. J. Guchelaar, 2004. Cardiotoxicity of cytotoxic drugs. Cancer Treatment Reviews, 30, 181−191.
4. Gianni, L. & C. Myers, 1992. The role of free radical formation in the cardiotoxicity of anthracycline. In: Cancer Treatment and the Heart, eds F. M. Muggia, M. D. Green & J. L. Speyer, Johns Hopkins University Press, Baltimore, pp. 9−46.
5. Link G, Tirosh R, Pinson A, Hershko C, 1996. Role of iron in the potentiation of anthracycline cardiotoxicity: identification of heart cell mitochondria as a major site of iron-anthracycline interaction. J Lab Clin Med., 127(3): 272-278.
6. Hoke Eileen M., Maylock Caroline A., Shacter Emily, 2005. Desferal inhibits breast tumor growth and does not interfere with the tumoricidal activity of doxorubicin.; Free radical biology & medicine, , vol. 39, no3, pp. 403-411.
7. Saad SY, Najjar TA and Al-Rikabi AC, 2001. The preventive role of deferoxamine against acute doxorubicin-induced cardiac, renal and hepatic toxicity in rats. Phar-

macol Res, 43: 211-218.
8. Hayakawa H, Komada Y, Hirayama M, Hori H, Ito M, Sakurai M, 2001. Plasma levels of natriuretic peptides in relation to doxorubicin-induced cardiotoxicity and cardiac function in children with cancer. Med Pediatr Oncol. Jul; 37 (1): 4-9.
9. Mavinkurve-Groothuis AM, Kapusta L, Nir A, Groot-Loonen J., 2008. The role of biomarkers in the early detection of anthracycline-induced cardiotoxicity in children: a review of the literature. Pediatr Hematol Oncol. Sep; 25 (7):655-664.
10. Tarnow I, Olsen LH, Kvart C, Hoglund K, Moesgaard SG, Kamstrup TS, Pedersen HD, Häggström J., 2009. Predictive value of natriuretic peptides in dogs with mitral valve disease. Vet J. May; 180 (2):195-201.
11. Misdorp, W., Else, R.W., Hellmen, E., Lipscomb, T.P, 1999. Histological classification of mammary tumors of the dog and the cat. vol. 7. Washington, DC: Armed Forces Institute of Pathology, American Registry of Pathology, 11–15.
12. Garcic A., 1979. Clin. Chem. Acta 94, 115-119.

ПРИМЕНА НА ЖЕЛЕЗЕН ХЕЛАТОР ДЕСФЕРИОКСАМИН КАЈ КУЧИЊА СО МАЛГНИ ТУМОРИ НА МЛЕЧНА ЖЛЕЗДА ТЕРАПИРАНИ СО ЕПИРУБИЦИН

Тодорова Ирина[1]

[1]*Катедра за Ветеринарна хирургија, Факултет за ветеринарна медицина, Тракиски Универзитет, Стара Загора, Бугарија*

e-mail: irkatodorova@abv.bg

АПСТРАКТ

Антрациклини се антиканцерогени лекови, најчесто користени при терапија на малигни тумори на млечна жлезда. Познато е дека антрациклините предизвикуваат кардиотоксичност, делумно поради нивната интеракција со железото. Преоптовареноста со железо ја поттикнува кардиотоксичноста на антрациклините. Десфериоксаминот (Desferal) е клинички одобрен железен хелатор за терапија при преоптовареност со железо. Целта на оваа студија беше да се истражи ефектот на железните хелатори за намалување на антрациклин-индуцираната кардиотоксичност кај кучиња со тумори на млечна жлезда третирани со хемотерапија со епирубицин и циклофосфамиди. За таа цел, ги испитавме серумско железо, вкупен врзувачки капацитет на железо (TIBC), сатурација на трансферин (Fe sat), латентен врзувачки капацитет на железо (LIBC) и атријален натриуретичен пептид (проАНП) како биомаркери за антрациклин-индуцирана кардиотоксичност. Истражувањето беше изведено на 7 кучки на возраст од 7-13 години и телесна тежина 7-32 кг, пацинети на Клиниката за мали животни на Факултетот за ветеринарна медицина, Стара Загора со хистопатолошки потврдени малигни тумори на млечна жлезда. Терапијата се состоеше од хируршко отстранување на туморот и хемотерапија со епирубицин и циклофосфамид поврзани со железо хелатор десферал. Имплементирањето на железен хелатор Десфериоксамин во тек на хемотерапијата беше поврзано со намалена концентрација на серумско железо и сатурација на трансферин (Re sat). Нивото на натриуретичниот пептид проАНП значително се намали кај кучињата со малигни тумори на млечна жлезда во тек на сложената терапија со железен хелатор Десфериоксамин. Имплементацијата на железен хелатор Десфериоксамин при хемотерапија го штити срцето од антрациклин-индуцираната кардитоксичност, евидентирано преку намалување на натриуретичниот пептид проАНП.

Клучни зборови: куче, тумори на млечна жлезда, хемотерапија, десфериоксамин, проАНП

UDC: 614.23:636.09]:005.23

THE ROLE OF COMMUNICATIONAL SKILLS IN SUCCESSFUL MANAGEMENT OF VETERINARY PRACTICE

Sekovska Blagica[1], Tosevska-Apostolova Milica[2]

[1]*Department of rural Economy and Management, Food institute, Faculty of Vetrinary Medicine, University Ss. Cyril and Methodius, Skopje, Macedonia,*
[2]*Faculty of Veterinary Medicine, University St. Cyril and Methodius, Skopje, Macedonia,*

ABSTRACT
For 40 years, medical researchers have been studying physician-patient interactions, but communication between vets and their clients come in the focus in last 5 years. For Macedonia, this topic is quiet new and without any research in it. Appropriate training programs can significantly change veterinary practitioners' communication knowledge, skills, and attitudes.
Many of these findings are applicable to the practice of veterinary medicine. Although research on veterinarian-client-patient communication is lacking in veterinary medicine, we accept that the trust and rapport that results from a healthy veterinarian-client-patient relationship has the potential to motivate clients to make appointments, show up on time, consent to treatment, follow recommendations, pay their bills on time, and refer other people. The end result is personal and professional success resulting from healthy long-term veterinarian-client-patient interactions. It is clear that a focus on interpersonal interactions in veterinary medicine is essential to the ongoing evolution of the profession.
Key words: communication, veterinary, management, practice

INTRODUCTION

The importance of communication skills in veterinary medicine is increasingly growing. Appropriate communication skills towards the client are of utmost importance in both companion animal practice and production animal field and consultancy work. The need for building a relationship with the client, alongside developing a structure for the consultation is widely recognized and applies to both types of veterinary practice. When talking about veterinary practices we can say that communication between the doctor and his client is essential. The importance of communication in veterinary medicine is an emerging topic, as evident in multiple influential studies published in the past 5 years. For instance, one of the six critical issues identified during focus group sessions of the KPMG LLP study is that ''while the scientific, technical, and clinical skills of the veterinary profession remain high, there is evidence that veterinarians lack management and communication skills necessary for success in private practice.'' The Brakke Management Study reported that many veterinarians are not earning up to their potential and suggested that a limiting behavior is due to the failure to use management practices proven to improve business performance (Shaw 2009). The biggest number of complain to the pet owners regard vets is caused by low level of communication, not because of low level of experts knowledge (Sethuraman 2001). Where is the main problem?? Vets are focused on medical problems and pet owners are focused on social and emotional problems. In this paper will be presented some tips on how vets can communicate more effectively with people at work, co-workers, subordinates, or superiors, but especially with clients or pets owners)

MATERIAL AND METHODS

Analysis of new literacy from the topic and interviews with pet owners regard their expectation from vets. A random small sample of 10 companion animal owners in Skopje was chosen. The sample was very small and our main idea was to identify main problems which pet owners in Macedonia have in their communication with vets and to detect whether these communicational problems are similar with those in international literacy.

RESULTS AND DISCUSSION

In veterinary medicine, various reports and papers have highlighted the relevance of communication skills in the context of veterinary education, clinical science and practice management]. Professional communication skills refer to the ability of the veterinarian to communicate appropriately and effectively with clients.. It has been described as a core clinical skill, as it will not only influence the client-veterinarian relationship, but also directly influence the success of the consultation and the following therapy or other intervention. Problems in interpersonal communication will affect the client-veterinarian relationship negatively. International experiences told us that one of the six critical issues identified in communicational problems [Shaw 2009] was that ''while the scientific, technical, and clinical skills of the veterinary profession remain high, there is evidence that veterinarians lack management and communication skills necessary for success in private practice.'' The Brakke Management Study reported that many veterinarians are not earning up to their potential and suggested that a limiting behavior was the failure to use management practices proven to improve business performance.

Communication skills are a vital component of interpersonal interactions.Three broad types of communication skills have been identified: content skills, process skills, and perceptual skills (Emanuel & Emanuel 1992). A ''gold standard'' does not exist for assessing veterinar-

ian-client interactions, nor is there an accepted definition of the ideal veterinarian-client relationship. In fact, under different clinical circumstances, different models may be appropriate

and effective. Flexibility is of utmost importance, and the choice of communication style should be tailored to the individual client and patient. In human medical practice and veterinary practice, the most common model for the physician-patient relationship is still paternalism. In this model, the veterinarian dominates the medical encounter, setting the agenda and goals for the visit, and the client's voice is diminished. The content of the discussion is predominantly biomedical, and the veterinarian plays the role of guardian of the patient and acts in the client's and patient's best interest. In contrast, it has been proposed that the optimal model for physician-patient relationships is relationship-centered care, which reflects a balance between physician paternalism and patient autonomy . Relationship-centered care is characterized as a partnership in which negotiation and shared decisionmaking are used to take the patient's perspective into consideration. The role of the physician is as an advisor or counselor. The term relationship-centered care seems to reflect the nature of the veterinarian-client-patient relationship, which is composed of the veterinarian-client relationship, the client-pet relationship, and the veterinarian-pet relationship. In veterinary medicine, relationship-centered care is characterized by a joint venture between the veterinarian and the client to provide optimal care for the animal. During the process of gathering information and client education, questions and information giving include lifestyle and social issues that may influence the pet's health. Respect for the client's perspective and interests, asking for the client's opinion, recognition of the client's expertise in caring for the pet.

Macedonian pet owners identify three themes related to veterinarian-client communication which they expect from the vets: 1.education of the clients (explaining important information, providing information up front, and providing information in various forms), 2. providing choices to the clients (providing pet owners with a range of options, being respectful of owners' decisions, and working in partnership with owners), 3.using two-way communication (using language clients understand, listening to what clients have to say, and asking the right questions). The pet owners ware complains on breakdowns in communication that affected the client's experience (owners feeling misinformed, because had not been given all options, and their concerns had not been heard). Very important question was challenges when communicating with clients regard money matters (monetary concerns, client misinformation, and time limitati ons).

The survey data clearly show that the survey subjects who are studied are believe that vets job is to explain which kind of therapy he will use in order to improve the health condition of their pet. They want him to offer a variety of alternatives that will make the treatment more effective. This means that they expect their vet to think about the implementation of traditional and alternative therapy. Moreover, the respondents consider that the level of hygiene should be kept really high and they expect the vets to be kind and generous. They show no lack of willingness to pay for the services when all their requirements are fulfilled.

Nonverbal communication which includes all behavioral signals between interacting individuals exclusive of verbal content and occurs in several modes is also very important. These behavioral signals include body language (facial expressions, gestures, body position, tension, touch); spatial relationships, including the distance between the veterinarian and client and objects that may act as potential barriers to communication (examination table, animal, computer, seating); paralanguage (voice tone, rate, rhythm, emphasis, volume); and autonomic responses, such as flushing, blanching, tearing, sweating, and changes in breathing pattern and pupil size, which are involuntary nonverbal responses and communicate underlying emotional responses.Being aware of our perceptions and how this can help in frustrating situations that commonly occur in practice. This will build team unity, increase profitability and compliance. Real life strategies and tools (laughter, emotion) will be reviewed to assist you in identifying ways to make your practice more effective and fun.

CONCLUSIONS

In this wired era, pet owners are much better informed, are over-represented as Internet users, and demand more from their veterinarians. With growing competition, current economic pressures, and convenience no longer being the main criterion for the selection of a veterinarian, they now have to actively reach out and compel prospective clients to join their teams. They need to find ways of conveying greater value and to showcase their unique selling propositions to encourage people to come through physical front door and to retain them as clients with new communication platforms like websites, social media, blogging. Comunications with clients is extremely important. In some countries exist special staff like Veterinary Communication Manager who should enhance company image among the veterinary channel, represent the company at selected Veterinary Medical Association and professional organization meetings, assist in preparation and delivery of technical presentations to internal and external audiences, develops the Veterinary Communication Strategy etc.

REFERENCES

1. Adams CL, Bonnett BN, Meek AH: Predictors of owner response to companion animal death Publication Type:Journal Article; Research Support, Non-U.S. Gov't, ISSN:0003-1488, UNITED STATES
2. DiMatteo MR, Taranta A, Friedman HS, Prince LM: Predicting patient satisfaction from physicians' nonverbal communication skills, Publication Type:Journal Article; Research Support, U.S. Gov't, P.H.S., ISSN:0025-7079,UNITED STATES
3. Emanuel EJ, Emanuel LJ. Four models of the physician-patient relationship. JAMA 1992;267:2221–6.
4. Seturman K.R. :"Komunikaciski ve[tini vo medicinskata praktika", Tabernakul, Skopje, 2010
5. Shaw JR, Bonnett BN, Adams CL, Roter DL:Veterinarian-client-patient communication patterns used during clinical appointments in companion animal practice, Publication Type:Journal Article; Research Support, Non-U.S. Gov't, ISSN:0003-1488, United States
6. Shaw JR, Adams CL, Bonnett BN, Larson S, Roter DL. J Am Vet Med Assoc. 2008 Nov 15; 233(10):1576-86.

УЛОГАТА НА КОМУНИКАЦИСКИТЕ ВЕШТИНИ ВО УСПЕШЕН МЕНАЏМЕНТ НА ВЕТЕРИНАРНАТА ПРАКСА

Сековска Благица[1], Тосевска -Апостолова Милица[2]

[1]Катедра за рурална економија и менеџмент, Факултет за ветеринарна медицина-Скопје, Универзитет „Св. Кирил и Методиј-Скопје“
[2]Факултет за ветеринарна медицина-Скопје, Универзитет „Св. Кирил и Методиј-Скопје“

АПСТРАКТ
Интеракцијата помеѓу пациентот и лекарот активно се проучува во изминативе 40 години, но комуникацијата помеѓу ветеринарот и неговиот клиент активно се истражува од пред неколку години. За Македонија, оваа тема е сосема нова и до сега не се правени никакви истражувања на неа. Соодветна обука од комуникологија би можела значајно да ги промени комуниколошките знаења и вештини на ветеринарните практичари.
Многу од сознанијата изнесени во овој труд се апликативни во ветеринарната пракса. Всушност, истражувањата на тема релацијата ветеринар-клиент-пациент недостасуваат во ветеринарната медицина, иако довербата во оваа релација има моќ да ги мотивира потенцијалните клиенти да закажуваат средби, да реагираат на време, да следат инструкции на време, да плајќаат на време и да раскажуваат на други луѓе за своите искуства. Крајниот резултат е лична и професионална сатисфакција која што резултира со долгогодишна ветеринар-клиент-пациент интеракција. Од трудот јасно се гледа дека ставањето на интерперсоналните вештини во фокусот на ветеринарната медицина е есенцијално за иден развој на ова професија.
Клучни зборови: комуникација, ветерина, менаџмент, пракса

UDC: 636.4.09:[616.98:578.833.3.083.3(497.7)

COMPARISON OF RESULTS OBTAINED WITH TWO DIFFERENT ELISA KITS FOR DETECTION OF ANTIBODIES AGAINST CLASSICAL SWINE FEVER VIRUS

Krstevski Kiril[1], Dzadzovski Igor[1], Mitrov Dine[1], Mrenoshki Slavcho[1], Acevski Sinisa[1], Cvetkovikj Iskra[1], Naletoski Ivancho[2]

[1]*Veterinary Institute, Faculty of Veterinary Medicine Skopje, University Ss Cyril and Methodius Skopje, Macedonia*
[2]*Animal Production and Health, International Atomic Energy Agency, Vienna, Austria*

ABSTRACT

Classical swine fever (CSF) is OIE listed disease with great economic importance. In Macedonia, control of this disease is achieved by vaccination. Different serological tests could be used to assess the level of protection in vaccinated population with ELISA being the most convenient for high throughput testings.

We have compared the results obtained with two commercial ELISA kits for detection of CSFV specific antibodies and determined the level of agreement between both tests to assess the validity and reliability of these tests in absence of gold standard reference test.

Analysis of the results showed perfect agreement between the tests, thus leading to conclusion that both commercial test kits are trustworthy and could be used for obtaining valid estimations for CSF seroprevalence.

Key words: Classical swine fever, vaccination, ELISA, antibody, kappa value

INTRODUCTION

Classical swine fever, formerly known as hog cholera, is a highly contagious viral disease of pigs that cause serious economic loss to the swine industry worldwide. (1) Epidemics of CSF can have a high cost for the national economy and therefore preventive measures in a form of control strategies aimed at preventing and limiting an incursion of CSF should be implemented.

Macedonia is among few countries in Europe where prevention and control of CSF is achieved by routine vaccination of pigs with live, attenuated vaccine (C-strain).

During the last quarter of 2011 a statistically representative sample taken from commercial pig farms in Macedonia was tested for determination of the vaccine coverage using commercial ELISA test kit (2). To check for the validity of the obtained results, part of the samples were tested using second commercial ELISA kit and level of agreement between two tests was determined. Kappa test was performed for determination of the level of agreement beyond that which may be obtained by chance (3)

Obtained results demonstrated highest possible agreement (almost perfect agreement) indicating that both commercial ELISA tests generate valid results for presence of CSFV specific antibodies in the pig sera.

MATERIAL AND METHODS

For the purposes of this study, 308 pig serum samples from 34 different commercial farms were randomly selected and tested for the presence of CSFV specific antibodies using two commercial ELISA kits available at the market:

- IDEXX CSFV Ab Test
- SYNBIOTICS SERELISA HCV Ab Mono Blocking.

All tested samples were originating from pigs vaccinated against CSFV and were part of the samples collected at the slaughtering line for determination of the vaccine coverage ()

Test procedures were performed as described in the appropriate protocols provided by the manufacturers. Optical densities (OD) of the samples and controls were measured at 450 nm using BDSL Immunoscan spectrophotometer. Results from each test run were considered as valid only if required criteria for validity based on obtained ODs for positive and negative controls were fulfilled. Interpretation of obtained results was based on calculation of Percentage of Positivity (PP) in IDEXX ELISA or Competition Percentage (CP) in SYNBIOTICS ELISA and their comparison with predefined cut-off values.

Obtained results from two tests were placed in the following table:

	IDEXX test		
SYNBIOTICS test	**POSITIVE**	**NEGATIVE**	**TOTAL**
POSITIVE	**a**	**b**	**a+b**
NEGATIVE	**c**	**d**	**c+d**
TOTAL	**a+c**	**b+d**	**N**

Table 1. *Table used for distribution of results; a=number of samples positive in both tests, b=number of samples positive in SYNBIOTICS test, but negative in IDEXX test, c=number of samples negative on SYNBIOTICS, but positive on IDEXX, d=number of samples negative in both tests.*

Kappa coefficient was calculated as ratio of the observed agreement beyond chance (OA) to the maximum possible agreement beyond chance (MA):

kappa = OA/MA,

OA and MA were calculated using following formulas:

OA = [(a+d)/n] – {[(a+b)/n * (a+c/n] – [(c+d)/n * [b+d)/n]}
MA = 1 - {[(a+b)/n * (a+c/n] – [(c+d)/n * [b+d)/n]}

RESULTS

144 (46.7%) of tested samples were found to be positive using IDEXX test kit whereas number of positive samples on SYNBIOTICS kit was 130 (42.2%). 7 (2.3%) samples were positive on SYNBIOTICS but negative on IDEXX and 21 (6.8%) samples were positive on IDEXX and negative on SYNBIOTICS test kit.

Both tests gave identical status for 280 (90.9%) samples, or observed agreement was 0.909. All results are presented in Table 2.

	IDEXX+	IDEXX-	TOTAL
SYNBIOTICS+	a **123**	b **7**	a+b **130**
SYNBIOTICS-	c **21**	d **157**	c+d **178**
TOTAL	a+c **144**	b+d **164**	N **308**

Table 2. *Comparison of results obtained on both ELISA test kits*

Expected agreement by chance (EP) was 0.505. Maximum possible agreement beyond chance was 0.481, while observed agreement beyond chance was 0.390. This gives kappa ratio of 0.811.

DISCUSSION

Macedonia is among few countries in Europe where prevention and control of CSF is achieved by routine vaccination of pigs with live, attenuated vaccine (C-strain). This vaccine is highly efficacious and mounts strong immunological response in vaccinated animals which could be detected by different serological tests (4); but could not be differentiated from the immunological response induced by natural infection with the virus. Despite this fact, when used in the healthy vaccinated population, these tests could provide a solid estimation for the achieved level of protection. For this purpose ELISA tests are preferred because they are rapid, easy to perform and could be used with high throughput.

Currently few manufacturers offer CSF antibody ELISAs on the market: IDEXX, Synbiotics and CEDI Diagnostics. European Union Reference Laboratory for CSF does not recommend a certain commercial product and validity of these tests should be evaluated at national level. (4) In the absence of golden standard assessment of agreement between the tests is indicative for test validity. (3,5) Using this approach, we have obtained 0.811 kappa value, which stands for the perfect agreement between the tests. This suggests that obtained results for determination of the vaccine coverage with above mentioned commercial ELISAs are reliable and valid. Nevertheless, due to possible cross reactions of CSFV with other pestiviruses (Border Disease Virus, Bovine Viral Diarrhea Virus), diagnostic performances of both tests (specificity and sensitivity) should be still evaluated using gold standard sera and compared with the performances of the virus neutralization test.

REFERENCES

1. World Organisation for Animal Health (OIE) (2008). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Ed. OIE, Paris.
2. Djadjovski I, Krstevski K, Mitrov D, Mrenoshki S, Acevski S, Cvetkovikj I, Celeska I, Kirandjiski T, Nakova E, O'Flaherty, R. (2012) Epidemiological situation, legal framework and laboratory diagnostic of classical swine fever in Republic of Macedonia. STOP-CSF, International Scientific Conference, Proceedings, Pp.137-147.
3. Thrusfield, M. (2003): Veterinary Epidemiology, 3rd edition, Blackwell Science Ltd., Diagnostic Testing, Pp 327-329.
4. Blome, S., Meindl-Böhmer, A., Loeffen, W., Thuer, B., Moennig, V. (2006) Assessment of classical swine fever diagnostics and vaccine performance. Rev. sci. tech. Off. int. Epiz., 2006, 25 (3), 1025-1038
5. Julius, S., Wright, C. C. (2005) The Kappa Statistic in Reliability Studies: Use, Interpretation, and Sample Size Requirements. PHYS THER. 85:257-268.
6. EU Diagnostic Manual for Classical Swine Fever Diagnosis: Technical Part. Third draft, June 2007
7. Floegel-Niesmann, G., Moennig, V. (2004). Quality management in reference tests for the diagnosis of classical swine fever. Rev. sci. tech. Off. int. Epiz., 23 (3), 895-903.
8. European Commission (2003) Diagnostic techniques and vaccines for foot-and-mouth disease, classical swine fever, avian influenza and some other important OIE List A Diseases. In Report of the Scientific Committee on Animal Health and Animal Welfare adopted 24-25th April 2003. European Commission, Directorate-General for Health and Consumer Protection, Brussels, 1-150

СПОРЕДБА НА РЕЗУЛТАТИТЕ ДОБИЕНИ СО ПРИМЕНА НА ДВА РАЗЛИЧНИ ELISA КИТОВИ ЗА ДЕТЕКЦИЈА НА АНТИТЕЛА ПРОТИВ ВИРУСОТ НА КЛАСИЧНА СВИНСКА ЧУМА

Крстевски Кирил[1], Џаџовски Игор[1], Митров Дине, Мреношки Славчо[1], Ацевски Синиша[1], Цветковиќ Искра[1], Налетоски Иванчо[2]

[1]Ветеринарен Институт, Факултет за ветеринарна медицина Скопје, Универзитет Св.Кирил и Методиј, Скопје, Македонија
[2]Оддел за здравствена заштита и продукција кај животните, Меѓународна агенција за атомска енергија, Виена, Австрија

АПСТРАКТ
Класичната свинска чума (КСЧ) е болест од листа на Меѓународната ОИЕ која има огромно економско значење. Во Македонија, контролата на оваа болест се спроведува со примена на вакцинација. За проценка на постигнатиот степен на заштита во вакцинираната популација може да се користат различни серолошки тестови, но ELISA тестовите се најпогодни за масовни тестирања.
Во нашата студија ги споредивме резултатите добиени со примена на два комерцијални ELISA китови наменети за детекција на специфични антитела против вирусот на КСЧ и го одредивме степенот на усогласност помеѓу нив. Со ова, во отсуство на референтен тест кој се смета за златен стандард, направивме проценка на валидноста и веродостојноста на резултатите кои ги даваат овие тестови.
Анализата на резултатите покажа дека постои перфектна усогласеност помеѓу тестови те, од што може да се заклучи дека и двата комерцијални ELSISA китови може да се користат за добивање валидни податоци за серопреваленцата на КСЧ.
Клучни зборови: Класична свинска чума, вакцинација, ELISA, kappa вредност

UDC: 636.2.09:[616.98:578.841.93(735.8)

BRUCELLA EPIDEMIOLOGY IN GEORGIA

Makaradze Levan, Mirtskhulava Merab, Giorgobiani Marina

Agrarian University, Faculty of Veterinary Medicine,
Institute of Sanitary Hygiene and Medical ecology, Tbilisi, Georgia

ABSTRACT
Epizootic situation of brucellosis was studied according to separate regions. Rose-Bengal test and ELISA were used for diagnosis. The research results within 2008-2010 years were show that in 2008, 2.959 blood sera of cattle were tested, out of which 325 appeared positive (10.98%), in 2009, 5.236 were tested, out of which 634 were positive (12.1%) and in 2010, 3.619 animals were tested, out of which 357 were positive (9.86%).
In 2009, 21.044 small ruminants (sheep) were tested, out of which 127 were positive (0.6%). In 2010, 10.200 sheep were tested, out of which 103 were positive (1.0%).
In 2008, 168 people were affected with brucellosis, in 2009 – 175 and in 2010 – 152. B. melitensis was a causative agent in all cases.
Key words: Georgia, brucellosis, cattle, sheep, Rose-Bengal, ELISA.

INTRODUCTION

Brucellosis is an infectious disease caused by the bacteria of the genus Brucella. It's also known as "Undulant fever", "Mediterranean fever" or "Malta fever" which is a zoonosis and the infection is almost invariably transmitted by direct or indirect contact with infected animals or their products. It affects people of all age groups and of both sexes. Although there has been great progress in controlling the disease in many countries, there still remain regions where the infection persists in domestic animals and, consequently, transmission to the human population frequently occurs. It is an important human disease in many parts of the world especially in the Mediterranean countries of Europe, north and east Africa, the Middle East, south and central Asia and Central and South America and yet it is often unrecognized and frequently goes unreported. There are only a few countries in the world that are officially free of the disease, although cases still occur in people returning from endemic countries .

MATERIAL AND METHODS

Various Brucella species affect sheep, goats, cattle, deer, elk, pigs, dogs, and several other animals. The most common agents of human disease are: B. melitensis, B. abortus, B. suis and B. canis in decreasing order. Epizootic situation of brucellosis was studied according to separate regions. Rose-Bengal test and ELISA were used for diagnosis.

RESULTS AND CONCLUSIONS

The research results within 2008-2010 years were given in the report (Pic #1).

Particularly, in 2008 2959 blood sera of cattle were tested, out of which 325 appeared positive (10,98%), in 2009 5236 were tested, out of which 634 were positive (12,1%) and in 2010 3619 animals were tested, out of which 357 were positive (9,86%).

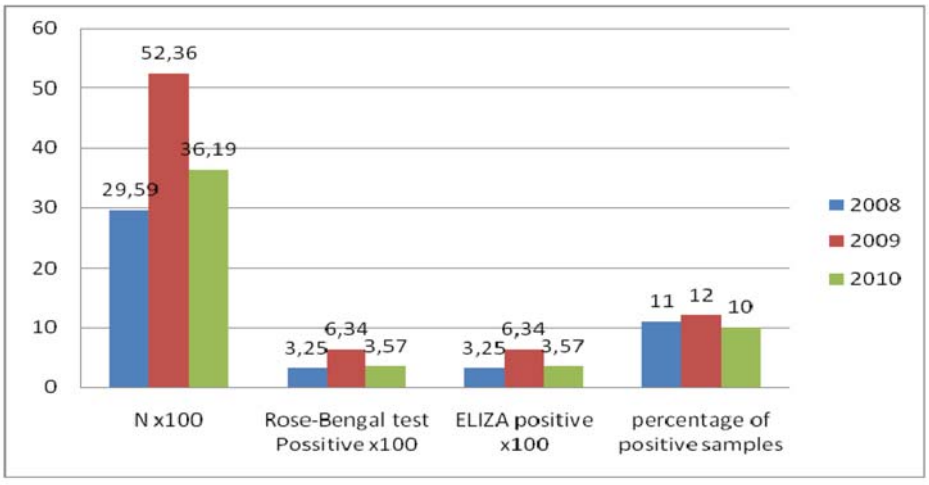


Figure 1. *Epizootic situation of brucellosis*

In 2009, 21044 small ruminants (sheep) were tested, out of which 127 were positive (0,6%). In 2010, 10200 sheep were tested, out of which 103 were positive (1,0%). In 2008, 168 people were affected with brucellosis, in 2009 – 175 and in 2010 – 152. B. melitensis was a causative agent in all cases. (Pic #2).

Control of brucellosis in humans via animal vaccination is a common method of control. The provinces with the highest level of disease are seen in the eastern part of the Republic of Georgia. This area is characterized as the most ethnically diverse and as having the majority of the sheep population. Sheep are the major carriers of B.

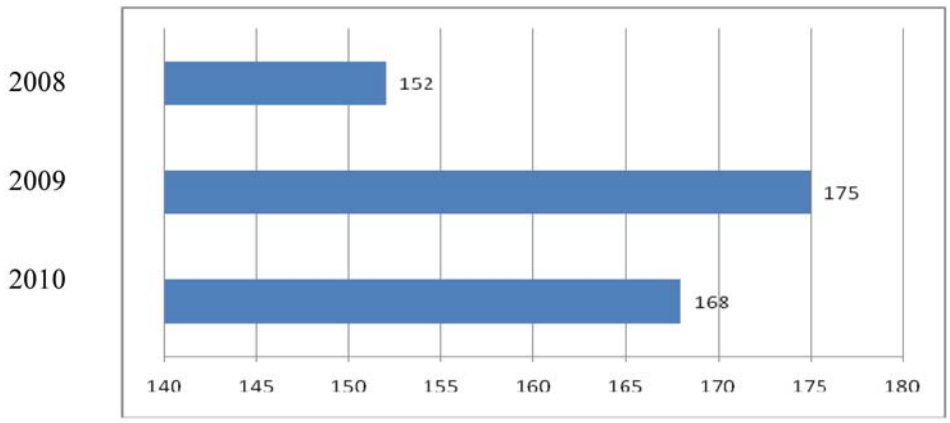


Figure 2.

Brucellosis is often a disease of rural communities associated with animal husbandry, which I the dominant form of agricultural production in the mountainous regions of Georgia. The prevalence of disease in domestic animals is an important predictor of disease in humans.

melitensis, the most common zoonotic pathogen of the Brucella spp. Kakheti province had the highest prevalence, followed by Mtskheta-Mtianeti, Kvemo Kartli and Shida Kartli.

ЕПИДЕМИОЛОГИЈА НА БРУЦЕЛОЗАТА ВО ГРУЗИЈА

Макарадзе Леван, Мирцкулава Мераб, Ґоргобиани Марина

Земјоделски универзитет, Факултет за ветеринарна медицина,*
Институт за санитарна хигиена и медицинска екологија, Тбилиси, Грузија

АПСТРАКТ
Епизоотиолошката состојба на бруцелозата беше проучувана по одделни региони. За дијагностика бе користени Розе-Бенгал тест и ЕЛИСА. Резултатите од истражувањето во периодот 2008-2010 година покажаа дека во 2008 година, беа тестирани 2.959 крвни серуми од говеда, од кои 325 покажаа позитивен резултат (10,98%), во 2009 година, беа тестирани 5.236 говеда, од кои 634 беа позитивни (12,1%) и во 2010 година, тестирани беа 3.619 животни, од кои 357 беа позитивни (9,86%).
Во 2009 година, беа тестирани 21.044 мали преживари (овци), од кои 127 беа позитивни (0,6%). Во 2010 година, 10.200 овци беа тестирани, од кои 103 беа позитивни (1,0%).
Во 2008 година, 168 луѓе беа заболени од бруцелоза, во 2009 година заболеа 175, а во 2010 година 152 заболени луѓе. Во сите случаи предизвикувачки агенс беше Бруцела мелитензис.
Клучни зборови: Грузија, бруцелоза, говеда, овци, Розе-Бенгал, ЕЛИСА.

UDC: 636.7.09:616.61-089.873

UNILATERAL NEPHRECTOMY AND URETERECTOMY IN DOG: CLINICAL CASE

Trenkoska-Spasovska Pandorce[1], Ilievska Ksenija[2], Trojacanec Plamen[2]

[1] *Veterinary Clinic, Dr. Naletoski, Skopje, R. Macedonia*
[2] *Department of Surgery, Orthopedics and Ophthalmology, Faculty of Veterinary Medicine, Skopje, R. Macedonia*

ABSTRACT
Kidney infection, trauma or urethral obstruction are the main cause of acute or chronic renal failure accompanied by moderate to severe dehydration, oliguria, anemia, electrolyte and acid-base abnormalities, elevated blood urea nitrogen and creatinine. Bacterial urinary tract infections are very common in female dogs and it can be located in the bladder, urethra, ureter or kidney. In most cases infection is ascendant. Renal cysts, hydronephrosis, pyonephrosis, neoplasm, trauma of the renal parenchyma are main indications for nephrectomy. The aim of this report is to present a successful recovery after unilateral nephrectomy and ureterectomy in 2,5 year old female Labrador suffering from pyonephrosis. The owner noticed yellowish vaginal discharge accompanied with polyuria, polydipsia and decreased appetite. After the clinical examination, elevated body temperature, depression, abdominal pain, were recorded. Ultrasonic inspection of the abdomen revealed an elongated, opalescent echogenic structure near the bladder and extremely enlarged and fluid filled right kidney. The patient was premedicated by i/m injection of acepromazine maleate. Surgical anesthesia was induced by i/v injection of propofol and maintained using isofluran. A midline abdominal incision from the xyphoid processus to the pubis revealed enlarged and elongated structure of the right ureter next to the bladder running cranially to the extremely enlarged right kidney. The enlarged ureter was carefully dissected and ligated. The kidney together with the ureter was removed after double ligation of the renal artery and vein. The wound was closed in standard manner. Hematological and renal tests, carried out on the 1st, 2nd and 3th week postoperatively, have shown successful recovery and improvement of renal filtration.
Key words: nefrectomy, ureterectomy

INTRODUCTION

The kidneys are paired, multifunctional organs with major role in maintaining of stable plasma composition by regulation the relationship between the intake and urinary loss of water (Michell, 1988). Besides excretion of the end-products of protein metabolism (mainly urea) and blood waste products, the kidneys produces hormones that stimulates bone marrow to produced red blood cells and also regulates the volume of extracellular fluid and sodium, potassium and calcium in the blood (Michell, 1988). Renal failure usually refers to a numerous clinical symptoms which occur when the kidneys are unable to maintain their function (excretory, regulatory and endocrine) resulting with retention of nitrogenous solids, disturbance in electrolyte, fluid and acid-base balance (Ettinger & Feldman, 2011). When 75% or more of the nephorne are non-functional renal failure can occur (Ettinger & Feldman, 2011). Urolithiasis, urinary stasis, micturition disorders, acquired or congenital defect of the bladder, immunosuppresion are only a few of predisposing factors contributing to renal diseases. Urinary tract infection (UTI) usually is a consequence of ascending bacteria migration from the genital organs and urethra to the bladder and can subsequently extended to the ureters and kidneys (Elliot & Grauer, 2007). Pyelonephritis usually refers on inflammation on renal pelvis and parenchyma whereas pyelitis applies on inflammation on renal pelvis only (Parry, 2005). In most cases pyelonephritis arise directly as ascendant infection from lower urinary tract but less frequently is hematogenous dissemination of infection (Parry, 2005). The clinical signs of the upper urinary tract infection depend of the duration of disease and degree of the renal parenchymal involvement (Bojrab, 1993). If the problem is unilateral, nephrectomy is usually the best choice with several consequences that results with hyperfunction and increased glomerular filtration rate per nephrone (Henderson and Leypold, 1989). Mild proteinuria is one of the consequences of the unilateral nephrectomy (Henderson and Leypold, 1989).

MATERIAL AND METHODS

A 2,5 years old female Labrador, 25 kg body weight was admitted to the clinic with a history of vomiting, loss of appetite, weight loss and frequent urination with yellowish-red vaginal discharge and elevated body temoperature that lasts for more than 7 days. The clinical examination have shown extremely elevated body temperature (41,0-41,8^{0}C), abdominal pain and anorexia. Hematological and biochemical examination were performed. Leucocytosis, mild erythropenia, decreased hemoglobin and hematocrite were observed the day before surgery. Blood urea nitrogen and creatinine were increased. The urine analysis showed proteinuria, hematuria and increased presence of sediment (table 1). The ultrasonographic examination revealed an elongated and dilated ureter and extremely enlarged right kidney (figure 1). The cortical tissue was extremely reduced, and the medular and pelvic space was replaced with hyperechoic fluctuating mass. Splenomegalia was also detected.

The patient was premedicated with normal pulse and respiration paramethers and elevated temperature (118 bpm, 42 rr and 40,2^{0}C). Sedation was achieved by i/m injection of 0,3 mg/kg Acepromazine maleat (Castran, Interchemie, Holland), while surgical anesthesia was induced with 5mg/kg Propofol (Braun, Melsungen) fol-

lowed by endotracheal intubation. The surgical plain of anesthesia was maintained with 2 -2.5 MAC Isofluran (isofluran CP, CP-Pharma). The patient was positioned in dorsal recumbency and ventral, midline abdominal incision was performed from the xiphoid to the pubis. Moistured sponges were placed on the edges of the abdominal incision. The laparotomy revealed grossly enlarged right ureter adhered to the cervical stump and extending cranialy. The extremely enlarged right kidney was identified in the right cranial quadrant of the abdominal cavity. The ureter was ligated with 2/0 PGA, separated from the cervical stump and futher dissected from the retroperitoneal space heading to the kidney. The peritoneum was grasped with Allis forceps, incised with scissors and pilled-off from the kidney. The renal artery and vein were identified on the dorsal surface of the hilus and double ligated separately with 2/0 PGA. The artery and vein were transected distaly to the ligature and the kidney was removed. The intestines were returned in the normal position and abdomen was closed with continiuos interrupted suture of 1/0 PGA. Intraoperative and postoperative analgesia was achieved by i/m injection of 2 mg/kg ketoprofen (Ketonal,). The skin was closed with continuous horizontal mattress sutures using 2/0 supramid. During the surgery, the dog received total of 250 ml saline and 50 mg/kg Lendacin (Cefrtiaxone) until removal of the sutures. Urination was monitored for 24 hours posteperatively. The activity and exercise were limited for three weeks after surgery and the sutures were removed 10 days after the surgery. A low-protein medical diet was prescribed.

The duration of surgery was approximately 3 hours with smooth recovery. The patient was discharged the next day after 24 hours of clinical observation. Three weeks after the surgery the hematology, serum concentrations of urea and creatinine and results from the urinanalysis have returned to normal values (table 1). The body temperature ranged 38,4-39,2^{0}C in the postoperative period.

Tabel 1. *Values obtained from complete blood count, biochemistry profile and urinanalysis before and after nephrectomy.*

Analysis (hemogram, renal function)	**Referent values**	**Day before surgery**	**1 week after surgery**	**2 weeks after surgery**	**3 weeks after surgery**
Erythrocytes (10^{12}/L)	5,5-8,0	4,86	4,92	5,9	5,9
Hemoglobin (g/L)	120,0-180,0	117	117	129	132
Hematocrite (%)	37,0-55,0	32,9	35,6	39,5	40,3
Leucocytes (10^9/L)	6,0-17,0	29,6	23,4	18,0	15,6
Urea (mmol/L)	3,1-9,2	17,9	14,9	8,7	6,2
Creatinine (µmol/L)	44,3-138,4	146	136	130,5	112,6
Urinanalysis					
Protein		++	+	-	-
Blood		+	-	-	-
Leucocytes		+	+	-	-

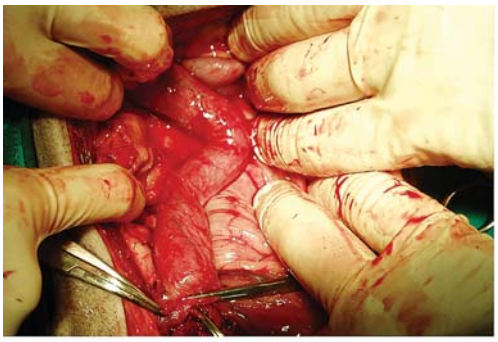


Figure 1. *Exposed dilated ureter after laparotomy.*

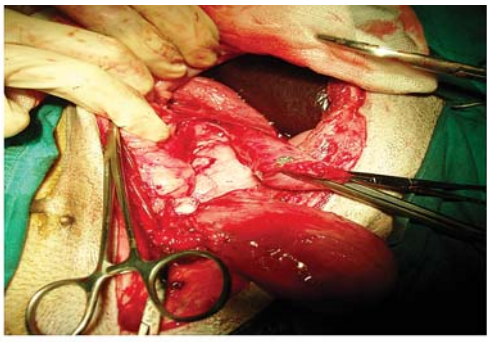


Figure 2. *Ligation and excision of the ureter. Enlarged spleen.*



Figure 3. *Dissected enlarged right kidney*

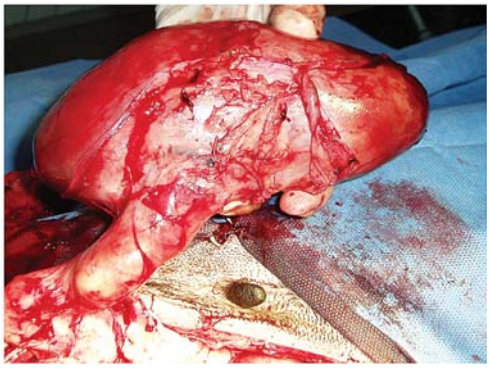


Figure 4. *Right kidney with the uretra after ligation and transection of the renal artery and vein.*

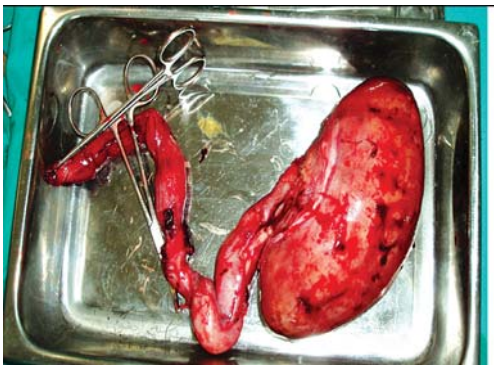


Figure 5. *Macroscopic view of the extirpated right kidney with the ureter – 23 cm length*



Figure 6. *Visible reduction of the renal medulla and purulent content from the kidney.*

CONCLUSIONS

Uretral obstruction and long term bladder infection increases the risk of hematogenous spread of infection to the kidney and can be a predisposing factor of pyonephrosis. The clinical signs and laboratory tests performed on the patient indicated an inflammatory process on the urinary tract. The ultrasonography revealed extremely enlarged kidney and distended ureter. Nephrectomy and ureterectomy were the only choice of treatment, despite the febrile state of the patient. The functional left kidney compensated the loss after the nephrectomy keeping normal levels of blood urea nitrogen and creatinine during the first 3 weeks after the surgery.

REFERENCES

1. Bojrab M.J. (1993): Disease Mechanism in Small animal Surgery, 441, Lea and Febiger
2. Elliot J., Grauer F.G. (2007): BSAVA Manual of Canine and Feline Nephrology and Urology, second edition
3. Ettinger S.J., Feldman E.C. (2011): Textbook of Veterinary Internal medicine, 7th edition, Vol. 2, Saunders
4. Henderson L.W. & Leypoldt J.K. (1989): Unilateral nephrectomy and glomerular solute transport in the dog, Kidney International, Vol. 54, 589-594
5. Michell A.R. (1988): Renal Disease in Dogs and Cats, Blackwell Publishing
6. Parry N.M. (2005): Pyelonephritis in Small animals, UK VET, Vol. 10, 1-5

УНИЛАТЕРАЛНА НЕФРЕКТОМИЈА И УРЕТЕРЕКТОМИЈА КАЈ КУЧЕ: КЛИНИЧКИ СЛУЧАЈ

Тренкоска-Спасовска Пандорче[1], Илиевска Ксенија[2], Тројачанец Пламен[2]

[1]Ветеринарна амбуланта Др. Налетоски, Скопје, Македонија
[2]Катедра по Хирургија, ортопедија и офталмологија, Факултет за ветеринарна медицина, Скопје, Македонија

АПСТРАКТ

Инфекција на бубрезите, траума или обструкција на уретралните патишта се една од причините за појава на акутна или хронична слабост на бубрезите придружени со умерена до изразена дехидрација, олигурија, анемија, електролитни и ацидо-базни абнормалности, зголемени вредности на уреа и креатинин. Бактериската инфекција на уринарниот тракт е честа појава кај женските кучиња и истата може да биде локализирана на мочниот меур, уретрата, уретерот или бубрегот. Во најголем број случаи инфекцијата е асцедентна. Цисти на бубрег, хидронефроза, пионефроза, неоплазми, траума на реналниот паренхим се едни од главните индикации за нефректомија. Целта на овој труд е да се прикаже успешна унилатерална нефректомија и уретеректомија кај кучка, раса лабрадор на возраст од 2,5 години поради пионефроза. Сопственикот забележал жолтеникав вагинален исцедок, придружен со симптоми на полиурија, полидипсија и намален апетит. По клиничкиот преглед беа забележани зголемена телесна температура, депресија и абдоминална болка. При ултразвучен преглед беше утврдена издолжена, опалесцентна ехогена структура во близина на мочниот меур и екстремно зголемен и исполнет со течност десен бубрег. Пациентот беше премедициран со и/м апликација на ацепромазин малеат. Хируршката анестезија беше индуцирана со и/в апликација на пропофол и одржувана со изофлуран. По абдоминална инцизија од ксифоидата до пубисот утврдена беше зголемена и издолжена структура на десниот уретер, почнувајќи од мочниот меур која се протегаше кранијално до екстремно зголемениот десен бубрег. Зголемениот уретер беше внимателно препариран и лигиран. Бубрегот заедно со уретерот беа отстранети по претходна двојна лигација на реналната артерија и вена. Раната беше затворена на стандарден начин. Успешното закрепнување како и подобрување на реналната филтрацијата беа потврдени со хематолошките и реналните тестови изведени 1та, 2та и 3та недела после операцијата.

Клучни зборови: нефректомија, уретеректомја

UDC: 636.7.09:616.711.6-001.5-089.84

CASE REPORT: MODIFIED SEGMENTAL SPINAL FIXATION TECHNIQUE FOR TREATMENT OF LUMBAR FRACTURE IN DOG

Pavlovski Damjan[1]

[1]*Veterinary Clinic "Animal Medica", Skopje, Macedonia*

ABSTRACT

A dog with a fracture of L-1 vertebra and symmetrical paralysis on the pelvic limbs was subjected to the surgical treatment using modified segmental spinal fixation technique. This technique provides a simple, versatile, and strong repair of vertebral fracture. Pin and wire application requires exposure of dorsal spinous processes and articular facets like in dorsal laminectomy procedures. After post-operatively analgesic, antibacterial treatment and appropriate physical therapy the function of the pelvic limbs is particularly improved. The Modified Segmental Spinal Fixation as a surgical technique has shown great results in the treatment of spinal traumatic injuries, it could be applied in various spinal injuries and is not limited to age and size of the patient. For fully recovery from the trauma a special post-operative treatment and care should be established.

INTRODUCTION

A 4 year male stray dog, mix breed (small terrier) was presented at the Veterinary clinic "Animal Medica" in Skopje, Macedonia. The dog lives in the yard of the owner. In the anamnesis the owner reported that the dog is unable to use his pelvic limbs. The dog didn't come for his regular meal for 3-4 days. On 16th of May it was found dragging his pelvic limbs and on the skin at the stifle area had deep laceration reaching almost the muscle tissue. The owner stated that the patient had not used one of the pelvic limb (not sure L or R) before it went missing for 3-4 days, what indicates progressive disease and possibility of deterioration of neurological symptoms due to spinal cord compression.

The examination showed evident neurological deficit with the following findings:

- Behavior, mental state and appetite were in normal range;
- Present symmetrical paralysis on the pelvic limbs;
- Sensory and reflex evaluation - absent deep pain perception on IV (fourth) toe - sciatic nerve, and moderately present on the I (first) toe - femoralis n. (both symmetrical). Completely absent skin sensorium on the lateral side of the limbs and moderately present on the medial side of the limbs and at the inguinal area. Proprioception, hopping reflex, as well as, visual and tactile placing were absent in both pelvic limbs. The existing patellar hyperreflexion on both sides indicated spinal cord injury at the level above L-4. There was absence of tail movement and partially present perianal sensorium. Pannicilus reflex absent in the line of L-3.
- Palpation on the abdomen demonstrated evident enlarged bladder and urine retention.

From the morphological aspect there was focal lesion at the L-1 level, indicating the following differential diagnosis: trauma, IVDD, FCEM and neoplasia. Ultrasound examination identified enlarged bladder, while the radiological examination determined enlarged radiopaque bladder and compressive trauma of the spinal cord resulting from the fracture of the body L-1 vertebra (image 1). Corresponding with the clinical findings the diagnosis *Fractura corpus vetrtebrae L1* was concluded.



Figure 1

Considering the type of the fracture and present sensorium, surgical stabilization of the fracture following dorsal decompression of the spinal cord affected area, and postoperative cage rest was suggested.

MATERIALS AND METHODS

The applied surgical technique for this case was *Modified Segmental Spinal Fixation*. This technique provides a simple, versatile, and strong repair of vertebral fracture. Pin and wire application requires exposure of dorsal spinous processes and articular facets as is it same for dorsal laminectomy procedures. Number and size of longitudinal and central pins used are dependent on size and activity of the patient and relative stability of the fracture. Generally, large patients with an unstable fracture are stabilized with relatively large central pins and a greater number of longitudinal pins (i.e., three to six pins). Moreover, central and longitudinal pin size may be varied and the length of longitudinal pins may be sequentially decreased to achieve a leaf spring effect. For further stiffness, muscular and tendinous attachments lateral to the articular facets are dissected free and a second set of pins is placed in a similar fashion lateral to the facets [1].

The dorsal laminectomy (image 2) is made with the incision over the dorsal midline to include two spinous processes cranial and caudal to the lesion. Using a periosteal elevator or small osteotome subperiosteally epaxial muscles are elevated from the dorsal spinous processes, laminae, articular facets, and pedicles of affected vertebrae. Gelpi retractors are used to facilitate gentle retraction of epaxial musculature during dissection. The exposed dorsal spinous processes are removed to the level of the dorsal lamina with large, single-action duckbill rongeurs. Using a pneumatic air drill the outer cortical (white) layer of bone is drilled from the lamina of both vertebrae. Articular processes of the cranial vertebrae are removed with the drill, working carefully so the cranial articular processes are left intact as much as possible. Drilling is continued to the dorsal lamina until the medullary layer of bone is encountered (it is red in appearance, soft, and easily drilled). This layer is carefully burred until the white inner cortical layer is visualized. The inner cortical layer is easily recognized in the midlaminar portion of each vertebral body; however, it becomes more difficult to recognize at the intervertebral space, where bone appears white throughout drilling. Before drilling over the intervertebral space, the interarcuate ligament is removed (i.e., ligamentum flavum and yellow ligament) using sharp dissection with a No. 11 Bard-Parker scalpel blade. A pneumatic bone is not used to drill because it tends to grab soft tissue and force the drill downward toward the vertebral canal. Drilling depth is estimated at the intervertebral space, first by reaching the inner cortical layer at the midlaminar portion of the vertebral body cranial and caudal to the interspace. Remaining bone at the intervertebral space is drilled to the same level. Once the inner cortical layers of both laminae have been reached, careful burring is used until soft periosteum can be palpated with a dental spatula. Burring is continued until periosteum is palpable over both vertebrae and the intervertebral space. Dental tool and Lempert rongeurs are used to gently penetrate periosteum, and carefully to prune away the remaining inner cortical layer. If necessary, the dorsal laminae can be undercut to gain additional exposure. A 2- to 3-mm diameter carbide or diamond burr is used to carefully drill away the inner layer of laminar bone [1].

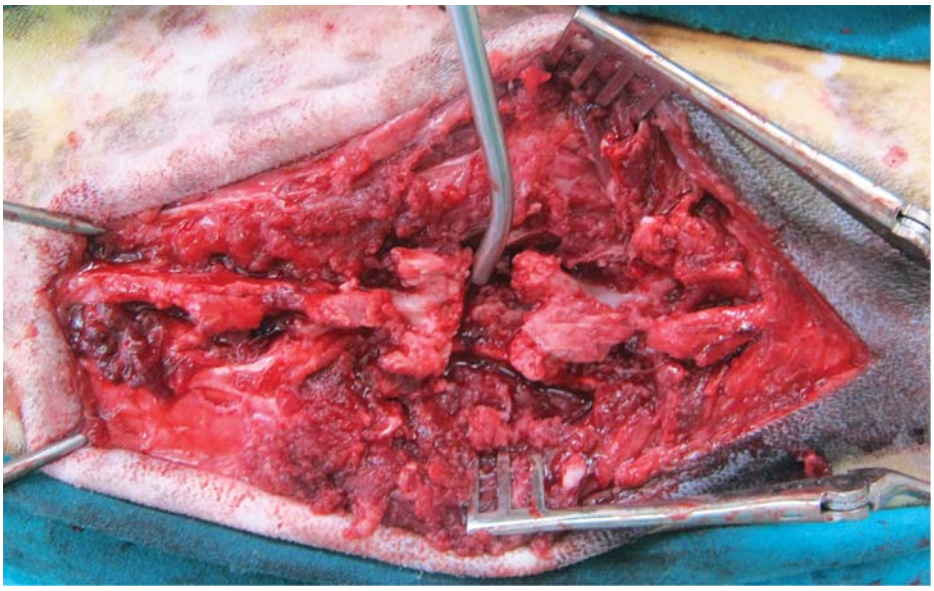


Figure 2

We exposed the two processes and articular facets, cranial and caudal to the fracture. First, a dorsal laminectomy was performed. The fracture was reduced and the fixation was maintained as previously described. Holes were drilled through the bases of articular facets, dorsal spinous processes, and tangentially through the dorsal lamina. The holes were large enough to accommodate 18- or 20-gauge orthopedic wire. Orthopedic wire was placed through each hole, leaving the ends long enough to wrap around several Steinmann pins. Two Steinmann pins were selected long enough to include two vertebrae cranial and caudal to the affected vertebra, called longitudinal pins. The ends of the longitudinal pins were bent at right angles to extend into the interspinous space. The longitudinal pins were placed in the space between dorsal spinous processes and articular facets. We posi-

tioned one central pin on each side of the dorsal spinous processes nearest the fracture. The ends of the central pins were bent to hook around the base of the dorsal spinous processes. The central and longitudinal pins were wired to the base of the articular facets, dorsal lamina, and dorsal spinous processes with the preplaced stands of orthopedic wire(image3). The surgical site was lavage with sterile saline, the devitalized muscle was debrided, the epaxial muscles were closed with a nonabsorbable monofilament suture - polypropylene, and subcutaneous tissue (PGA 1-0) and skin (Silk 2-0) was routinely closed.

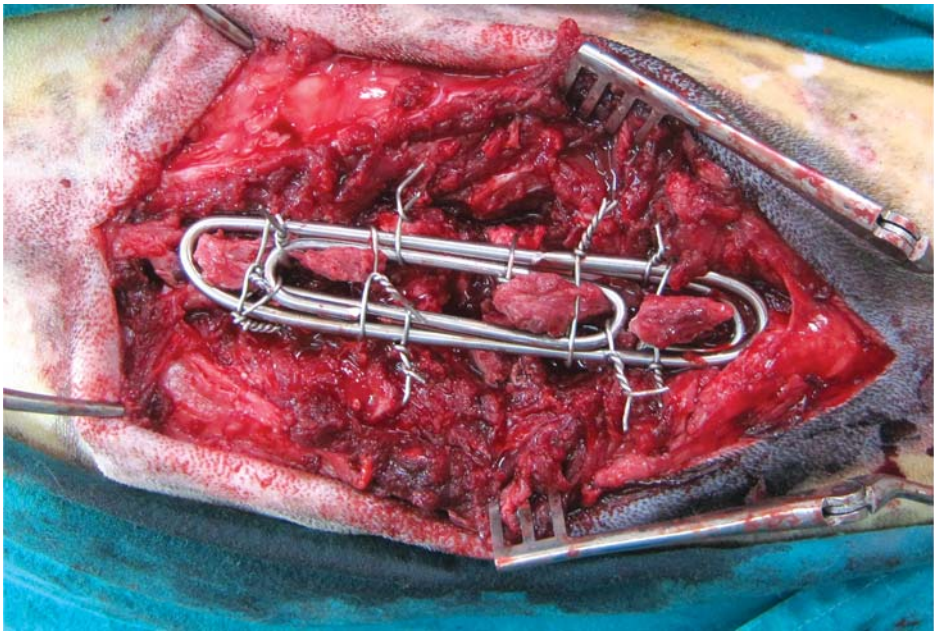


Figure 3

Regarding the anesthesia method, the patient was sedated with Xylazine, Fentanyl and Diazepam. Ketamine was used in premedication. The anesthesia was maintained with CRI mixture of Lidokaine, Fentanyl and Ketamine.

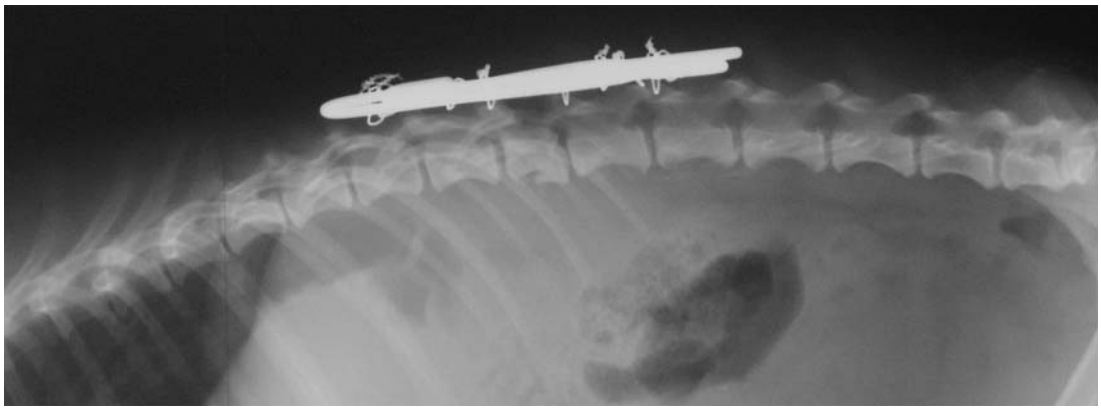


Figure 4



Figure 5

RESULTS

Following the surgery the patient was treated with Meloxicam at dose 0.2 mg/kg and Tramadol 5mg/kg q12. Tramadol was continued with the same dose for seven days, while Meloxicam was terminated. The antibacterial treatment (Amoxicilin + clavulante acid 20mg/kg q12) was applied for 2 weeks.

Additionally, the patient was put in cage rest for 2 weeks to minimize the activity, preventing the displacement of the fixator or further damage to the spinal cord. After the second week the patient started with confined short leash walks for the next 2 weeks. During the whole post-operative period, the bladder was manually emptied. In order to minimize the mechanical force to the implant until the bone started to heal, a physical therapy was suggested with passive range of motion and careful assisted standing and walking.

One week postoperatively the patient rip of the orthopedic wire from the T-12 spinous process, there wasn't any destabilization of the fracture. The patient started to gain relative control (still not fully controlled) over urinating and defecation, 6 weeks post operatively. The function of the pelvic limbs is particularly improved. The patient is still under physical therapy and additionally swimming exercises are introduced. Because the nerve tissue has long period of regeneration, the final improvement of the patient state is expected after 6 months with the fully effect of the treatment.

CONCLUSIONS

The Modified Segmental Spinal Fixation as a surgical technique has shown great results in the treatment of spinal traumatic injuries. This technique is not limited by the location of the spinal traumas or by the age and size of the animal. The procedure is easy to perform with no special equipment, providing a full recovery from the injury. Although, pin migration and fatigue fracture of orthopedic wire or pins may occur. Therefore special post-operative treatment should be performed in order to expect high results from this surgical technique.

REFERENCES

1. Theresa Welch Fossum (2007). *Small animal surgery*, Third ed. Mosby, Elsevier, St. Louis, Missouri, p1632

КЛИНИЧКИ СЛУЧАЈ: МОДИФИЦИРАНА СЕГМЕНТАЛНА ТЕХНИКА НА СПИНАЛНА ФИКСАЦИЈА ЗА ТРЕТМАН НА ЛУМБАЛНА ФРАКТУРА КАЈ КУЧЕ

Павловски Дамјан[1]

[1]Ветеринарна амбуланта "Анимал Медика", Скопје, Македонија

АПСТРАКТ

Куче со фрактура на Л-1 и симетрична парализа на задните екстремитети беше подложено на хируршки третман со примена на модифицирана сегментална техника на спинална фиксација. Оваа техника овозможува едноставна, хармонична и силна репарација на вертебралната фрактура. Апликацијата на пин и жица имаат потреба од експозиција на дорзалните спинални израстоци и артикуларните плочи слично како и при дорзална ламинектомија. По постоперативната апликација на аналгетици, антибактериската терапија и соодветна физикална терапија функцијата на задните екстремитети е значително подобрена. Модифицираната сегментална спинална фиксација како хируршка техника покажала одлични резултати при третман на спинални трауматски повреди, и истата може да се примени кај различни спинални повреди без ограничување во однос на возраста и големината на пациентот. За целосно опоравување од траумата, треба да се воспостави специјален пост-оперативен третман и нега.

UDC: 597.552.512-123.4:616-006.66

INTESTINAL ADENOCARCINOMA IN RAINBOW TROUT (*Oncorhynchus mykiss*)

Gombač Mitja[1], Seničar Marija[2], Švara Tanja[1], Žižek Suzana[1], Hristovski Mišo[3], Pogačnik Milan[1]

[1]*Institute of pathology, Forensic and Administrative Veterinary Medicine, Veterinary Faculty, Ljubljana, Slovenia*
[2]*Insitute for Breeding and Health Care of Game, Fish and Bees, Veterinary Faculty, Ljubljana, Slovenia*
[3]*Department of Biology and Pathology of Fish, Bees and Wild Animals, Faculty of Veterinary Medicine-Skopje, Ss.Cyril and Methodius, Skopje, Macedonia*

ABSTRACT
Intestinal tumours are uncommon in fish. To date, intestinal adenocarcinoma has been described in blue gularis (*Fundulopanchax sjostedti*) in Atlantic salmon (*Salmo salar*) and in rainbow trout (*Oncorhynchus mykiss*).
Intestinal adenocarcinoma with metastases into the mesentery in 4-year-old rainbow trout from a Slovene hatchery is described. A lump was felt in the posterior part of the abdominal cavity during stripping. The trout was euthanized and submitted for a necropsy, where a firm, whitish, irregularly lobular mass originating from the intestine was noticed. Histologically, the intestinal mass showed a prominent proliferation of tall columnar neoplastic epithelial cells arranged in dense irregular islands and papillotubular protuberances with metastases into the mesentery. The intestinal mucosa was severely chronically inflamed. The intestinal mass was *histopathologically* diagnosed as intestinal adenocarcinoma.
Key words: intestinal adenocarcinoma, rainbow trout, fish

INTRODUCTION

Although intestinal tumours are commonly described in domestic animals, they are rarely and only recently reported in fish (1). They usually show poor abilities for metastasis (2). To date, intestinal carcinoma has been described in zebrafish (*Danio rerio*) (3), blue gularis (*Fundulopanchax sjostedti*) (1) Atlantic salmon (*Salmo salar*) and in rainbow trout (*Oncorhynchus mykiss*) (2).

We report a single case of intestinal adenocarcinoma with metastases to mesentery in 4-year-old rainbow trout from a Slovene hatchery.

MATERIALS AND METHODS

A 4-year-old rainbow trout from a Slovene hatchery, in which a small lump was felt in the abdominal cavity during the stripping, was euthanized with an overdose of 2-phenoxyethanol (Fluka) and submitted for a necropsy. Samples from the mass originating from the intestine as well as samples from intestine, gills, kidney, spleen, liver, gonads and heart were immediately put in 10% buffered formalin for 24 hours at room temperature and were routinely paraffin embedded. Four µm thick sections were stained with haematoxylin and eosin (HE) and examined under a light microscope.

RESULTS

Anamnesis

A 4-year-old rainbow trout with a small lump in the posterior part of the abdominal cavity, noticed not before the first routinely performed stripping, originated from a Slovene hatchery with 2000 breeding 4-year old rainbow trouts. The trout was apparently healthy and there were no signs of clinical diseases or visible abnormalities in any of the other trouts. The animals are feed the approved commercial food for broodstock.

Macroscopic examination

The trout was in good body condition, measuring 55cm and weighing 2.07 kg. In coelomic cavity, a firm, whitish, irregularly lobular, apparently un-encapsulated mass, measuring 3.5 x 2.5 x 2 cm, originating from the posterior part of the intestine was noticed. The mass caused a partial occlusion of the lumen of the affected part of the intestine. No changes were noticed in other organs and tissues.

Microscopic examination

The intestinal mass showed a prominent proliferation of tall columnar neoplastic epithelial cells arranged in dense irregular islands and papillotubular protuberances infiltrating the submucosa. The neoplastic cells exhibited round to slightly oval nuclei with prominent nucleoli and abundant cytoplasm. Mitotic figures were rare. The stroma was scant. Small multifocal metastases were seen in the mesentery but not in other examined organs. The intestinal mucosa was severely chronically inflamed. The intestinal mass was histopathologically diagnosed as intestinal adenocarcinoma.

CONCLUSIONS

Intestinal adenocarinomas in fish are rare and they usually have poor ability for metastasis (1). To date, only metastases to the liver in Atlantic salmons are reported (2). Etiologic factors which are supposed to cause intestinal adenocarcinomas are poorly understood. Reported possible causes of intestinal adenocarcinomas

were: in 3-year-old blue gularis from an aquarium an advanced age of the fish (1), in 10.62% of Norwegian farmed 4-year-old Atlantic salmons and rainbow trouts the approved commercial diet (2) and in two zebrafish the nematode *Pseudocapillaria tomentosa* (3). Intestinal adenocarcinoma with metastases into mesentery in Slovene rainbow trout was accidentally discovered during stripping, so the cause remains unknown. Regular examinations of rainbow trouts from the hatchery in which the adenocarcinoma was diagnosed will be performed and if new cases of intestinal tumors are found, further investigations will be undertaken.

REFERENCES

1. Magi GE, Di Cicco E, Rossi G (2008). Spontaneus intestinal adenocarcinoma in a blue gularis *Fundulopanchax sjostedti*: an immunohistochemical study. Fish Pathology, 43(3), 128-131.
2. Dale OB, Torud B, Kvellestad A, Koppang HS, Koppang EO (2009). From chronic feed-induced intestinal inflammation to adenocrcinoma with metastases in salmonid fish. Cancer Research, 69(10), 4355-4362.
3. Kent MI, Bishop-Stewart JK, Matthews JL, Spitsbergen JM (2002). *Pseudocapillaria tomentosa*, a nematode pathogen, and associated neoplsams of zebrafish (*Danio rerio*) kept in research colonies. Comparative Medicine, 52(4), 354-358.

ЦРЕВЕН АДЕНОКАРЦИНОМ КАЈ ВИНОЖИТНА ПАСТРМКА (*ONCORHYNCHUS MYKISS*)

Гомбач Митја[1], Сеничар Марија[2], Швара Тања[1], Жижек Сузана[1], Христовски Мишо[3], Погачник Милан[1]

[1]Институт за патологија, судска и административна ветеринарна медицина, Ветеринарен факултет, Љубљана, Словенија,
[2]Институт за одгледување и здравствена заштита нариби, пчели и диви животни, Ветеринарен факултет, Љубљана, Словенија,
[3]Катедра за биологија и патологија риби, пчели и дивеч, Факултет за ветеринарна медицина Скопје, Универзитет „Св. Кирил и Методиј“Скопје, Македонија

АПСТРАКТ

Цревните тумори не се вообичаени кај рибите. До денес, цревниот аденокарцином е опишан кај златниот фазан (*Fundulopanchax sjostedti*), атлантскиот лосос (*Salmo salar*) и виножитната пастрмка (*Oncorhynchus mykiss*).

Опишан е цревен аденокарцином со метастази во мезентериумот кај 4 години стара виножитна пастрмка од словенско мрестилиште. За време на измолзување почуствувана е грутка во задниот дел на стомачната шуплина. Пастрмката е еутаназирана и оподложена на некропсија, при што е забележана цврста, белузлава неправилна топчеста маса потекнувајќи од цревата. При хистолошкото испитување, на цревната маса се утврди силна пролиферација на високо стеблести неопластични епителни клетки организирани во густи непшравилни островчиња и папилотубуларни израстоци со метастази во мезентериумот. Цревната мукоза беше силно воспалена со хроничен тек. Цревната маса, со патохистолошко испитување дијагностицирана е како цревен аденокарцином.

Клучни зборови: цревен аденокарцином, виножитна пастрмка, риба

UDC: 636.7.09:618.1-006.6-085.277

CANINE TRANSMISSIBLE VENERAL TUMOR – SURGICAL TREATMENT AND CHEMOTHERAPY: CASE REPORT

Atanaskova Petrov Elena[1], Nikolovski Goran[1], Ilievska Ksenija[2], Trojacanec Plamen[2], Celeska Irena[3], Velev Romel[4]

[1]Departement of Internal Diseases in Small Animals and Horses, Faculty of Veterinary Medicine - Skopje, R. Macedonia,
[2]Department of Surgery, Orthopedics and Ophthalmology, Faculty of Veterinary Medicine - Skopje, R. Macedonia,
[3]Department of Pathophysiology, Faculty of Veterinary Medicine - Skopje, R. Macedonia,
[4]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine - Skopje, R. Macedonia,

ABSTRACT

Canine transmissible venereal tumor (TVT) is a benign reticulo-endothelial tumor of the dog that mainly affects the external genitalia and occasionally the internal genitalia. The etiology appears to be cell transplant from affected to unaffected dogs. As it is usually transmitted during coitus, it mainly occurs in young, sexually mature animals. Gross findings of small nodule like lesions which bleed are the most consistent clinical finding. Smears made from the tumor reveal round cells with vacuoles and mitotic figures. The tumor is many times self limiting and vincristine sulfate is currently considered the most effective therapy. The aim of this study is to present a clinical case and efficiency of a combined surgical and chemotherapy treatment in a 3.5 years old male castrated dog with previous history of TVT. After the surgical treatment, chemotherapy was conducted with Vincristine sulfate (doses 0.025 mg/kg i.v. once weekly) for 4 consecutive weeks. During this period regular blood samples were taken for hematological examination. Side effects such as gastrointestinal upset, depression, hair loss and decrease in appetite were observed in the patient after the third treatment.This treatment resulted in complete remission of the TVT.

Key words: transmissible venereal tumor, surgical treatment, chemotherapy, vincristine sulphate, dog.

INTRODUCTION

Canine transmissible venereal tumor (TVT), also known as infectious sarcoma, venereal granuloma, transmissible lymphosarcoma or Sticker tumor is an important contagious neoplasm that commonly attacks the reproductive tract. This tumor widely spreads in free-roaming dogs. According to its localization, TVT can be classified into genital and extragenital (1). Genital TVT is transmitted via natural mating while extragenital TVT is occurred by social contact, like sniffing or licking. The clinical presentations for TVT are visible cauliflower-like mass in genital area or on skin surface with the presence of bloody discharge, ocular or nasal deformation from tumor invasion (2).

Most commonly used treatments for TVT are: surgery, radiation or chemotherapy**.** Surgical removal does not only provide unsatisfactory response but also causes tumor recurrence. Vincristine sulfate has been widely accepted as an efficient single chemotherapeutic agent for treatment of TVT (2). Vincristine sulphate is the salt of an alkaloid obtained from the common periwinkle (Vincarosa livia). It is also known as leurocristine. It has a wider application in human medicine in combined chemotherapy with other anti-tumour drugs. Vincristine sulfate acts by binding to tubulin dimer which is necessary for mitosis of spindle fibers, contributing to cellular division arrested in metaphase stage. The typical course of vincristine treatment is four to eight week of intravenous administration at 0.5 to 0.7 mg/m2 body surface area (BSA) or 0.025 mg/kg body weight (BW) (1; 3). However, side effects usually occur when the combined chemotherapeutics are used and recurrence is seen in cases treated by surgical removal (1).

The aim of this report is to demonstrate the efficiency of the combined surgical intervention and chemotherapy treatment, with minimal side effects to the patient.

MATERIAL AND METHOD

Case history and diagnosis. In November, 2011 3.5 year old male, mixed breed dog was admitted to the University Veterinary Hospital in Skopje, Republic of Macedonia with a history of bloody discharge from the prepuce. A year ago transmissible venereal tumor (TVT) had been diagnosed. The tumor has been removed surgically and the patient was castrated.

Physical examination revealed excellent general condition. Mild pain and diffuse swelling of the penis during palpation were noticed. A cauliflower red mass, located on the posterior portion of the penis around the bulbous was presented.

The mass was diagnosed as TVT by exfoliated cell cytology. The samples were smeared and stained with a commercial modified Giemsa staining. The cytology showed round-tooval shaped cells with increased ration between nucleus and cytoplasm, dense nucleolus and intracytoplasmic vacuoles suggesting TVT. Hematological and blood chemistry profile including blood urea nitrogen, creatinine and alkaline phosphatase were analyzed and defined as in normal range before the treatment started.

Treatment. Due to the recurrence of the TVT our treatment of choice was combination of surgical treatment with chemotherapy.

Surgical treatment. Premedication was achieved by i/m application of 0.2 mg/kg acepromazine maleat (Castran, Interchemie, Holland) while as induction and maintenance of anesthesia was achieved by i/v administration of 4 mg/

kg propofol (Propofol 1%, braun, Melsungen). The hair was clipped and prepuce and penis were lavaged with antiseptic solution. In order to prevent potential damage of the urethra, an indwelling urethral catheter was placed. Due to the size and localization of the tumor mass a lateral incision of mucocutaneous junction of the prepucium was made. Surgical excision of the penis mucosa along with the tumor mass was made (Figure 1). Part of a corpus spongiousum was excised in order to remove the all tumor (Figure 2). Interrupted absorbable suture 3/0 polyglicolid acid was placed to aposse the mucosa. Skin incision was closed with interrupted 2/0 polipropilen suture (Figure 3). The tumor was large, cauliflower red mass (Figure 4). The sutures were removed at the beginning of chemotherapy treatment (Figure 5).



Figure 1. *TVT around the bulbous of the penis.*

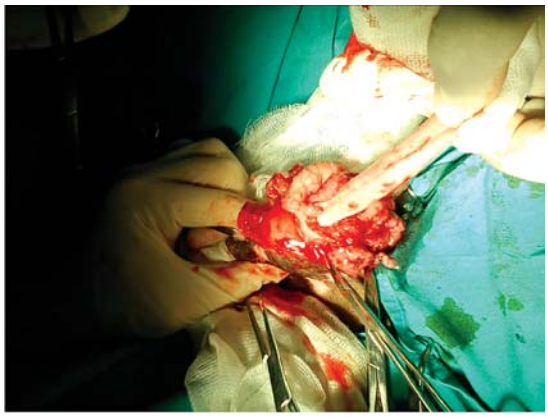


Figure 2. *Corpus spongiousum excised in order to remove the all tumor.*

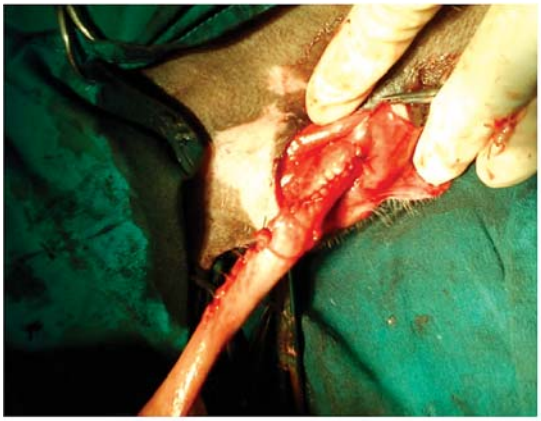


Figure 3. *Interrupted absorbable suture 3/0 polyglicolid acid was placed to aposse the mucosa.*

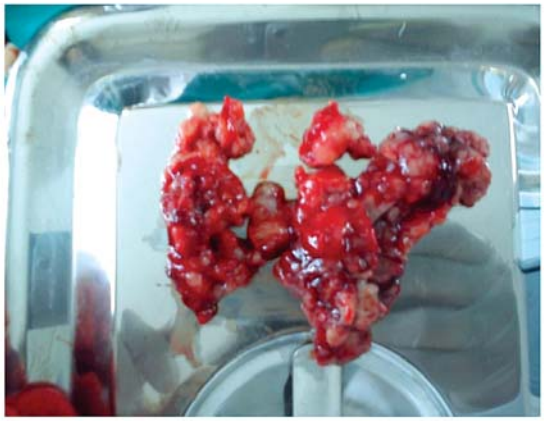


Figure 4. *The removed tumor mass*



Figure 5. *Prepucium after removal of the sutures*

Chemotherapy. A week after the surgery, chemotherapy was started with vincristine sulphate (Sindovin 1mg, Sindan, Romania) at doses 0.025 mg/kg by slow intravenous injection once a week, for 4 consecutive weeks. Supportive therapy was prescribed (vitamins and antiemetic). After the third treatment with vincristine sulfate, the patient showed side effects.

Hematologic examination. Blood samples were collected every week for hematological examination during the treatment program. Pre-treatment values were compared with those of post-treatment values recorded at day 7, 15, 23.

RESULTS

Clinical evaluation of the patient was made every week during the treatment. After the third treatment with vincristine, patient revealed mild anorexia, nausea and hair dullness. These symptoms disappeared a week after the last reatment, accompanied by significant improvement of the general health patient condition. During the all therapy weakly blood samples were taken for hematology examination. The results from the hematologic examination are present in table 1. There were no significant changes in the complete blood count during the treatment.

Table 1. *Hematological examination, results pre-treatment and during the treatment.*

			Findings			
Parameter	Unit	Referent values	Pre- treatment	Day 7	Day 15	Day 23
RBC	* 10^{12}/L	5.5-8.5	6.16	6.05	7.01	6.78
PVC	%	37-55	44.9	45.6	52.0	49.9
Hb	g/dl	12-18	16.1	13.6	16.4	15.7
MCV	fL	60-77	72.9	75.5	74.2	73.5
MCH	Pg	19-25	26.1	22.4	23.4	23.2
MCHC	%	32-36	35.9	29.8	31.5	31.5
PLT	* 10^9/L	200-500	306	147	379	301
WBC	* 10^9/L	6.0-17.0	11.1	15.6	11.3	10.3

After the last treatment, the patient was brought to the hospital every month for regular check ups. Six months after the treatment no visible side effect from the chemotherapy treatment were observed.

DISCUSSION

In our case we successfully combined the surgery and chemotherapy treatment with reversible mild side effects. Similarly, other authors used combined treatment of surgery and chemotherapy with vincristine sulphate. They have noticed 100% regression. They have also concluded that this is the most effective treatment, with minimal side effects (4).

In cases of generalized TVT, standard surgical removal is not the best option. If the tumour is resectable, electrosurgical excision or cryosurgical treatment (5) is desirable because the tumour can be easily transplanted in the surgical wounds when traditional operative methods are used. Furthermore, recurrence following traditional surgery is not uncommon. Minimal recurrence was noticed when castration or ovario-hysterectomy was combined along with chemotherapy (6). During the surgery on male dogs, maximal care should be taken to avoid eventual damage of the urethra. If the urethral ori¢ce is involved, an indwelling urethral catheter should be used until the area has healed.

Vincristine sulphate at the rate of 0.025 mg/kg body weight intravenously at weekly intervals on 3 to 4 occasions is the most effective, safe and convenient chemotherapeutic agent, giving a better survival time even in TVT patients with extra genital metastasis, according to many authors (7, 8, 9).

TVT is the most prevalent neoplasia of the external genitalia in dogs from tropical and sub-tropical areas. A bloody discharge is the most commonly observed clinical symptom by the owner's. Diagnosis is based on typical clinical and cytological findings. (2) It is very important to make an early diagnosis and starting the treatment of the tumor in order to prevent further transmission to other dogs.

REFERENCES

1. Das, U. and Das, A.M. 2000. Review of canine transmissible venereal tumor sarcoma. Vet Res Comm. 24: 545-556.
2. Mello Martins, M.I., Ferreira de Souza, F., and Gobello, C. 2005. The canine transmissible venereal tumor: etiology, pathology, diagnosis and treatment. In: Recent Advances in SmallAnimal Reproduction. P.W. Concannon, G. England, J. Veretgegen, C. Linde-Forsberg (eds). International Veterinary Information Service, Ithaca NY (www.ivis.org) Apr 25, 2005: A1233.0405.
3. Kunakornsawat, S., Imsilp, K., Yatbanthoong, N., Ratanapob, N., Supsavhad, W., Sreesampan, S., and Nuklang, G. 2009. Vincristine chemotherapeutic treatment for transmissible venereal tumor in 60 dogs. KPS J. 8(2): 79-91.
4. Athar M., Suhail A., Muhammad G., Shakoor A.and Azim F. 2001. Pakistan Vet. J., 21 (1): 39-43.
5. Idowu, A.L.. Cryosurgery of canine transmissible venereal tumour. Tropical Veterinarian, 3, 74-78 Jackson, C., 1944. The cytology of the contagious (venereal) tumour of the dog. Onderstepoort Journal of Veterinary Science, Animal Industries, 20: 1985, 97-118.
6. Pandey, S.K., Chandpuria, V.P., Bhargava, M.K. and Tiwari, S.K., 1989. Incidence, treatment, approach and metastasis of canine transmissible venereal sarcoma. Indian Journal of Animal Sciences, 59, 510- 513.

7. Das, A.K., Das, U. and Das, D., 1991. A clinical report on the efficacy of vincristine on canine transmissible venereal sarcoma. Indian Veterinary Journal, 68, 575-576.
8. Maiti, S.K., Roy, S., Ali, S.L. and Ghosh, R.C., 1995. Therapeutic management of TVT with vincristine in a dog - a case report. Indian Veterinary Journal, 72, 614-615.
9. Singh, J., Pangaonkara, G.R., Singla, V.K., Kochhar, H.P.S. and Nanda, A.S., 1997. Efect of geriforte supplementation on blood chemistry in transmissible venereal tumour afected dogs treated with vincristine sulphate. Indian Veterinary Journal, 74, 420-421

ТРАНСМИСИВЕН ВЕНЕРИЧЕН ТУМОР КАЈ КУЧИЊА – ХИРУРШКИ ТРЕТМАН И ХЕМОТЕРАПИЈА: КЛИНИЧКИ СЛУЧАЈ

Атанаскова Петров Елена[1], Николовски Горан[1], Илиевска Ксенија[2], Тројачанец Пламен[2], Целеска Ирена[3], Велев Ромел[4]

[1]Катедра за внатрешни болести кај милници и копитари, Факултет за Ветеринарна Медицина - Скопје, Р. Македонија,
[2]Катедра за хирургија, ортопедија и офталммологија, Факултет за Ветеринарна Медицина - Скопје, Р. Македонија,
[3]Катедра за патофизиологија, Факултет за Ветеринарна Медицина - Скопје, Р. Македонија,
[4]Катедра за фармакологија и токсикологија, Факултет за Ветеринарна Медицина - Скопје, Р. Македонија.

АПСТРАКТ
Трансмисивен венеричен тумор кај кучиња (ТВТ) е бениген ретикуло-ендотелен тумор кој најчесто ги зафаќа надворешните полови органи, а поретко и внатрепните полови органи. Начинот на негово пренесување е со трансплатација на клетки од заразено на здраво животно. Најчесто се пренесува при парење, па затоа се појавува кај млади, полово зрели животни. Најчестиот клинички наод е присуство на мали лезии во вид на јазолчиња кои крварат. Размаските направени од ткивото на туморот откриваат округли клетки со вакуоли и митотски форми. Во многу случаи туморот може да биде со самоограничувачки карактер и винкристин сулфатот се смета за најефикасен за негова терапија. Целта на ова истражување е да се прикаже еден клинички случај и ефикасноста на комбинираната терапија од хируршко отстранување и хемотерапија кај 3.5 годишно, кастрирано машко куче, со претходна историја на TVT. После хируршкото отстранување, започната е хемотерапија со Винкристин сулфат (во доза од 0.025 мг/кг и/в, еднаш неделно) во текот на 4 недели. За време на терапијата, се вршени редовни анализи на крвната слика. После третата доза се забележани несакани ефекти како гасторинтестинални пореметувања, депресија, опаѓање на влакна и намален апетит. Овој терапевтски протокол резултира во комплетно отстранување на туморот.
Клучни зборови: трансмисивен венеричен тумор, хируршко отстранување, хемотерапија, винкристин сулфат, куче.

UDC: 636.7.09:[616.98:579.881.3

A CASE REPORT OF CANINE MONOCYTIC EHRLICHIOSIS IN 6 YEARS OLD MALE SIBERIAN HUSKY

Dimeski Z.[1], Josheski M.[1] and Trajanoska B.[1]

[1]*Veterinary clinic and pet shop "Pet-Land"Prilep, Macedonia*

ABSTRACT

The etiologic agent of canine monocytic ehrlichiosis (CME), the rickettsia Ehrlichia canis (E. canis), is a small pleomorphic gram-negative coccoid bacteria that parasitizes in circulating monocytes intracytoplasmically in clusters of organisms called morulae.[1] Ehrlichia canis is the most common etiological agent in dogs and is known to cause canine monocytic ehrlichiosis, a systemic disorder manifested by fever, hemorrhagic tendencies associated with thrombocytopenia and platelet dysfunction and non-regenerative anemia.[2] The disease is transmitted through the saliva of the brown dog tick, Rhipicephalus sanguineus, and has a worldwide distribution. Canine monocytic ehrlichiosis is manifested by a wide variety of clinical signs that can be categorized into acute, subclinical and chronic phases, although in endemically infected countries it is difficult to classify clinical cases into such distinct stages. In this case is described the diagnosis and treatment of canine monocytic ehrlichiosis in a male dog of Siberian Husky`s breed, aged about 6 years, with signs of epistaxis, depresion, weight lose and nasal discharge. Disease progressed from subclinical to chronical, with no evidence of E. canis morulae in mononuclear cells. After negative result of intranuclear morulae in the blood smear, a computed tomography and magnet resonance imaging was made for differential diagnosis of canine monocytic ehrlihiosis and intranasal tumors.

Key words: canine monocytic ehrlichiosis, siberian husky, ehrlichiosis, CME.

INTRODUCTION

In this case is described the diagnosis and treatment of canine monocytic ehrlichiosis in a male dog of Siberian Husky`s breed, aged about 6 years, with signs of epistaxis, depresion, weight lose and nasal discharge. Canine monocytic ehrlichiosis is a disease, caused by the obligate, intracytoplasmatic parasite Ehrlichia canis, with transmission through the saliva of the brown dog tick, *Rhipicephalus sanguineus.*

The genus Ehrlichia, because of certain genetic affinities between its species, has been divided into three genogroups. Genogroup I incorporates three species i.e. E. canis, E. chaffeensis, and E. ewingii. Genogroup II includes E. phagocytophila, E. equi, and the human granulocytic ehrlichiosis (HGE) agent. Genogroup III covers two species: E. sennetsu and E. risticii. The name of each genogroup is consistent with the name of the first species described. Ehrlichia canis is the most common etiological agent in dogs and is known to cause canine monocytic ehrlichiosis (CME), a systemic disorder manifested by fever, hemorrhagic tendencies associated with thrombocytopenia and platelet dysfunction, and non-regenerative anemia.

Ehrlichia canis is an obligate, intracytoplasmic parasitic disease that affects the canidae and is the causative agent of canine monocytic ehrlichiosis. The disease is also known as canine rickettsiosis, canine hemorrhagic fever, tracker dog disease, canine tick typhus, Nairobi bleeding disorder and tropical canine pancytopenia, names representing different aspects of the same disease. The disease was first described in Algeria in 1935 by *Donatien and Lestoquard*.[3] Since then, it has been reported worldwide, causing extensive morbidity and mortality among domestic dogs and other canids. At present, it is widely distributed around the world, particularly in tropical and subtropical areas.The disease is transmitted through the saliva of the brown dog tick, *Rhipicephalus sanguineus*, and has a worldwide distribution. Recently it was also shown to be experimentally transmitted by *Dermacentor variabilis*, the American dog tick.[4] Throughout feeding, ticks inject Ehrlichia canis-contaminated salivary gland secretions into the feeding site. Once an animal is infected, the syndrome progresses through several phases: acute, subclinical and chronic. Each stage can be characterized by an assortment of clinical and hematologic abnormalities. The most prevalent hematological abnormality in all stages of the disease is thrombocytopenia, approximately 84% of all cases.[5]

The vector tick contaminates the feeding sites with salivary secretions during blood sucking. The incubation period of E. canis varies from 8 to 20 days, during which time the organisms multiply in macrophages of the monocytic phagocytic system throughout the body, especially in the liver, spleen and lymph nodes. The incubation period for tick-borne fever is 5 to 14 days in naturally infected animals, and 2 to 6 days after experimental transmission in blood.[4] Canine monocytic ehrlichiosis is manifested by a wide variety of clinical signs that can be categorized into acute (1–3 weeks), subclinical (average 11 weeks) and chronic phases, although in endemically infected countries it is difficult to classify clinical cases into such distinct stages. The disease causes significant changes in hematological parameters along with bone marrow suppression and/or destruction and depending of the stage of the disease is associated with a deficiency in production of one or more blood elements.[6]

Clinical signs associated with ehrlichiosis are vague, including fever, anorexia, depression, weight loss, ocular and nasal discharge, lymphadenomegaly, hepatomegaly, and splenomegaly. Bleeding tendencies (petechia, ec-

chymoses, and epistaxis) may be present. When a dog is infected by E. canis, the disease may progress through three subsequent phases: acute, subclinical and chronic. Each phase is characterized by various degrees of clinical and hematologic abnormalities. The severity of clinical signs in the acute phase vary from mild to severe, and the symptoms include non-specific clinical signs including fever, anorexia, weight losses, depression, dyspnea, ocular disturbances, petechiae, ecchymoses and epistaxis as well as neurological disorders. Thrombocytopenia, mild anemia, and mild leucopenia are among the hematological abnormalities seen in the acute phase. The disease may progress to the subclinical phase, lasting for years in the absence of an appropriate treatment protocol. The diseased animals in the subclinical phase appear clinically healthy although mild thrombocytopenia may still exist. The chronic phase in its severe form is associated with pancytopenia that results from bone marrow hypoplasia and deficiency in bone marrow derived blood elements. Dogs with pancytopenia suffer from severe nonregenerative anemia, leucopenia, and thrombocytopenia. The diseased dogs in this stage do not respond to antibiotic treatment and subsequently die of secondary infections and bleeding.[6]

Some dogs that recover clinically from the acute phase remain subclinically infected for months or years. During the subclinical stage, an infected dog can clear the parasite, remain infected but asymptomatic, or develop chronic disease. There may also be progressive deterioration in the hematologic values during this stage. The conditions leading to the development of the chronic phase are unknown. Bleeding disorders occur frequently, and may result in pale mucous membranes, petechiae, ecchymoses, epistaxis, hematuria or melena. Pancytopenia can occur, and may lead to secondary infections. Death can occur as a consequence of hemorrhages or secondary infections.[4]

For unknown reasons the disease may progresses and enters the chronic stage during which the animal may develop severe pancytopenia, as well as secondary pulmonary hemorrhage, thromboembolism, hepatomegaly, splenomegaly, renal and reproductive disease, polyarthritis, anterior uveitis, retinal disorders, meningoencephalitis, and death as a result of hypotensive shock.[6]

Immunocompetent dogs may eliminate the infection during subclinical period, but some will eventually develop the chronic phase of the disease, characterized by severe bone marrow aplasia (myelosuppression), peripheral blood pancytopenia and high mortality due to septicemia and/or severe bleeding.[7]

Depression, anorexia, mucosal pallor, bleeding tendency, fever or hypothermia, lymphadenomegaly, splenomegaly and ocular abnormalities are prominent clinical manifestations in the spontaneous myelosuppressive canine monocytic ehrlichiosis. For instance, bleeding diathesis is more common and severe in the chronic phase of canine monocytic ehrlichiosis. It is mainly expressed as superficial bleeding such as cutaneous and mucosal petechiae and ecchymoses, hyphaema, epistaxis, haematuria, melena and prolonged bleeding from venipuncture sites due to the impairment of primary haemostasis.[7]

Despite a variety of clinical and hematologic abnormalities identified in dogs infected with E. canis, a definitive diagnosis can be challenging. Diagnosis is usually made on the basis of a combination of clinical signs, hematologic abnormalities and serologic findings. Several methods exist to correctly diagnose a case of canine monocytic ehrlichiosis. Examination of a peripheral blood smear for the presence of E. canis morulae in mononuclear cells is a viable option of diagnosis. Morulae are most often found in the acute phase of the disease. However, the sensitivity of this diagnostic method is poor since the morulae are found in only 4% of the positive cases.[8]

Ehrlichiosis is usually treated with the tetracycline antibiotics. In dogs, chloramphenicol and other drugs are also used occasionally. One report described the successful treatment of a dog with severe chronic canine monocytic ehrlichiosis, using a combination of hematopoietic growth factors, low dose vincristine, doxycycline and glucocorticoids.[4] Doxycycline, a semi-synthetic tetracycline, has been the first-line drug in the treatment of canine monocytic ehrlichiosis. While in the acute canine monocytic ehrlichiosis doxycycline has been shown to be very effective in eliminating the infection, its effectiveness in the suclinical and chronic E. canis infection is still controversial. There is currently limited evidence based justification for using other tetracyclines (minocycline, tetracycline, oxytetracycline), chloramphenicol, enrofloxacin, or imidocarb dipropionate in the treatment of E. canis infection. Other drugs with known efficacy against E. canis include tetracycline hydrochloride, oxytetracycline, minocycline and chloramphenicol. Supportive treatment should include multi-vitamin supplements. In severe cases blood transfusions should be given.[7]

Ehrlichiosis can be prevented by controlling the tick vectors.The prognosis depends on the stage of the disease. Dogs in the acute stage of ehrlichiosis usually respond within 24 to 72 hours to treatment, and the prognosis is favorable. Dogs in the subclinical stage may require prolonged treatment. Canine monocytic ehrlichiosis is difficult to cure once it reached the chronic stage.[4]

MATERIALS AND METHODS

Complete blood count was performed in blood samples in EDTA tubes using an automatic blood analyzer calibrated specifically for canine blood. After performed complete blood count and continued bleeding from the nose, computed tomography and magnetic resonance imaging were performed to make a differential diagnosis from tumors in the nasal cavity.

RESULTS AND DISCUSSION

After performing a complete blood count, the blood examination demonstrated severe non-regenerative anemia [packed cell volume (PCV): 9.6%] and low hemoglobin level (3.2g/dl), thrombocytopenia, leukocytosis and monocytosis. The blood smear doesn`t gave positive result of intracytoplasmic morulae. Diagnosing of ehrlichiosis through blood smear analysis is difficult because intracytoplasmic morulae are only occasionally seen during the acute phase of the disease.[3]

Serological tests (dot-ELISA and IFA) are the method most commonly used for veterinary diagnosis. Serological detection of E. canis antibodies can be done through indirect immunofluorescence antibody (IFA) test, which is considered the serological ''gold standard'', or using commercial serological tests for E. canis immunoglobulin-G (IgG) antibodies.[9] However, the fact that an animal is seropositive does not mean that it is sick. Since the presence of antibodies reveals exposure to the agent, PCR may help in reaching a diagnostic conclusion. The IFA test was negative in this case.

Table 1. *Hematological finding in the 6 year-old Siberian husky, on the day of receiving in the clinic and 3 days after the initial treatment.*

Analysis	Results on day 0	Results after 3 day of treatment	Reference range
Erytrocytes	1.48	2.14	6.5±0.45*100.000/µl
Leucocytes	58.9	42.7	8.5±1.12*10^3/µl
Trombocytes	107	92	450±17.61*10^3/µl
Hemoglobin	3.2	6.0	16±1.12 g/dl
HCT	9.6	17.9	40.0±2.42%
Lymphocytes %	30.0	32.7	3.4±0.15*10^3/µl
Monocytes %	8.2	5.3	1-5%
Segmented granulocytes	61.8	62	60-77%
MCV	81.3	83.6	60-70 fl
MCH	27.1	28.0	12.0-30.0pg
MCHC	33.3	33.5	30-35%

Baba K. et al. (2011) in the study of 19 dog, 18 had mild anemia [packed cell volume (PCV): 29.5%], leukocytosis (25,600/µl) with a left shift, thrombocytopenia (78,000/µl), hyperproteinemia (8.8 g/dl), and increased levels of blood urea nitrogen (BUN: 74.5 mg/dl) and alkaline phosphatase (ALP: 2,073 IU/l). Complete blood count (CBC) and serum biochemical findings included non-regenerative anemia (PCV: 21%), thrombocytopenia (27,000/µl), hypoalbuminemia (1.5 g/dl), and increased levels of BUN (50.2 mg/dl), ALP (1,090 IU/l), and C-reactive protein (CRP: >20 mg/dl).[2]

Waner T. and Harrus S. (2000) found that thrombocytopenia is the most common and consistent hematological finding in acute canine monocytic ehrlichiosis. A concurrent significant increase in the mean platelet volume is also usually seen reflecting active thrombopoiesis. Mild leukopenia and mild anemia (usually normocytic, normochromic, non-regenerative) commonly occur in the acute stage of the disease. Mild thrombocytopenia is a common finding in the subclinical stage of the disease. Numerous diseases can result in thrombocytopenia. These include immune-mediated thrombocytopenia, neoplastic processes, inflammatory diseases or other infectious agents. A decline in the neutrophil counts may occur. Erythrocyte parameters are not normally affected during this stage of the disease. Severe thrombocytopenia, leukopenia and anemia are most frequently seen during the chronic stage of canine monocytic ehrlichiosis. Severe pancytopenia is the hallmark of the severe chronic phase, occurring as a result of suppressed hypocellular bone marrow.[1]

In one study of 19 dogs with chronic (myelosuppressive) naturally-occurring perfomed by Mylonakis M., Siarkou V. and Koutinas A. (2010), shown that most commonly found clinical manifestations of canine monocytic ehrlichiosis are depression, bleeding diathesis, mucosal pallor and anorexia (Table 1). Most commonly found haematology abnormalities in 19 dogs with chronic (myelosuppressive) naturally-occurring are trombocytopenia, anemia, lymphopenia, leucopenia, neutropenia and panleucopenia (Table 2).[7]

Table 2. *Common clinical abnormalities in 19 dogs with chronic (myelosuppressive) naturally-occurring canine monocytic ehrlichiosis.*

linical manifestation	No. with finding/No. tested (%)
Depression	19/19 (100)
Bleeding diathesis	19/19 (100)
Mucosal pallor	18/19 (95)
Anorexia	18/19 (95)
Fever	10/19 (53)
Lymphadenomegaly	9/19 (47)
Weight loss	6/19 (32)
Splenomegaly	6/19 (32)
Hypothermia	5/19 (26)
Ocular discharge	2/19 (11)
Respiratory distress	4/19 (21)
Uveitis	4/19 (21)
Tick infestation	3/19 (16)

***Table 3**. Common haematological abnormalities in 19 dogs with chronic (myelosuppressive) naturally-occurring canine monocytic ehrlichiosis.*

Haematology	No. with finding/No. tested (%)
Thrombocytopenia	19/19 (100)
Anaemia	19/19 (100)
Lymphopenia	18/19 (95)
Leucopenia	17/19 (89)
Neutropenia	17/19 (89)
Pancytopenia	17/19 (89)

Computed tomography and magnetic resonance imaging were performed to make a differential diagnosis from tumors in the nasal cavity and meningoencephalitis in dogs with canine monocytic ehrlichiosis. Computed tomography show soft-tissue proliferation in the left nasal cavity with complete bone base in the cavity. There weren`t evidence of bone destruction, so differential diagnosis was made from intranasal neoplasia (Picture 1 and 2).

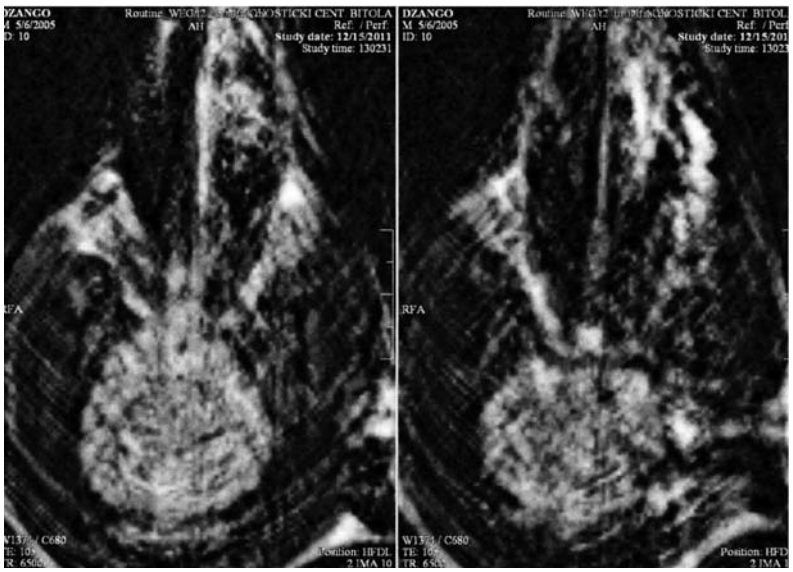


***Figure 1.** Soft-tissue proliferation, 4 cm long in the left nasal cavity with complete bone base in the cavity, with normal pneumatisation of other sinusal cavities.*

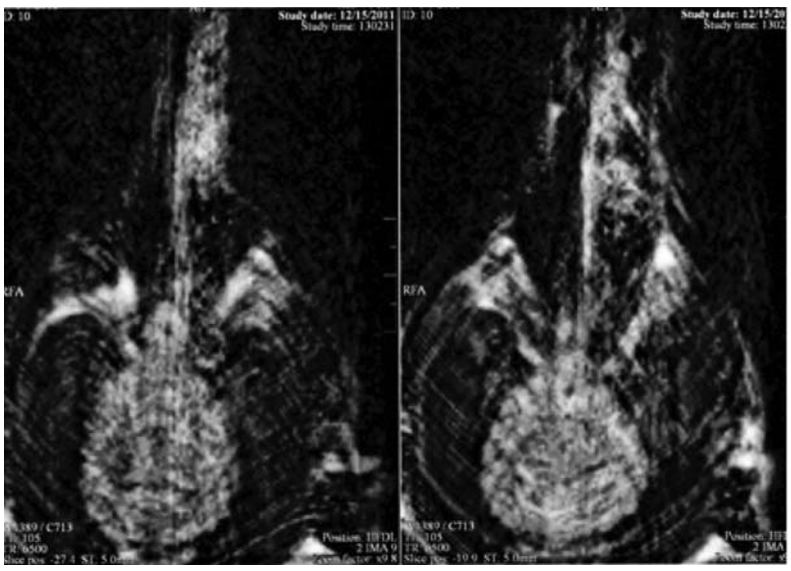


***Figure* 2.** *Soft-tissue proliferation in the left nasal cavity with complete bone base in the cavity.*

Magnetic resonance imaging was performed after computed tomography. MRI shown soft-tissue proliferation, 2 cm from the opening of the nasal cavity in caudal direction. This tissue was long 4-5cm and there weren`t signs of bone proliferation and destruction. So, the intranasal neoplasia, such chondrosarcoma and osteosarcoma were differentiated from this tissue. Other changes in the brain, such meningitis and meningoencephalitis were not presented (Picture 3).

Lang G. L. et al. (2011) found with MRI various meningoencephlitides in dogs, with areas of abnormal brain signal intensity (T2 hyperintense ± T1 hypointense), contrast enhancement of brain parenchyma and meninges, loss of cortical gray/white matter demarcation, mass effect, and space occupying lesions. In this case, there weren`t any changes in the brain.[10]

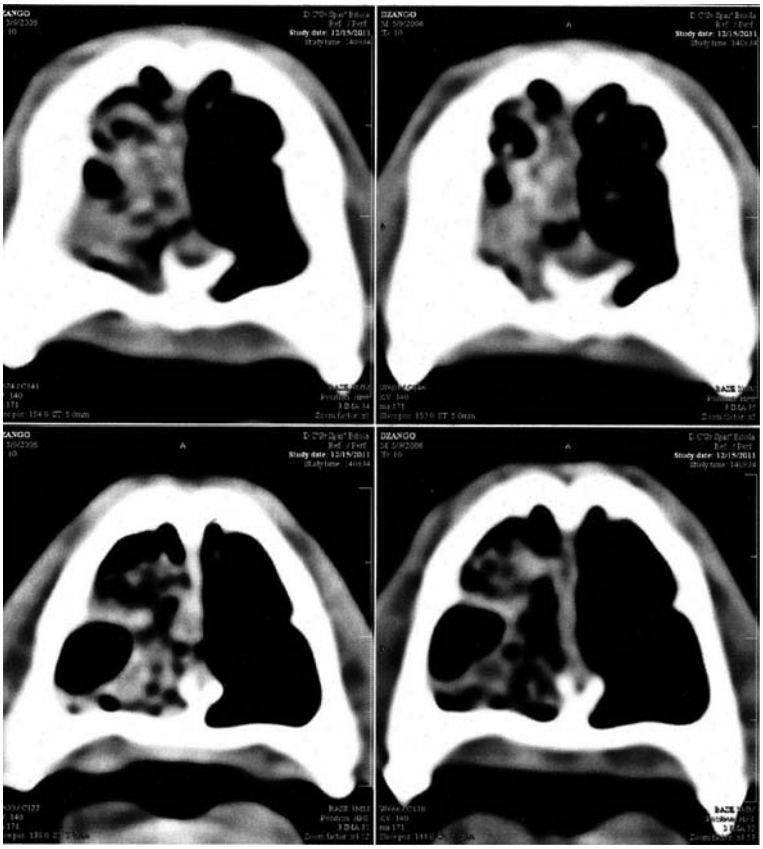


Figure *3.* *MRI from 6-years old Siberian husky presented with epistaxis and bleeding diathesis, with intranasal soft-tissue mass, long 4-5 cm.*

Ehrlichiosis is usually treated with the tetracycline antibiotics.[4] After treatment with antibiotics based on tetracyclines, like Limoxin 1ml per 15 kg body weight, transfusion of blood from other dogs and high doses of vitamin K (0.5 ml per day) and vitamin C (2ml per day), the bleeding from the nose stopped and the dog react well on the treatment. After 2 months of treatment, the bleeding appear again, with more intensive signs of canine monocytic ehrlichiosis. Pets owner wanted dog to be euthanized.

CONCLUSION

Ehrlichia canis is the most common etiological agent in dogs and is known to cause canine monocytic ehrlichiosis, a systemic disorder manifested by fever, hemorrhagic tendencies associated with thrombocytopenia and platelet dysfunction, and non-regenerative anemia. The disease may progress through three subsequent phases: acute, subclinical and chronic. Each phase is characterized by various degrees of clinical and hematologic abnormalities. Most common hematological findings in dogs with canine monocytic ehrlichiosis are non-regenerative anemia and low hemoglobin level, thrombocytopenia, leukocytosis and monocytosis. Mild thrombocytopenia is a common finding in the subclinical stage of the disease. Diagnosis is usually made on the basis of a combination of clinical signs, hematologic abnormalities and serologic findings. Examination of a peripheral blood smear for the presence of E. canis morulae in mononuclear cells is a viable option of diagnosis. Diagnosing of ehrlichiosis through blood smear analysis is difficult because intracytoplasmic morulae are only occasionally seen during the acute phase of the disease. Serological tests (dot-ELISA and IFA) are the method most commonly used for veterinary diagnosis. However, the fact that an animal is seropositive does not mean that it is sick. The use of computed tomography and magnetic resonance imaging is useful in differential diagnosis of canine monocytic ehrlichiosis and epistaxis in the diagnosis of intranasal neoplasia in dogs.

REFERENCES

1. Waner T. and Harrus S. (2000); "Canine Monocytic Ehrlichiosis (CME)"; In Recent Advances in Canine Infectious Diseases. International Veterinary Information Service;
2. Baba K. et al. (2011); "Ehrlichia canis infection in two dogs that emigrated from endemic areas"; The Journal of Veterinary Medical Science;
3. Leiva M., Naranjo C. and Peña T. M. (2005); "Ocular signs of canine monocytic ehrlichiosis: a retrospective study in dogs from Barcelona, Spain"; Veterinary Ophthalmology 8, 6, 387–393
4. Ehrlichiosis-Institute for International Cooperation in Animal Biologics, an OIE Collaborating Center Iowa State University College of Veterinary Medicine and Center for Food Security and Public Health, College of Veterinary Medicine Iowa State University;
5. Tuna E. G. and Ulutas B. (2009); "Prevalence of Ehrlichia canis infection in trombocytopenic dogs"; Lucrări Stiinifice Medicină Veterinară Vol. XLII, 2009, Timisoara;
6. Yağci B. Ba. et al. (2010); "The spread of canine monocytic ehrlichiosis in Turkey to Central Anatolia"; Israel Journal of veterinary medicine Volume 65 (1);
7. Mylonakis M., Siarkou V. and Koutinas A. (2010); "Myelosupressive caninemonogytic ehrlichiosis (Ehrlichia canis): An update on the pathogenesis, diagnosis and management"; Israel Journal of the veterinary medicine; Volume 65 (4) 2010;
8. Bulla C. et al. (2004); "The relationship between the degree of thrombocytopenia and infection with Ehrlichia canis in an endemic area"; Vet. Res. 35 (2004) 141–146;
9. Muhairwa P. A. et al. (2012); "Seroprevalence and factors affecting canine monocytic ehrlichiosis and canine brucellosis in Tanzania"; Res. Opin. Anim. Vet. Sci., 2012, 2(3), 181-188.
10. Lang G. L. et al. (2011); "Magnetic Resonance Imaging Lesions in the Central Nervous System of a Dog with Canine Monocytic Ehrlichiosis"; Case Reports in Veterinary Medicine Volume 2011, Article ID 379627, 5 pages;

ПРИКАЗ НА СЛУЧАЈ НА КУЧЕШКА МОНОЦИТНА ЕРЛИХИОЗА КАЈ 6 ГОДИНИ СТАР МАШКИ СИБИРСКИ ХАСКИ

Димески З.,[1] Јошески М.[1] и Трајаноска Б[1]

[1]Ветеринарна клиника и пет шоп "Пет-Ленд" Прилеп, Македонија

АПСТРАКТ

Етиолошкиот причинител на кучешката моноцитна ерлихиоза (CME), рикецијата Ehrlichia canis (E. canis), е мала плеоморфна грам негативна кокоидна бактерија која паразитира интрацитоплазматски во циркулирачките моноцити во групи на организми наречени морули. Ehrlichia canis е најчест етиолошки причинител кај кучиња и е познат по предизвикување на кучешката моноцитна ерлихиоза, системско нарушување кое се манифестира со треска, хеморагични тенденции поврзани со тромбоцитопениа и тромбоцитна дисфункција и нерегенеративна анемија. Болеста се пренесува преку плунката на кафениот кучешки крлеж**,** Rhipicephalus sanguineus и е широко распространет во светот. Кучешката моноцитна ерлихиоза се манифестира со широк варијаритет на клинички знаци кои може да се категоризираат во акутна, субклиничка и хронична фаза иаково ендемично инфицирани земји тешко е да се класифицираат клиничките случаи со така специфични фази. Во овој случај опишана е дијагнозата и третманот на кучешка моноцитна ерлихиоза кај машко куче од расата сибирски хаски, околу 6 години старо со знаци на епистакса, депресија, губење на телесната маса и носен исцедок. Болеста напредува од субклиничка до хронична без присуство на морули во мононуклеарните клетки на E.canis. После негативниот резултат за присуството на интрануклеарните морули во крвниот размаз, беше направено сликање со компјутерска томографија и магнетна резонанса за да се направи диференцијална дијагноза помеѓу кучешката моноцитна ерлихиоза и интраназалните тумори.

Клучни зборови: кучешка моноцитна ерлихиоза, сибирски хаски, ерлихиоза, CME

UDC: 636.8.09:616-006.327

LABORATORY DIAGNOSTIC OF CAT LIP FIBROMA - CASE REPORT

Ulčar Igor[1], Pavlovski Damjan[2], Celeska Irena[1]

[1]*Department for Pathophysiology, Faculty for Veterinary Medicine, Skopje, Macedonia*
[2]*Animal Medica, Veterynary Clinic, Skopje, Macedonia*

e-mail: iulcar@fvm.ukim.edu.mk

ABSTRACT

The mantion of the word neoplasma describe abnormal growths in the body. Fibromas are common benign tumor of connective tissue, well-defined, solid, solitary and firm. This is usually uncommon tumor in adult cats. It is present as a solitary lesion at the different part of the body. Grossly, it is firm to soft, well circumscribed, hairless, dome shaped or pendiculated. Fibromas may be attached to epidermis. Cytollogicaly, variable numbrs od spindle or fusiform cells with small uniform, dense oval nuclei occur individualiy or occasionally in small bundles. Generally few cells exfoliate into cytologic preparation. Cytoplasm is lightly basophilic and cell borders are poorly defined as they form cytoplasimic tails and opposite sides of the nucleus. Amorphus eosinophilic material representing intracellular collagen protein wcich may be associated with neoplastic cells. Easy to cure with surgery. Fibromas can occur in the dermis or subcutaneous tissue. Often fine needle aspiration is performed

Key words: cat, mass, fine needle aspiration.

INTRODUCTION

The words neoplasia or neoplasm are the proper terms used to describe any new and abnormal growth in the body (1,6). These words do not describe how "dangerous" a growth may be, only that an abnormal growth does exist. The word tumor is sometimes used instead of the word neoplasm (2). More correctly, the word tumor describes a swelling of/in a tissue (10). The mention of the words tumor or cancer cause most people to think of the death of their pet (3,4). Despite tremendous progress in the treatment of tumors, most people unfortunately still find their minds drawn into the worst thoughts possible (5). It is important to know that many tumors of domestic cats can be cured with the pet living normally for years after treatment. Neoplasms originating in the fibroblasts are common in cats, where they comprise 24 to 33 percent of all tumors of the skin and subcutis (7). Fibromas are the bening counterpart of spindle cell tumors. Fibromas occur predominantely in adult cats and there is no sex and breed predisposition in appearing. Also, there in no predilection and specific anatomic site for appearing the tumors. They usually growth as subcutaneous or dermoepidermal/subcutaneous oval masses with smooth surface. The word benign means not malignant, and that the chances for recovery are favorable. It may also be used to describe a neoplasm that is not likely to spread to other parts of the body (8,9).

MATERIAL AND METHOD

The male castrated cat 7 years old was admitted in the clinic. According the anamnestic data, the swelling of the lip was noticed one month before adimission, with small growth rate, but there in no noticeable changes in cat bechaviour. Complete physical exam was done and attention was payed at the firm mass size in diameter 5mm on the labia on the mandibula, at the right side. Palpation of the lumps show firm mass. The mass was located in the subcutis and was not attached to the base. Complete laboratory exeminations were done, blood sample was taken for complete blood count (Myndray, China), and serum for biochemical parameters (Human, Germany) was examined with wet biochemistry (Stat Fax 3300, INC, Awarenwss technology, USA). Fine-needle aspiration (FNA) cytology is usually diagnostic and test usually requires no anesthesia. Needle was introduced in the skinned lesions and a cells were collected for staining and preparation of cytological smear (Diff Quck, Merck KGaA, Germany). The cat was examined of Feline Leucemia Virus (FLeV) and Feline Immunodeficiency Virus (FIP) with rapid test (Antigen, Korea) and the tests were negative. The tumor was surgically removed. After six months at the same place appired the same firm mass at the same place. As the first time FNA was done and fibroma durum was diagnosed. Two weeks after surgical removing the cat recovered completly and back to previous normal life.

RESULTS AND CONCLUSIONS

Parametars for CBC did not deviate from reference intervals (shown table 1). Biochemistry analysis were in physiological ranges (shown table 2). Oral examination did not show any changes in oral cavity.



Figure 1. *Cat lip fibroma*



Figure 2. *Afrer surgical treatment*

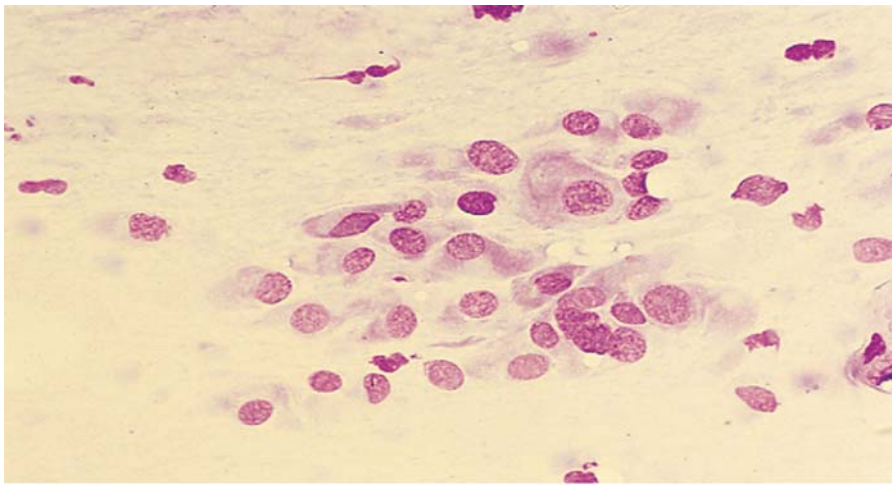


Figure 3. Cytology features

Table 1. *Hematological parameters*

Parameters	Measure unit	Reference values	Result
RBC	* 10 ^{12} / L	5-10	6.23
WBC	* 10 ^{9} / L	5.5-19.5	8.35
PCV	%	30-45	39
Hb	g / dl	10-15	13
MCV	fL	39-55	62
MCH	pg	12.5-17.5	20.8
MCHC	%	30-36	33
PLT	* 10 ^{9}/L	300-800	264

Table 2. *Biochemistry parameters*

Parameters	Measure unit	Reference values	Result
ALT	U/L	8.3-52.5	21.6
AST	U/L	9.2-39.5	19.4
ALKP	U/L	12.0-65.1	34.8
Glucose	mmol/l	3.4-6.9	4.1
Total protein	g/l	57.5-79.6	64.8
Albumine	g/l	24.5-37.5	28.4
Creatinin	µmol/l	48.6-165.0	98.3
Urea	mmol/l	5.5-11.1	6.6
Calcium	mmol/l	2.0-2.7	2.2

Cytology features showed low cell populations of spindle cells with elongate shape, with oval and elongated nuclei. Citoplasm tend to “stream” at the end of the cell making tail or point. The cells were high differentiate, with similar size and shape. The niclei are round to oval with a smooth to lacy chromatin pattern and contain 1 small, round indistinct nucleoli. Small vacuoles are seen in the cytoplasm. There in no imflamatory cells components. The cytology diagnosis is fibroma with good prognosis. Diferential diagnosis was lymphoma, mastocytoma, hystiocitoma. Complete surgical excision of fibromas is curative. Fibroma appired as the results of chronic irritation of premolar upper teeth.

REFERENCES

1. Birchard, SJ; Sherding, RG (eds.) Saunders Manual of Small Animal Practice. W.B. Saunders Co. Philadelphia, PA; 1994.
2. Carlson, D. & Giffin, J.M. (2008). “Skin Lumps or Bumps in Cats.” Pets.WebMD.com.
3. Griffin, C; Kwochka, K; Macdonald, J. Current Veterinary Dermatology. Mosby Publications. Linn, MO; 1993.
4. Marsalla, R. (2010). “ Contact Dermatitis in Cats,« »Contact Dermatitis in Dogs,« “Acne in Dogs” and “Acne in Cats.” PetPlace.com.
5. McKeever, PJ; Harvey, RG. Skin Diseases of the Dog and Cat. Iowa State University Press. Ames, Iowa; 1998.
6. Nash, H. (2010). “ Causes of Solid-Appearing Lumps and Bumps on the Skin in Cats ” and “ Causes of Solid-Appearing Lumps & Bumps on the Skin in Dogs.” PetEducation.com.
7. Paterson, S. Skin Diseases of the Cat. Blackwell Science Ltd. London, England; 2000.
8. Paterson, S. Skin Diseases of the Dog. Blackwell Science Ltd. London, England; 1998.
9. Pets.WebMD.com. (2007). “Skin Lumps or Bumps in Dogs.”
10. Thompson, M. (2010). “Skin Growth, Lump, Swelling or Mass in Cats.” PetPlace.com

ЛАБОРАТОРИСКА ДИЈАГНОСТИКА НА ФИБРОМ – КЛИНИЧКИ СЛУЧАЈ

Улчар Игор[1], Павловски Дамјан[2], Целеска Ирена[1]

[1]*Катедра за патофизиологија, Факултет за ветеринарна медицина, Скопје, Македонија*
[2]*Анимал Медика, ветеринарна Клиника, Скопје, Македонија*
e-mail: iulcar@fvm.ukim.edu.mk

АПСТРАКТ

Неоплзмата е термин кој објаснува абнормален раст во телото. Фибромите се чести бенигни тумори на сврзното ткиво, кои се добро диференцирани, тврди, единечни и цврсти. Овие обично се поретки тумори кај возрасните мачки. Тие се претставени како единечни лезии на на различни делови на телото. Обично се цврсти до меки, оделени од околината, без влакна, во вид на купола или висечки. Цитолошки карактеристики се различен број на мезенхимални клетки со мало единечно густо и овално јадро или понекогаш малку завиткано. Воглавно само малку клетки ексфолираат на цитолошкиот препарат. Цитоплазмата е светло плава, а клеточните граници се слабо диференцирани формирајќи опашки на спротивните страни на јадрата. Аморфен еозинофилен матерјал претставува интрацеуларниот колагенски протеин кој моќе да се јави кај неопластичните клетки. Овој тумор е лесен за лечење со хирушка интервенција Фибромите можат да се појават на кожата или во поткожното ткиво. Често се дијагностицира со апирација ао тенка игла.

Клучни зборови: мачка, клеточна маса, аспирација со тенка игла.

UDC: 636.7.09:[616.24-033.2-073.75:616.718.4-006.34

RADIOLOGICAL DIAGNOSTIC OF LUNG METASTASIS WITH FEMUR OSTEOSARCOMA ORIGIN

Acevski Sinisha[1], Mitrov Dine[1], Krstevski Kiril[2],
Dzadzovski Igor[2], Janevski Aleksandar[2], Velkovski Dimche[3].

[1]*Department of Diagnostic Imaging, Faculty of Veterinary Medicine, Ss. Cyril and Methodius University in Skopje, Macedonia*
[2]*Department of Animal Health, Faculty of Veterinary Medicine, Ss. Cyril and Methodius University in Skopje, Macedonia*
[3]*"Veterinary Centre" Skopje, Macedonia*

ABSTRACT
In this case, described is the occurrence of osteosarcoma of the right rear leg and metastases to the lungs, of the dog race Shar Mountain Shepherd Dog (Sharplaninec), about 10 years old. Osteosarcoma is a highly metastatic tumor that usually affects the lungs even before the first clinical signs (rarely the liver, lymph nodes, other bones, etc), and when the dog's lameness and radiological changes of the bone are noticed, it is already too late for any further interventions.
This radiological examination of the lungs parenchyma during metastatic changes is often used as the first method (cytological examination is a second method) because the affected metastatic cells that leaves the primary tumor gets at the lungs.
The female dog was taken on radiological imaging, with clinical signs manifest: lameness on the right rear limb during long active movement, accompanied with causal discomfort due to pain. The diagnosis was supplemented by radiological imaging of the thorax as well, for possible existence of metastatic changes in the lung tissue.
For radiological diagnostic of the osteosarcoma, the dog was in lateral recumbence and right profile body placement (LL-ML and SD).
The tumor is localized to the distal metaphysis of the femur. Metastases who have left the primary tumor through the vena cava has reached back into the lungs where they form secondary tumorous change.
After diagnosis, the appropriate drug therapy was given to the dog, application of corticosteroid preparations and analgesics for pain relief and chemotherapeutics for possible reduction of the aggressiveness of the tumor.
The owner of the dog was advised by his veterinarian, not to amputate the leg with coxofemoral disarticulacion. Three months after the radiological diagnosis of osteosarcoma, the dog is still in relatively good health condition and is relatively active.
Key words: osteosarcoma, lung metastasis, radiological diagnostic.

INTRODUCTION

In this case, described is the occurrence of osteosarcoma of the right rear leg and metastases to the lungs, of the dog race Shar Mountain Shepherd Dog (Sharplaninec), about 10 years old. Although this tumor occurs in older dogs from 7-9 years, it is possible, but rarely to occur in dogs aged 1-2 years (4, 1, 2). Osteosarcoma as a malignant disease usually is with a primary character, and male dogs have negligible affinity towards it (2). Also, this tumor is more common on the epiphyses of the front limbs, unlike the long bones of the hind limbs (Figure 1) (4, 1). Characteristic of this tumor is its aggressiveness (1). It is a highly metastatic tumor that usually affects the lungs even before the first clinical signs (rarely the liver, lymph nodes, other bones, etc), and when the dog's lameness and radiological changes of the bone are noticed, it is already too late for any further interventions (1).

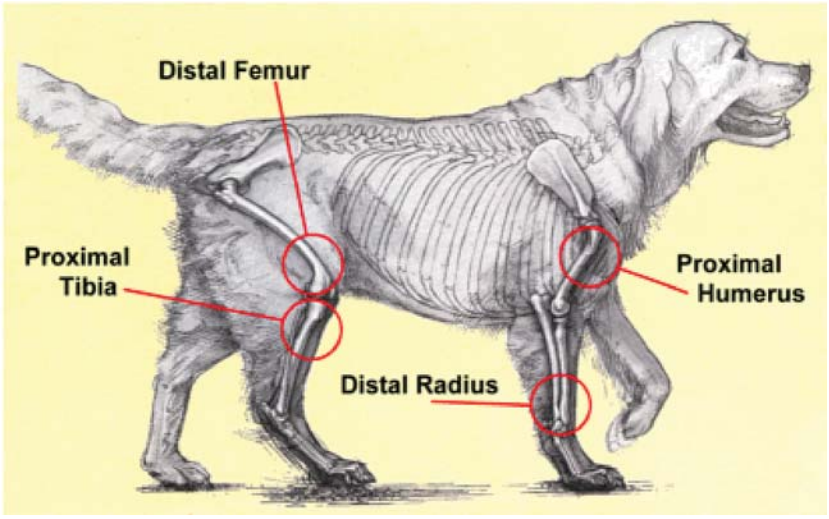


Figure 1. *(The most common sites for osteosarcoma development. Source: http://www.marvistavet.com/html/body_canine_osteosarcoma.html*

MATERIALS AND METHODS

As a metastases detection method, diagnostic radiological imaging was used. This radiological examination of the lungs parenchyma during metastatic changes is often used as the first method (cytological examination is a second method) because the affected metastatic cells that leaves the primary tumor gets at the lungs (3).

For radiological diagnostic of the osteosarcoma, the dog was put in the trocho position (lateral recumbency) with mediolateral (ML) view of the rear leg. For the chest x-ray, the dog was also in lateral position, right profile body placement (LL-SD), which is most practical and appropriate for detection of lung metastases (3, 6).

As a test method initial radiography was used. Imaging was performed on 24x30 cm x-ray films, with an average exposure of 60 kV with 20/0.12 mAs, and the thoracic cavity (lungs) on 30x40 cm x-ray films with an average exposure of 85 kV with 12/0.08 mAs.

RESULTS

The female dog was taken on radiological imaging, with clinical signs manifest: lameness on the right rear limb during long active movement, accompanied with causal discomfort due to pain. Before the radiography, the pain was also confirmed by palpation on the swelling of the leg with manual passive movements of the limb (2).

The diagnosis was supplemented by radiological imaging of the thorax as well, for possible existence of metastatic changes in the lung tissue. Furthermore, the radiographs of the lungs was diagnosed and confirmed by the Professor of Visual Diagnostic methods (3).

DISCUSSION AND CONCLUSION

This case, for us, it was confirmation that the most common occurrence of primary osteosarcoma is in medium size and large breeds of dogs within their middle age, unlike the previous case of the same diagnosis at 2 years old dog. Also, by radiological imaging of the lungs we diagnosed the presence of metastases of sarcoma in the lungs (5).

The tumor is localized to the distal metaphysis of the femur (4, 1). Malignant process very rarely affects articular cartilage, and as you can see, it has not affected the joint surfaces of the knee wrist and the knee cap (patella) (4, 1). In a month time, after the owner first noticed his dog is limping, the bone destruction progressed to the extent of occurrence of various distinct mottled and mosaic destruction in the cortical and medullary part of the affected distal epiphysis of the femur. On the border with the healthy bone tissue, it shows newly formed spined bone formations which are normally placed in relation to longitudinal axis of the bone, completing the cortical part of bone, elevations of the periosteum due to subperiosteal hemorrhage -Codman's triangle (production of new bone tissue in such circumstances or amorphous models of mineralized matrix as a result of periostal response) and loss of the fine trabecular drawing in the metaphyseal bone (Figure 2) (1, 8, 9, 2, 7, 5, 6).



Figure 2.

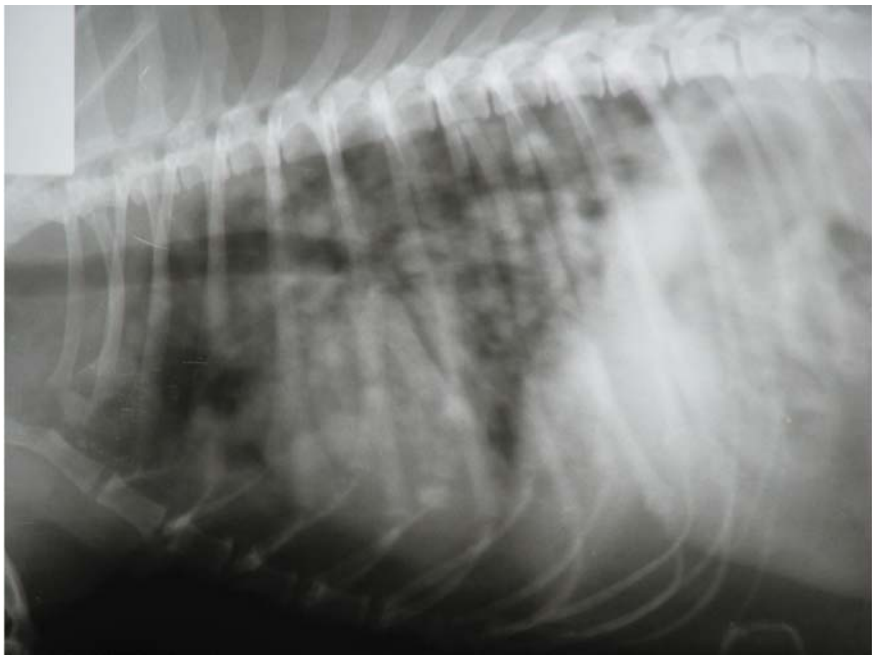


Figure 3.

Metastases who have left the primary tumor through the vena cava has reached back into the lungs where they form secondary tumorous change (3). Changes of multiple pulmonary metastases are disseminated and well-outlined nodular bundles in size over 6-8 mm in diameter, which are well noticed and overlapping with the shadow of the lung drawing, shadow of the heart and with one another (Figure 3) (3, 8, 10, 2, 7, 5, 6).

After diagnosis, the appropriate drug therapy was given to the dog, application of corticosteroid preparations and analgesics for pain relief and chemotherapeutics for possible reduction of the aggressiveness of the tumor (2).

The owner of the dog was advised by his veterinarian, not to amputate the leg with coxofemoral disarticulacion because there was no presence of pathological fractures of the epiphysis changed femur and the dog has a positive reaction to the curative treatment of the primary tumor.

Three months after the radiological diagnosis of osteosarcoma, the dog is still in relatively good health condition and is relatively active, but is avoiding the use of the leg and it is a lot more tired. The stable condition of the dog is a result of the good ethics and discipline of the owner, through his respect and adherence to the advices of the veterinary doctor.

REFERENCES

1. Mensur Šehić: BOLESTI KOSTI, ZGLOBOVA, TETIVA I ŽIVČANOG SUSTAVA U DOMAĆIH ŽIVOTINJA, Rentgenografija, Ultrasonografija, Kompjutorizirana Tomografija i Magnetska Rezonancija, Pregled Radova, Zagreb, 2004.
2. Stephen J. Ettinger, Edward C. Feldman: Textbook of Veterinary Internal Medicine, 6th Edition.
3. Mensur Šehić: BOLESTI ORGANA I ORGANSKIH SUSTAVA ABDOMENA I TORAKSA U DOMAĆIH ŽIVOTINJA, Rentgenografija, Ultrasonografija, Kompjutorizirana Tomografija, Pregled Radova, Zagreb, 2004.
4. Mensur Šehić: KLINIČKA RENTGENOLOGIJA U VETERINARSKOJ MEDICINI, Zagreb, 2002.
5. Живко Филипов: ВЕТЕРИНАРНА РЕНТГЕНОЛОГИЯ, Стара загора 1999 г.
6. Kealy J. Kevin, McAlister Hester: Diagnostic Radiology and Ultrasonography of the Dog and Cat, Third Edition
7. Ruth Denis, Robert M. Kirberger, Frances Barr, Robert H. Wrigley: Handbook of SMALL ANIMAL RADIOLOGY and ULTRASOUND, Techniques and Differential Diagnoses, Second Edition.
8. William D. Carlson: VETERINARY RADIOLOGY, Second Edition.
9. Donald E. Thrall: TEXTBOOK OF Veterinary Diagnostic Radiology, 2nd Edition
10. Vladimir Butković, Mensur Šehić: OSNOVE SPECIJALNE VETERINARSKE RENDGENOLOGIJE, Beograd, 1990.

РАДИОЛОШКА ДИЈАГНОСТИКА НА МЕТАСТАЗИ ВО БЕЛИТЕ ДРОБОВИ СО ПОТЕКЛО ОД ОСТЕОСАРКОМ НА ФЕМУР

Ацевски Синиша[1], Митров Дине[1], Крстевски Кирил[2],
Цаџовски Игор[2], Јаневски Александар[2], Велковски Димче[3]

[1]Катедра за Визуелни дијагностички методи, Факултет за ветеринарна медицина,
Универзитет „Св Кирил и Методиј“ во Скопје, Македонија
[2]Катедра за Здравствена заштита, Факултет за ветеринарна медицина,
Универзитет „Св Кирил и Методиј“ во Скопје, Македонија
[3]„Ветеринарен Центар“ Скопје, Македонија

АПСТРАКТ

Во овој случај, опишана е појава на остеосарком на задната десна нога со метастази на белите дробови, кај куче од расата Шарпаланински овчар, на возраст од околу 10 години. Остеосаркомот е високо метастатски тумор кој најчесто ги зафаќа белите дробови (поретко црн дроб, лимфни јазли, други коски и сл.) пред појавата на првите клинички знаци, а кога ќе се забележи кривењето на кучето и радиолошките промени на коските, веќе е доцна за било какви понатамошни интервенции. Ваквото рентгенографско испитување на белодробниот паренхим на присуство на метастатски промени често се употребува како прва метода (цитолошкото испитување е втора метода), бидејќи метастатските туморозни клетки кои го напуштаат примарниот тумор најчесто доспеваат до белите дробови.

Кучката беше донесена на радиолошка дијагностика, со клинички знаци: повремено кривење (хромост) со задната десна нога при долготрајно движење на кучето, но и последична нерасположеност како резултат на болката после шетањето.

Дијагнозата беше дополнета и со контролно радиолошко снимање и на тораксот, заради евентуално присуство на метастатски промени во белодробното ткиво.

За радиолошка дијагностика на остеосаркомот, кучето беше во легната положба со десна странична поставеност на телото (LL-ML и SD).

Туморот е локализиран на дисталната метафиза на фемурот. Метастазите кои што го напуштиле примарниот тумор, преку задната шуплива вена доспеале во белите дробови каде што формирале секундарни туморозни промени.

После поставувањето на дијагнозата, кај кучката се отпочна со соодветна медикаментозна терапија, апликација на аналгетици и кортикостероидни препарати за намалување на болките и хемотерапевтици за евентуално намалување и на агресивноста на туморот.

Сопственикот на кучето беше советуван да не се ампутира ногата на кучето со коксофеморална дисартикулација. Три месеци по радиолошкото дијагностицирање на остеосаркомот, кучката е уште во релативно добра здравствена кондиција и е релативно активна.

Клучни зборови: остеосарком, белодробни метастази, радиолошка дијагностика.

UDC: 639.3.043.2:597.551.2

EFFECTS OF DIETARY VITASIL® ON GROWTH PERFORMANCE OF CARP (*Cyprinus caprio*)

Atanasoff Alexander[1], Ivanov Veselin[2], Nikolov Galin[3], Zhelyazkov Georgi[3], Petrova Biliana[4]

Trakia University, Faculty of Veterinary medicine, Department of Animal husbandry, Stara Zagora, Bulgaria
[2]Trakia University, Faculty of Medicine, Department of Biochemical, Stara Zagora, Bulgaria
[3]Trakia University, Faculty of Agriculture, Department of Biology and Aquaculture, Stara Zagora, Bulgaria
[4]Trakia University, Faculty of Veterinary medicine, Department of Surgery, Stara Zagora, Bulgaria

*Author for correspondence:hmi_atanasoff@mail.bg

ABSTRACT
Problem statement: We investigated the effects of Vitasil® supplementation on growth performance of the carp (*Cyprinus caprio*). Two hundred and forty carp with initial average weight 63,3 ±0,2 g were divided into three groups and reared in nine concrete tanks (1 m x 1 m x 1 m).
Approach: The animals were fed with 3 diets: The feeds were I basal diet only (control), II standard trout diet (with high content of protein and fat), and III trout diet with 5% Vitasil®.
Results: The results show that dietary Vitasil® levels significantly influenced the growth of the carp compared with the control group, average weight gain (AWG) in all treatment groups, body weight gain (BWG), specific growth rate (SGR) and feed conversion ratio (FCR) in fish fed with diets supplemented with 5% Vitasil® were significantly increased (P<0.001).
Conclusion: In general, with the supplementation of Vitasil®, particularly at dose of 5%, the growth performance can be improved effectively.
Key words: Vitasil®, growth performance, carp.

INTRODUCTION

Aquaculture is a rapidly growing industry worldwide. One of the most widely cultured species in the world is common carp (Heydarnejad, 2012). The use of supplements in carp culture has become inevitable for the success of fish culture (Shahzadi et al., 2006). There has been heightened research in developing new dietary supplementation strategies in which various health- and growth-promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been evaluated (Denev et al., 2009). The supplements using plays an important role in intensive and super-intensive fish culture system. It also offers best means of fish production within shortest possible time (Afzal et al., 2008). Several artificial feedstuffs of plant (byproducts) are useful to formulate the feed for different developmental stages of carp. On of them is silymarin, a purified extract of seeds of milk thistle (*Silybum marianum* L., *Asteraceae*), contains flavolignans like silybinin (60–70%) along with isosilybin (5%), silydianin (10%), and silychristin (5%) (Saller et al., 2001; Khan et al., 2006). Silybin has structural similarity with steroidal hormones and thereby acts in protein synthesis (Kosina et al., 2002). Such potential was attributed to its ability to maintain the cell fluidity (Adhikari et al., 2010), to enhanced protein and DNA synthesis, and to its anti-inflammatory ability (Dehmlow et al., 1996) and ability to modulate the hepatic detoxification machinery (Baer-Dubowska et al., 1998).

The main objective of this study was to compare the growth performance of carp, with and without Vitasil® supplement, in super intensive culture system.

MATERIALS AND METHODS

Experimental Site

The study was carried out in the Aquaculture base of the Faculty of Agriculture, Trakia University, Bulgaria.

Experimental System and Fish

The experimental fish, carp (*Cyprinus caprio*) were procured from the freshwater fish farm Tundja73, Nikolaevo, Bulgaria. They were acclimatized for five (5) days before the commencement of the experiment. This was done in order for the fish to empty their stomach content and to force them to adjust to the new diet.

The feeding trial was conducted in concrete tanks (1x1x1 m) were properly washed, disinfected and rinsed with clean water. The fish were stocked at a density of 26 in each of the tank containing 1000 L of dechlorinated bore water. Adequate level of oxygen in each tank was maintained through aeration.

Diet Formulation and Preparation

The calculated daily ration was supplied in five equal rations: 08:00; 10:00; 12:00, 14:00 and 16.00 h. This ration level was determined near to satiety according to rearing conditions.

Table 1. *Used diets*

Content	Soprofish 32/7	Soprofish 45/13	(NRC, 1993)
Protein, (%)	min. 32	min. 38	30 – 35
Fat, (%)	min. 10	min. 7	5 – 10
Fiber, (%)	max. 45	max. 4	5
Ash, (%)	max. 10	max. 10	8
Ca, (%)	min. 1,2	min. 1,6	1,7
P, (%)	min. 0,8	min. 1,2	1,6

Experimental Procedure

For the purpose of this study the formulated diet was divided into three portions designated feed 1, 2 & 3. The nine tanks of 3 replicates were used as follows for the experiment. Feed I: standard artificial carp feed, Feed II: standard artificial trout feed (high level of protein and fat in fish diet) and Feed III: standard artificial trout feed with 5% supplemental of Vitasil®.

The experiment was run for 9 months all analyses for proximate composition were determined according to the methods of Todorov et al., (2011). Water temperature (°C), dissolved oxygen, (mg/l), nitrate (ppt) and active acidity, (pH) were demand for 24 h with a digital thermometer while dissolved oxygen, nitrate concentration and pH were determined using WTW-oxi330, HANNA HI 98312 and REDOX Zac plus.

Measurement of growth parameters

The growth parameters were measured according to the methods described by Todorov (1983). Before measurement, the fish were anesthetized with clove oil (40 mg L^{-1}). Growth performance was calculated as follows:

Mean weight gain (**MWG**) was calculated as the difference between the initial and final weight divided by the number of the surviving fish at the end of the culture period.

$$\textbf{MWG} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Number of surviving fish}} \times 100$$

Average daily growth (**ADG**) was calculated as the difference between the final weight and the initial weight divided by the number of days i.e. the experimental period.

$$\textbf{ADG} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Number of days}}$$

Body weight gain (**BWG**) was calculated as the difference between the final weight and the initial weight divided by the initial weight.

$$\textbf{BWG} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Initial body weight}} \times 100$$

Specific growth rate (**SGR**) (%/day): This is the relationship of the difference in the weight of the fish within the experimental period. Where t is the period of culture in days, ln W_0 is the natural logarithm of the weight of the fish at the beginning of the experiment, and ln W_t is the natural logarithm of the weight of the fish at day t. (W_0 and W_t are in gram).

$$\textbf{SGR} = \frac{\text{Natural logarithm final body weight} - \text{Natural logarithm initial body weight}}{\text{Number of days}} \times 100$$

Feed conversion ratio **(FCR)** was determined by dividing the total weight of the food given by the total increase in weight gained by the fish over a period of time while feed intake (FI) was calculated as the addition of daily mean feed intake of the fish during the period.

$$\textbf{FGR} = \frac{\text{Dry weight of ingested food}}{\text{Wet weight of produced fish}} \times 100$$

Statistical analysis

All data were expressed as mean ± SD. Data were subjected to analysis of variance (STATISTICA 6) to compare differences among individual means.

RESULTS

Table 2. *Effect of different experimental feed on growth performance*

	Initial weight	Final weight	Total weight gain
Feed I (control)	63.91±2.43	134.04±0.13[a]	70.13±2.56
Feed II (trout feed)	64.66±5.37	157.90±10.33[b]	93.24±13.49
Feed III (trout feed + Vitasil®)	65.21±0.64	181.15±4.28[ab]	115.94±4.49

**Values with the same superscript in the vertical columns are significantly different from each other (p<0.05).*

Table 3. *Over all weight gain (g) with and without with and without Vitasil® supplement*

	Feed I	Feed II	Feed III
Mean weight gain	1.67±0.06	1.63±0.24	1.84±0.07
Average daily growth	0.29±0.01[a]	0.39±0.06	0.49±0.02[a]
Body weight gain	1.09	1.44	1.77
Specific growth rate	3.1±0.02[b]	3.7±0.05	4.2±0.06[b]
Feed conversion ratio	1.70±0.08[cd]	1.44±0.04[c]	1.41±0.04[d]

**Values with different alphabets within a column differ significantly from each other (p<0.001).*

CONCLUSION

Supplement using is known to increase the carrying capacity of culture systems and can enhance fish production by several folds. In the present study, when comparing growth of fish with and without supplement feeding, a significantly higher (P<0.05) final weight of 181.15±4.28 g was achieved in group with supplement versus another two groups without supplements. The average daily weight of carp was also significantly higher (P<0.05) than without supplement feeding (0.49±0.02 g) at the end of the experiment. The feed conversion ratio (FCR) is another good tool to compute the acceptability of supplement in fish feeding experiment. Better FCR was found in the third group Feed III 1.41±0.04

The control weighing revealed intensive body weight gains of fish representing three feeding groups. The increments, however, were significantly higher in fish of group Feed II and Feed III, fed high protein and fat feed, which, could have been expected considering high energy value of this type of feed. Nevertheless, relatively large gains of body weight in the fish of group Feed III may constitutes an evidence that bioflavonoid is also able to influence of digest and assimilate process in carp.

The present study indicates that supplement plays an important role for better growth of carp and can increase the fish production. It can easily be interpreted that the supplementation of Vitasil®, particularly at dose of 5%, the growth performance can be improved effectively.

REFERENCES

1. Adhikari, M., R. Arora, R. Chawla, J. Sharma, A.S. Dhaker, D. Gupta, N. Dubey, R. Kumar, V. Ivanov, V. Gadjeva, R. Gevrenova, R.K. Sharma, 2010. Evaluation of silymarin as a promising radioprotector. *Z Naturforsch C*. 65(5):337-346
2. Afzal, M., A. Rab, N. Akhtar, I. Ahmed, M. F. Khan, M. Qayyum, 2008. Growth performance of Bighead carp *Aristichthys nobilis* (Richardson) in monoculture system with and without supplementary feeding. *Pak. Vet. J.,* 28 (2): 57-62.
3. Baer-Dubowska, W., H. Szafer, V. Krajkakuzniak, 1998. Inhibition of murine hepatic cytochrome P450 activities by natural and synthetic phenolic compounds. *Xenobiotica* 28, 735-743.
4. Dehmlow, C., N. Murawski, H. De Groot, 1996. Scavenging of reactive oxygen species and inhibition of arachidonic acid metabolism by silibinin in human cells. *Life Sci.* 18, 1591-1600.
5. Denev SA., Y. Staykov**,** R. Moutafchieva, G. Bee, 2009. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. *International Aquatic Research* (1): 1-29.
6. Heydarnejad, M.S., 2012. Survival and growth of common carp (*Cyprinus carpio* L.) exposed to different water pH levels. *Turk. J. Vet. Anim. Sci.* 36(3): 245-249.
7. Khan, S.A., B. Ahmad, T. Alam, 2006. Synthesis and antihepatotoxic activity of some new chalcones containing 1,4-dioxane ring system. *Pak. J. Pharm. Sci.* 4, 290-294.
8. Kosina, V., R. Kren, F. Gebhardt, J. Grambal, D. Ulrichova, 2002. Antioxidant properties of silybin glycosides. *Phytother. Res.* 16, 533-539.
9. Saller R., R.Meier, R. Brignoli, 2001. The use of silymarin in the treatment of liver diseases. Drugs **61**, 2035-2063.
10. Shahzadi T., M. Salim, U. Kalsoom and K. Shahzad, 2006. Growth performance and feed conversion ratio (FCR) of hybrid fingerlings (*Catla catla* X *Labeo rohita*) fed on cottonseed meal, sunflower meal and bone meal. *Pakistan Vet. J.,* 26(4): 163-166.
11. N. Todorov, A. Ilchev, S. Chobanova, D. Girginov, G. Ganchev, D. Penkov, Z. Shindarska, G. Mihailova, J. Naidenova, K. Nedelkov, A. Atanasov, 2010. Manual of animal nutrition. *Publ. "Iztok-Zapad" – Sofia,* ISBN: 978-954-321-733-5.

ЕФЕКТИ ОД ИСХРАНАТА СО ВИТАСИЛ® ВРЗ РАСТОТ КАЈ КРАП (*Cyprinus caprio*)

Атанасоф Александар[1], Иванов Веселин[2], Николов Галин[3], Желјазков Георги[3], Петрова Билјана[4]

[1]Тракиски Универзитет, Факултет за ветеринарна медицина, Катедра за Сточарство, Стара Загора, Бугарија
[2] Тракиски Универзитет, Медицински факултет, Катедра за Биохемија, Стара Загора, Бугарија
[3] Тракиски Универзитет, Земјоделски Факултет, Катедра за Биологија и Аквакултура, Стара Загора, Бугарија
[4] Тракиски Универзитет, Факултет за ветеринарна медицина, Катедра за Хирургија, Стара Загора, Бугарија

*Автор за контакт: hmi_atanasoff@mail.bg

АПСТРАКТ

Опис на проблемот: Ние ги истражувавме ефектите на Витасил® како додаток за ефикасност во растот на крапот (Cyprinus caprio). Двесте и четириесет крапови со почетна просечна тежина 63,3 ±0,2 гр. беа поделени во три групи и се одгледуваа во девет бетонски базени (1м. х 1 м. х 1 м.).

Метод: Животните беа хранети на 3 начини на исхрана: Хранењето беше: 1 -само базална исхрана (контрола), 2 -стандардна исхрана за пастрмки (со висока содржина на протеини и масти) и 3 -исхрана за пастрмки со 5% Витасил®.

Резултати: Резултатите покажаа дека исхраната со Витасил® значително влијаеше врз растот на краповите во споредба со контролната група. Просечната тежина (AWG) во сите третирани групи, телесната маса (BWG), специфичната стапка на раст (SGR) и соодносот на конверзија на храната (FCR) кај рибите што се хранат со 5% Витасил® беа значително зголемени ($P<0.001$).

Заклучок: Во принцип, со дополнување на Витасил®, особено во доза од 5%, ефективно може да се подобрат перформансите на растот.

Клучни зборови: Витасил®, ефикасност во раст, крап.

FOOD SAFETY
AND
VETERINARY PUBLIC HEALTH

Plenary Lecture

UDC: 614.31
608.3:664

MODERN CHALLENGES IN FOOD HYGIENE/SAFETY AND THE RESPONSES FROM VETERINARY EDUCATION - EAEVE, EBVS AND ECVPH

Buncic Sava[1]

[1]*Department of Veterinary Medicine, Faculty of Agriculture, Novi Sad, Serbia*

ABSTRACT

The responsibilities of "food safety controllers" in modern times have increasingly shifted from the traditional "end-product control" towards longitudinally integrated safety assurance. If veterinarians wish and intend to continue to play a major role in ensuring food safety, they have to adjust their knowledge and professional skills so to be able to deal with more complex and multi-disciplinary problems and issues. This necessitates the restructuring of university training programs, in order to provide a starting competence in this area for veterinary graduates or a subpopulation thereof. Having recognized this, the European Association of Veterinary Establishments for Veterinary Education (EAEVE) recently instituted a Working Group to analyze the current situation, with a view to produce Standard Operating Procedures allowing fair and transparent evaluations of Universities/Faculties constituting their membership, and in concurrence with the explicit European legislation on professional qualifications deemed necessary for this veterinary discipline. This paper summarizes the main conclusions and recommendations of said Working Group, conceived so as to contribute to international efforts to optimize veterinary training in FH/VPH. Also, the roles of European Board for Veterinary Specialization (EBVS) its European College of Veterinary Public Health (ECVPH) in achieving, certifying and maintaining the specialist skills in the veterinary public health area are briefly outlined. This paper is based on, and contains parts of, related recent publications (enlisted) in which this author was involved.

Key words: Food Hygiene; Food safety; Veterinary public health; Veterinary education; Veterinary specialization

INTRODUCTION

Modern food hygiene/safety challenges and assurance

In modern times, the main food safety risks are zoonotic, bacterial foodborne pathogens. Their effective control requires longitudinal and integrated ("meat chain") approach and use of risk analysis and GMP/GHP/HACCP principles (Buncic, 2006). Food safety hazards can enter the food chain at various stages and multiple points; hence there is no just one single point in the food chain where the safety of food could be reliably and entirely ensured. Rather, multiple measures aimed at preventing or reducing food safety hazards have to be implemented at multiple points along the food chain in a coordinated manner. This aspect will be illustrated here by using example of the meat chain.

In the *pre-harvest phase* (on-farm), the global "recycling" of microbial pathogens can be reduced through agricultural land- and animal by-products management. Pathogen faecal shedding by farm animals can be reduced by using antimicrobial treatments of animal feeds, introducing new animals only from controlled sources, biosecurity, ensuring optimal animal welfare (stress management) and hygienic animal husbandry, as well as by using prebiotics-, probiotics- and/or competitive exclusion-, and vaccination-related concepts. In the *harvest phase* (at-abattoir), minimizing animal transport and lairaging duration reduces cross-contamination *via* vehicles/pens. The "logistic slaughter" approach can be used: slaughtering higher-risk batches of animals separately from lower-risk ones. The batch risk ranking is based on the Food Chain Information (FCI) concept, including consideration of data from on-farm monitoring and surveillance of zoonotic agents, farm quality assurance and herd health plans. At-abattoir antimicrobial measures include hygienic slaughter and dressing, effective sanitation, and potential use of skin- and/or carcass-decontamination treatments. In the *post-harvest phase* (processing-storage-distribution-consumers), meat products can be grouped into those that receive a bactericidal step and those that do not. The latter can be further divided into those not allowing pathogens' growth and those allowing it. In no-bactericidal-step products, microbial controls rely on the "hurdle" concept. Overall, the risk reduction measures at post-harvest phase include effective cleaning-sanitation of all meat-related premises, uninterrupted cold chain, bactericidal steps wherever possible, prevention of cross-contamination during further handling and/or food preparation, post-cooking holding at >60^{o}C or <5^{o}C, and food hygiene education of consumers.

It should be kept in mind that the main focus of the meat safety system can differ between pathogens. While some pathogens (e.g. *Salmonella* spp*., Campylobacter* spp., *Y. enterocolitica* and VTEC) are most efficiently controlled by the main interventions applied in primary production combined with optimization of the slaughter hygiene, the main controls for others (e.g. ubiquitous *L. monocytogene*s) are focused on the post-harvest stage.

Today, the main responsibility for food safety rests with producers whose responsibilities include compliance with regulatory requirements (e.g. EC-Hygiene Pack legislation) and implementation of GMP/GHP/HACCP-based systems. Governments have a more advisory, and official controls- and audit-orientated role. Nevertheless, all HACCP-based meat safety systems implemented by the industry at various phases of the food chain are subject to regulatory verification and auditing. In the EU, the regulation (EC 2073/2005) introduced

"Process Hygiene" and "Food Safety" criteria for some microorganisms and certain foods, but those should be considered together with other aspects of legislation including HACCP-based checks and official controls.

This modern approach to food safety, inherently, means that both the nature of the regulatory controls and the skills of the regulatory controller have to be adapted accordingly. In the new situation, the official veterinarians can no longer claim all the controllers' positions simply by referring to the law: they have no "inherited" rights to public health tasks that are on offer today. Rather, veterinarians have to prove "anew" that they are up to new regulatory tasks. Unless veterinarians convincingly show that their competence represents significant added value over and beyond the minimum legal requirements on food control, other professions already associated - in one way or the other - with the food chain will have little difficulty taking over. This development has, once again, kindled a dispute between various professional groups about whether or not veterinarians are optimally equipped for performing all necessary control tasks. Hence, unless the veterinary profession is willing to give up its significant role in public health assurance, it seems well-advised to shape up in those areas where competence may be lacking and prove their own capacity to provide consumers with the assurance and reassurance they are demanding. This will only be successfully achieved when veterinarians follow a preventive veterinary medicine approach at the animal population level, base their considerations and decisions on risk analysis, are effective in auditing food safety systems in the context of the food chain (inherently complex and multidisciplinary), and remain aware of the social, trade and political consequences of their decisions.

<u>Current</u> professional qualifications required for "Official Veterinarians" in the EU

As reviewed by the expert group (Smulders et al., 2012) and presented below, education and competency requirements by the Requirements Since introduction of the Regulation 854/2004 (EC, 2004), it is no longer up to individual Member States to entirely follow their own strategies in the education of official veterinarians. It should be noted, however, that the indicated qualification requirements (Annex I, section III, chapter IV) are limited to the fresh meat area and do not relate to other foods, although the definition of "official veterinarian" as given in the Regulation 854/2004 Chapter IV: Professional Qualifications, A. Official Veterinarians (EC, 2004) indeed implies his/her involvement in ensuring the hygiene of *all* foods. According to this Regulation, the competent authority may appoint only veterinarians who have passed a test meeting the following requirements for official veterinarians:

1. national and community legislation on veterinary public health, food safety, animal health, animal welfare and pharmaceutical substances;
2. principles of the common agricultural policy, market measures, export refunds and fraud detection (including the global context: WTO, SPS, Codex Alimentarius, OIE);
3. essentials of food processing and food technology;
4. principles, concepts and methods of Good Manufacturing Practice and quality management;
5. pre-harvest quality management (Good Farming Practices);
6. promotion and use of food hygiene, food related safety (Good Hygiene Practices);
7. principles, concepts and methods of risk analysis
8. principles, concepts and methods of HACCP, use of HACCP throughout the food production chain;
9. prevention and control of food-borne hazards related to human health;
10. population dynamics of infection and intoxication;
11. diagnostic epidemiology;
12. monitoring and surveillance systems;
13. auditing and regulatory assessment of food safety management systems;
14. principles and diagnostic applications of modern testing methods;
15. information and communication technology as related to veterinary public health;
16. data-handling and application of biostatistics;
17. investigations of outbreaks of food-borne diseases in humans;
18. relevant aspects concerning TSE's;
19. animal welfare at the level of production, transport and slaughter;
20. environmental issues related to food production (including waste management);
21. precautionary principle and consumer concerns, and
22. principles of training of personnel working in the production chain.

Furthermore, Regulation EC854/2004-related requirements for official veterinarians also include that each official veterinarian is to undergo practical training for a probationary period of at least 200 hours before starting to work independently. During this period the probationer is to work under the supervision of existing official veterinarians in slaughterhouses, cutting plants, inspection posts for fresh meat and on holdings. The training is to concern the auditing of food safety management systems in particular. The competent authority may arrange for different tests to take account of candidates' background. However, when the competent authority is satisfied that the candidate has acquired all the required knowledge as part of a university degree, or through continuing education resulting in a postgraduate qualification, it may wave the requirement for a test.

It should also be noted that Regulation 854/2004 defines the ultimate knowledge required *at postgraduate level* (which, inherently, may be acquired either *at University courses* or *Continuing Education courses*) and decidedly does not imply that all the points should necessarily be dealt with in full detail *during undergraduate veterinary training*. Consequently, establishments for veterinary education now *must*:

- make sure that the scientific basis ('Day 1 skills') is provided for all students and for all listed elements; and
- clearly identify where postgraduate training should take over.

"<u>New</u>" profile of veterinarians required for "<u>new</u>" food hygiene/safety assurance systems

As discussed by the expert group (Smulders et al., 2012) and presented below, it is very important to keep in mind that any legislative definition of the qualifications and competencies required for "official veterinarian" can only reflect the public health- and food safety-related problems, scientific knowledge and control strategies that

existed at the time when the legislation was formulated. As the latter aspects always - sometimes rather rapidly - further evolve over time, it is logical that the legislative definitions need to be re-visited periodically and updated as necessary. For example, extensive activities of both the European Commission and the European Food Safety Authority are ongoing as this paper is being prepared, with the aim of fundamental modernization of meat inspection and with an immediate possibility that related legislation framework will require substantial modification in the relatively near future.

In the past, the tasks of controlling whether and how the whole chain of events leading to the conversion of animal to food met the FH/VPH requirements indicated above were allocated exclusively to veterinarians. This particularly related to traditional meat inspection, which indeed had an impressive historical record of successfully detecting and eliminating from the food chain the causative agents of classical zoonotic diseases. However, nowadays, the public health relevance of those zoonoses has faded in Europe because the zoonoses themselves have been eradicated or are now very rare, whilst meat inspection (using macroscopic techniques) has proved to be unable to detect the hazards causing food borne diseases of main current concern (e.g. *Salmonella*, *Campylobacter*, VTEC, *Toxoplasma gondii*, etc). Instead, the latter hazards can be controlled only through a range of preventative and technology- or process hygiene-based measures applied at multiple steps of the meat chain in a coordinated way. This essentially represents a "meat safety assurance" approach, which differs fundamentally from the traditional routines as defined by classical "meat inspection" concepts. The aforementioned changes have led to a situation where the traditional meat inspection practices - still largely followed today - are actually primarily beneficial for the detection of animal health hazards and for observing indicators of poor animal welfare, rather than serving a principal public health purpose. To change the situation and strengthen the public health relevance of meat inspection, intensive EU/EFSA activities on modernization of meat inspection system are ongoing, and the envisaged changes will particularly include:

– the main role of "official controller" will be primarily of risk manager nature and not any longer primarily of "macroscopic meat examiner" nature;

– the work of the risk manager will be based on analysis of complex and comprehensive data and information from the food (meat) chain including both farms and abattoirs; and

– all decisions related to the use of animals for food production, the process of their conversion to food (meat), and the appropriateness of the food (meat) for human consumption, will be based on risk analysis.

Veterinarians with their strong emphasis on biomedicine and their focus on the pathogenesis and epidemiology of (zoonotic) diseases, and on toxicology, plus their understanding of food production and food hygiene should stand out as experts in (veterinary) public health and in solving problems along the food chain. Obviously, this assumption will only remain valid provided the entire food production chain is comprehensively considered in veterinary education and indeed, this is a prominent feature of the curriculum in some veterinary faculties in Europe. Therefore, it cannot be stressed enough that such a 'longitudinal approach' remains in place or is introduced in curricula when missing. In addition to special food hygiene studies, prospective veterinary food hygienists should be trained in personnel management and constructive interaction skills. Also, leadership education must be included in veterinary undergraduate curricula, as most graduates pursuing a food hygiene career (including those active in meat inspection) will inevitably be engaged in managing other employees (Lundén et al., 2007; Maijala and Korkeala, 2008). Furthermore, veterinarians work in multidisciplinary teams, and thus good communication skills are essential. Unfortunately, as yet, these elements are not offered in many veterinary curricula in Europe. Hence, significant curriculum changes are needed to meet current and future demands, by specifically addressing the significance of improved social interaction and job motivation and including the associated practical training (Maijala and Korkeala, 2008).

Veterinary food hygiene/safety education in the EU at undergraduate level

European Association of Establishments in Veterinary Education (EAEVE) is the main European body that overseas, evaluates and accredits veterinary faculties in Europe. Since its establishment (1988), a total of 97 veterinary training establishments have become members and have requested evaluations by visitation teams, consisting of experts in the aforementioned veterinary disciplines. To support the evaluations, EAEVE has established basic Standard Operating Procedures (SOPs) which vary in their degree of detail. During the EAEVE General Assembly in Hannover (2009), the latest version of the SOPs was issued, which defines the requirements - to be monitored by evaluators of the Food Hygiene/ Veterinary Public Health (FH/VPH) elements, during their site visits - as: "Adequate knowledge of the hygiene and technology involved in the production, manufacture and putting into circulation of animal foodstuffs of animal origin, intended for human consumption …….including the relevant legislation".

Arguably, the definition is of little assistance as long as the term 'adequate' is not further specified in terms of: a) generally accepted curricular elements; b) 'weighing' of their various constituting components; and c) mode of knowledge transfer. As a consequence, much of the evaluation is actually left in the hands of the individual experts. Understandably, it is not always and entirely clear how and exactly on which basis an individual evaluator identified issues to be given a priority. Hence, the absence of more detailed, properly discussed and agreed evaluation guidelines is frustrating for the evaluator, as well as for the evaluated faculty when conducting self-evaluation exercises and assessing if their chosen approach is up to par. Consequently, EAEVE decided to make available more detailed guidelines. For that purpose, EAEVE nominated a working group (WG) comprising all authors of the publication by Smulders et al. (2012), and gave it a task to elaborate guidelines to remedy, which would be subsequently adopted by the EAEVE. The main points from the WG's analysis and recommendations are summarised below.

The optimal and most effective place for Food Hygiene training in the veterinary curriculum is at the final stage, i.e. after undergraduate students have been confronted with the clinics and have a better understanding of animal disease, basic epidemiology

and on-farm disease prevention options. The extent of the Food Hygiene training (in terms of proportion of the total veterinary curriculum) should range from a minimum of 12 to 15%, depending on national, regional or faculty interests and preferences. All elements listed should be provided BOTH in theory (lectures, seminars) AND (wherever it is meaningful to do so) *via* practicals, (extramural) internships and excursions. For further details on subjects and topics to be included in the food hygiene teaching, the reader is directed to the publication by Smulders et al. (2012).

Establishments for veterinary education provide graduates with the scientific basis (Day 1 skills) allowing them to undertake a career in Veterinary Public Health, after they have engaged in the necessary postgraduate training, which should be provided by the Competent Authority, as stipulated in European legislation (see above). Hence the primary responsibility for ensuring that the training of the Official Veterinarian as required by the 22 points described in Chapter IV of Regulation EC 854/2004 is undertaken lies with the Competent Authority, not with the veterinary faculty. Should the former consider it desirable or more efficient to largely delegate such (in essence 'postgraduate') training to establishments for veterinary education (i.e. to be included in the regular undergraduate training programme) it is stressed that the suggested 12 to 15% minimum training will then not suffice; this will inevitably lead to, perhaps unfair, criticism addressed to veterinary faculties that they deliver 'relatively incompetent' graduates. The also WG recognized that in some European countries there appears to be insufficient practical training in Food Microbiology. As their scope and methodology fundamentally differ (general) Veterinary Microbiology cannot substitute for Food Microbiology and the latter should preferably be taught by a specialised food microbiologist.

Veterinary food hygiene/safety education in the EU at specialist level

Veterinary European Board of Veterinary Education (EBVS) is the main European body that overseas, evaluates and accredits veterinary faculties in Europe organization formed by members and observers. The main objectives of the EBVS include to:

– recognise and monitor Veterinary Specialty Colleges in Europe;

– award the title of "European Veterinary Specialist in (name of specialty)" to veterinary specialists who meet the EBVS criteria;

– maintain a register of specialists recognised by the EBVS;

– provide information on specialisation in veterinary medicine in Europe to authorities, private organisations, veterinarians and owners of animal.

Within EBVS umbrella, there are 23 Specialist Colleges currently present in Europe. The credibility and quality control of all European Colleges is governed by EBVS, whose Board and Executive Committee make great efforts to ensure a level standard between all Colleges. EBVS defines guidelines for the recognition of Specialist areas, coordinate the foundation and monitor the performance and activity of each College, maintaining a register of College and ensuring regular revalidation, in order to ensure the quality of all European qualified veterinary Specialists (also called „Diplomates"). The way to become a Diplomate is to have completed an appropriate period of post graduate training or experience, as defined by each College and subsequently to undertake a residency programme for at least 3 years, which may only be done at a training institution which has been formally recognized and accredited by the College. Training institutions are generally (but not necessarily) located at a University. A standard resident must work for at least 2.5-3 years (depending on the length of the pre-requisite training) full time in close contact with a diplomate, and must publish at least 2 papers in scientific refereed journals during this time. After becoming Diplomate, each specialists is periodically re-validated, so to ensure that only those who remain active at a Specialist level, retain their Specialist qualifications. Re-validation is transparent process, where each diplomate is obliged to score in excess of 100 points in each 10 year period. Points are gained by publications in International Scientific per reviewed journals, by actively serving on College committees, by lecturing at national and international scientific meetings, by training residents etc.

One of Colleges under EBVS umbrella is the European College of Veterinary Public Health (ECVPH), which deals with veterinary specialisation in two areas: **Food Science** and **Population Medicine**. There is a growing demand for veterinary specialists in national veterinary services (private or governmental) and in (industrial) research institutes, in pre- and post-harvest monitoring and surveillance regarding food of animal origin, and food safety assurance throughout the production chain. This integrated approach is intended to contribute to and facilitate the formation of multidisciplinary teams of specialists in the fields of concern. The main ECVPH aims and objectives include:

– promotion of integrated, multidisciplinary approach towards analysis, control and prevention of hazards related to human and animal health;

– stablishing guidelines for post-graduate education and training prerequisites to become a specialist in Population Medicine or Food Science;

– examining and authenticating veterinarians as specialists in Veterinary Public Health in order to serve the livestock population (at both herd, region and national level), the livestock owners and the general public.

Concluding remarks

For the veterinary profession in Europe to maintain its reputation of contributing significantly to public health assurance it is essential that the establishments for veterinary education critically review their curricula in accordance with current societal demands. This inevitably includes upgrading the position of this important curricular element, Food Hygiene/Veterinary Public Health and ensuring that a minimum European standard along the lines described in this paper is established at each faculty, so as to guarantee that fresh graduates possess the starting competence to engage in a FH/VPH career and to do so in every European Member State.

Furthermore, in view of the legally established liberty of 'free movement' of veterinary professionals across European borders, it appears important for the reputation of our profession that the European legislator would make additional legal arrangements securing that only graduates from those establishments of veterinary

education whose curricula adhere to minimum requirements such as those presented here are recognised as being competent in the FH/VPH area.

REFERENCES

1. Buncic S., Forbes N. (2012) Evropski specijalista u veterinarskoj medicini: zašto i kako postati? Vet. Glasnik, submitted
2. Buncic S. (2006) Integrated Food Safety and Veterinary Public Health. CABI International Publishing, Wallingford, Oxfordshire, UK
3. EC (2004). Regulation (EC) No. 854/2004 of the European Parliament and of the Council of 29 April, laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption; Chapter IV 'Professional Qualifications'. Official Journal of the European Union, L 155/206
4. EC (2005). Directive 2005/36/EC of the European Parliament and the Council of 7 September 2005 on the recognition of professional qualifications, Official Journal of the European Union, L 255/27
5. Korkeala, H., Lindström, M., Fredriksson-Ahomaa, M. (2003), Food hygienic research and education in veterinary schools: the presence and the future. Arch Lebensmittelhyg 54: 146-150
6. Lundén J., Björkroth, J., Korkeala, H. (2007). Meat inspection education in Finnish veterinary curriculum. J. Vet. Med. Educ. 34: 205-211
7. Maijala, R., Korkeala, H. (2008) Reviewing the undergraduate veterinary curriculum in Finland for control tasks in veterinary public health. J. Vet. Med. Educ. 35: 241-249.
8. Smulders J.F.M, Buncic S., Fehlhaber K., Huey H., Korkeala H., Prieto M., Steinhauserova I. (2012) Towards Harmonization of the European Food Hygiene/Veterinary Public Health Curriculum. J. Vet. Med. Ed. 39(2), 169-179

СОВРЕМЕНИТЕ ПРЕДИЗВИЦИ ВО ХИГИЕНАТА/БЕЗБЕДНОСТА НА ХРАНА И ПОВРАТНАТА РЕАКЦИЈА ОД ВЕТЕРИНАРНОТО ОБРАЗОВАНИЕ -EAEVE, EBVS И ECVPH

Бунчиќ Сава[1]

[1]*Катедра за Ветеринарна медицина, Земјоделски Факултет, Нови Сад, Србија*

АПСТРАКТ

Одговорностите на "контролорите на безбедноста на храната" во современите времиња сè повеќе се префрлуваат од традиционалната "контрола на финалниот производот" кон должински интегрирано осигурување на безбедност. Доколку ветеринарите сакаат и имаат намера да продолжат да играат главна улога во осигурувањето на безбедноста на храната, тие треба да го прилагодат нивното знаење и професионални способности, за да бидат во можност да се справат со покомплексни и мулти-дисциплинарни проблеми и прашања. Ова укажува на неопходноста на преструктуирањето на универзитетските програми за обука, со цел да се обезбеди почетна компетентност од оваа област на дипломираните ветеринарни студенти или нивните субпопулации. Препознавајќи го ова, Европската асоцијација на Ветеринарни установи за Ветеринарно образование (EAEVE) неодамна воспостави работна група за да ја анализира тековната ситуација, со цел да се направат Стандардни оперативни процедури кои ќе овозможат фер и транспарентни проценки на универзитетите/факултетите кои го сочинуваат нивното членство, и во согласност со експлицитното Европско законодавство за професионални квалификации ги сметаат за неопходни за оваа ветеринарна дисциплина. Овој труд ги сумира главните заклучоци и препораки од претходно опишаната работна група, конципирано со цел да се придонесе кон меѓународните напори за оптимизирање на ветеринарната обука во FH/VPH. Исто така, накратко се наведени улогите на Европската Одбор за Ветеринарна специјализација (EBVS) и неговиот Европски колеџ за Ветеринарно јавно здравство (ECVPH) за постигнување, сертифицирање и одржување на специјалистичките способности во областа на Ветеринарното јавно здравство. Овој труд се базира на и содржи делови од, поврзани неодамнешни публикации (наведени), во кои што беше вклучен овој автор.

Клучни зборови: хигиена на храна, безбедност на храна, ветеринарно јавно здравство, ветеринарно образование, ветеринарна специјализација

Plenary Lecture UDC: 637.5.028

EFFECT OF MODIFIED ATMOSPHERE PACKAGING ON EXTENSION OF FOOD SHELF LIFE

Milijasevic Milan[1], Matekalo-Sverak Vesna[1], Babic Jelena[1]

[1]*Institute of meat hygiene and technology, Belgrade, Serbia*

ABSTRACT
Meat packaging is the most dynamic area of meat technology today and it continues to configure future of this branch of food industry. Retail meat packaging should fulfil certain technological and hygienic demands, as well as demands such as attractive apperance, appropriate meat color, consumer's acceptability, etc. Consumers are also very sensitive with regards to the use of food additives in industry. The demand for easily available fresh food is more present which makes food safety and availability of all kinds of foodstuffs of great importance. During the last two decades modified atmosphere packaging (MAP) has become a dominant retail meat packaging technology. Main reasons that stimulate MAP development are continuous increase in consumption of fresh meat, an increase of urban population and exhausting of natural food resources. The display of meat in plastic materials allows consumer evaluation of the product in an attractive, hygienic and convenient package. The purpose of this technology is to prolong shelf life of foodstuffs by preventing or inhibiting biochemical reactions (fat oxidation, metmyoglobine formation), growth of spoilage bacteria and degree of product respiration. MAP techniques are now used on a wide range of fresh or chilled foods, including raw and cooked meats and poultry, fish, fresh pasta, fruit and vegetables, coffee, tea and bakery products. For some of these, MAP is the major packaging method used. There are decisive economic advantages for the particular companies using MAP. This technology opens up new markets and offers the possibility of successfully establishing new products and thus extending the product range. This paper reviews in critical maner the most important aspects of packaging various foodstuffs in modified atmosphere.
Key words: modified atmosphere packaging, meat, foodstuffs, gases

"This paper is a result of the research within the projects TR 31011 and III 46009 financed by the Ministry of Education and Science, Republic of Sebia".

INTRODUCTION

The shelf life of perishable foods as meat, poultry, fish, fruits and vegetables and bakery products is limited in the presence of normal air by two principal factors - the chemical effect of atmospheric oxygen and the growth of aerobic spoilage microorganisms. These factors either individually or in association with one another bring about changes in odour, flavour, colour and texture leading to an overall deterioration in quality. Chilled storage will slow down these undesirable changes but will not necessarily extend the shelf life sufficiently for retail distribution and display purposes. Food spoilage is defined as changes that make a product unacceptable for human consumption. Such changes can include visible bacterial growth, slime formation, physical damage or off-odour. The process collectively known as food spoilage is very complex event, in wich a combination of microbial and biochemical or chemical activities interact.

Packaging of food now performs beyond the conventional protection properties and provides many functions for the contained product (Han, 2005). During the last two decades modified atmosphere packaging (MAP) has become a dominant retail meat packaging technology (Robertson, 1993). Main reason that stimulate MAP development are continuous increase in consumption of fresh meat, an increase of urban population and exhausting of natural food resources. Developments in packaging materials and technologies have made the application of modified atmosphere packaging on a larger scale to meat and meat products feasible (Brody, 1989). Packaging a perishable product in an atmosphere which has been modified so that its composition is other than that of air is termed as MAP. This refers to a system where the normal atmosphere, assumed to be approximatelly 78% N_2, 21% O_2, and <1% CO_2 is intentionally changed to some other identified gas composition. MAP is replacement of air in a pack with a single gas or mixture of gases; the proportion of each component is fixed when the mixture is introduced. This allows the preservation of the fresh state of the food product without the temperature or chemical treatments used by competitive preservation techniques, such as canning, freezing, dehydration and other processes. Because this system is used in a closed packaging system, the atmosphere once changed cannot be monitored or controlled. Maintenance of the correct gas mixture injected into MA packs is essential to ensure product quality, appeareance and shelf life extension. For these reasons routine gas analyses of MA packs should be included as part of the process control. Analysis of the gases within MA packs can indicate faults with seal integrity, MAP materials, MAP machinery or gas mixing prior the flushing. The main purposes of MAP of meat and meat products, but also the other foodstuffs, is two fold: to ensure the microbiological shelf life and the sensory quality of the product, including the color, odor and palatability. Many meat packaging systems currently exist, each with different attributes and applications. These systems range from overwrap packaging for short-term chilled storage and retail display, to a diversity of specified modified atmosphere packaging systems for longer-term chilled storage and display, to vacuum packaging, bulk gas flushing or MAP systems using 100% carbon dioxide for long term chilled storage. Preservation using MAP has been known for more than 100 years, but not

commercially used until the latter part of the 20th century (Brody, 1998). MAP was first used as an extension of the shelf-life of apples by storing them in atmospheres with reduced oxygen and increased carbon dioxide concentrations. In the 1930s it was used as modified atmosphere storage to transport fruit in the holds of ships. However, the technique was not introduced commercially for retail packs until the early 1970s. MAP techniques are now used on a wide range of fresh or chilled foods reflecting the increase in consumer demand for longer shelf life foods and less use of preservatives. Trough the use of natural gases and adequate packaging materials and machines, the quality of foodstuffs is maintained and their shelf life enhanced. During the last decades, MAP of various food products has been well studied and documented (Martinez et al., 2006; Ozogul et al., 2000).

Gases used in MAP technology

Oxygen

Oxygen has important role in MAP, especially in packaging of fresh meat (Martinez et al., 2006). The color of fresh meat is determined by the condition of myoglobin in the meat. When an anaerobic atmosphere is applied, myoglobin will be transformed to metmyoglobin, producing a brown color which is undesirable for consumers. It is therefore essential that oxygen is included into the applied gas atmosphere when fresh meat is packaged. This will ensure the myoglobin is oxygenated, resulting in an attractive bright red color (Church, 1993). O_2 is fairly active molecule and is associated with the process of the oxidation, i.e. the change of the chemical state of some biological molecules. The chemical breakdown of lipids is the primary process in dry or in dehydrated foodstuffs and in high fat fish. This is due to the oxydation of unsaturated fats in the presence of atmospheric oxygen, causing the product to turn rancid. Reduced oxygen concentration within the package could prevent or slow down oxidative reactions such as lipid rancidity in meats, fish and bakery foods, which would result in off odors and flavors, or the browning reaction in cut fresh fruits due to the action of polyphenol oxidase. However, complete absence of oxygen is not good either. For example, gaseous mixture used for fresh meat usually contains 80% oxygen in order to maintain the fresh bright red color. More importantly, extremely low level of oxygen would foster the growth of pathogen like *Clostridium botulinum.* Packaging must have appropriately low oxygen/gas permeability as well as tight sealings, otherwise too much gas can penetrate. The share of residual oxygen in each package should be less than 1-2%. In the case of higher oxygen values, MAP cannot be used to its best advantage as far as oxidation protection is concerned. The exceptions occur where oxygen is needed for fruit and vegetable respiration, color retention as in the case of red meat or to avoid anaerobic conditions in white fish.

Carbon dioxide

Carbon dioxide is the most important gas in the field of MAP technology. Carbon dioxide is a quite active gas as opposed to the inertness of nitrogen. This gas can inhibit the growth of several types of microorganisms, especially those that cause slime and off-odors in refrigerated foods. Carbon dioxide is both water and lipid soluble and although it is not a bactericide or fungicide, carbon dioxide has bacteriostatic and fungistatic properties. The bacteriostatic and fungistatic properties of carbon dioxide have been widely recognized since the 1920s and was used in shipments of beef, mutton and lamb from Australia and New Zealand to England. It's solubility increases with decreasing temperature and higher food pH. For maximum antimicrobial effect, the storage temperature of MAP product should be kept as low as possible. The absorption of CO_2 is highly dependent on the moisture and fat content of the product. Greater than 99% of the gaseous carbon dioxide exists as dissolved gas and less than 1% as carbonic acid (H_2CO_3). The owerall effect of carbon dioxide on microorganisms is an extension of the lag phase of growth and a decrease in the growth rate during the logarithmic growth phase. The precise mechanism of its action is still a subject of considerable interest and is not as well understood as mechanisms of other external factors, such as a_w and pH. Some speculate that it may be a simple lowering of pH within the cells of some organisms or it may inhibit specific metabolic pathways. Carbon dioxide probablly exerts its influence upon a cell by affecting particular enzymatic reactions. The primary sites where CO_2 exerts its effects are the enzymatic carboxylation and decarboxylation reactions, although inhibition of other enzymes has also been reported. Carbon dioxide probable inhibits microbial activity by effectively dissolving into the food´s liquid and fat phase, thereby reducing its pH, and by penetrating biological membranes, causing changes in permeability and function. What usually happens to perishable products stored in elevated levels of carbon dioxide is that there is a change not only in the numbers of microorganisms, but also a change in the types of organisms present. Very often this shift is from gram-negative types to gram-positive bacteria such as *Streptococci* and *Lactobacilli* (Brody, 1989). Most microorganisms such as mould and the most common aerobic bacteria are strongly affected by carbon dioxide. The growth of anaerobic microorganisms, on the other hand, is less affected by this gas atmosphere. Intensity of CO_2 activity depends on concentration of gas, initial bacterial contamination of foods, storage temperature and nature of packaged food (Reddy et al., 1991). Carbon dioxide also has the advantage that it is relatively nontoxic to humans. If carbon dioxide, in a concentration which allows it to unfold its bacteriostatic effect, is part of the modified atmosphere, the minimum concentration of this gas should be 20%.

Nitrogen

Nitrogen has been used in MAP for many years due to its inert property. It is an inert tasteless gas. It can dispace oxygen in MAP, thus extend the shelf life. It prevents fat rancidity and inhibits the growth of aerobic microorganisms. Moreover, use of nitrogen in MAP can prevent package collapse due to its low solubility in both water and fat phase of foods. The gas has no direct effects on color of food. Also, the role of nitrogen in MAP is to act as filler gas and keeps flexible packages from developing a vacuum.

Although other gases such as nitrous and nitric oxides, carbon monoxide, sulphur dioxide, ethylene, chlorine as well as ozone and propylene oxide have been investigated, they have not been applied commercially due to safety, regulatory and cost considerations.

The optimisation of packaging is a decisive factor for the efficiency of MAP. The packaging must have appropriately low oxygen/gas permeability as well as tight sealings, otherwise too much gas can penetrate.

Microbial safety of MAP

Growth and survival of spoilage and pathogenic microorganisms are affected by MAP. In anaerobic atmospheres with high levels of CO_2, lactic acid bacteria dominate the microflora in meat, particulary at low chill temperatures. The limitation of the shelf life of meat stored in conditions with access to air, or in atmospheres containing high O_2, is frequently caused by growth of the spoilage flora, *Brochothrix termosphacta* and *Pseudomonas spp*., which both grow at low storage temperatures. The microbial spoilage of fresh meat is dictated by the initial microbial quality of the meats, types of organisms present initially, product storage temperature, time and package conditions, including the gaseous atmospheres in the MAP products. The levels and types of microflora in MAP is dynamic, depending on the prevailing atmospheres and their lenghts of time. Presence of aerobic conditions results in growth of mainly aerobic psychrotrophic microorganisms, *Acinetobacter, Moraxella* and *Pseudomonas.* Growth of *Pseudomonas* results in production of off-odors. In anaerobic conditions, the aerobic spoilage types are inhibited and spoilage is primarily due to anaerobic, aerotolerant lactobacilli. In high pH meats the facultative anaerobic types, *Enterobacter, Brochothrix thermospachta* and *Alteromonas putrifaciens* will predominate. Growth of these microorganisms will result in sour, putrid, sulfurous odors at higher populations.

Major concern of MAP in the food industry is possibility that strains of *Clostridium botulinum* types B, E and F are able to grow and produce toxins under these conditions. *C.botulinum* type E is concern because of its ability to grow at temperatures as low as +3°C. Use of carbon dioxide concentrations >45% may provide a degree of safety in terms of delaying *C. botulinum* toxigenesis. Spores of *C. botulinum* are resistant to freezing and can survive in frozen storage indefinitely. Botulinal toxins present in foods are also tolerant to freezing and retain their activity.

Fresh meat is often contaminated with the pathogens *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophila* and they all are able to grow at temperatures used for storage of pork, lamb and poultry in MAP. *L. monocytogenes* has emerged as a pathogen of concern to the meat industry since the 1990s. This microorganism is psychrotrophic, facultatively anaerobic, and widely distributed in the enviroment. This bacteria does not compete well with the normal spoilage flora of the meat. *L. monocytogenes* does not grow in high pH fresh meat at <5°C. Presence of very low levels of oxygen or vacuum packaging and storage at 0, 5 and 10°C does allow its growth.

Carbon dioxide inhibits the growth of *Y. enterocolitica* at refrigeration temperatures. *Y. enterocolitica* is more prevalent in pork and pork products. The presence of this pathogen on fresh meat can result of growth and may present microbiological risk when stored under vacuum and MAP.

Aeromonas hydrophila can grow on high pH meat in vacuum, while in CO_2 enriched atmospheres its growth is inhibited. Storage of pork loin slices at +1°C under MAP increased the shelf-life to >16 days, along with control of *A. hydrophila*.

Packaging of meat

Consumers perceive meat with bright red color as "fresh" and therefore safe to consume. The variables that influence the shelf life properties of packaged fresh meat are product, gas mixture, package and headspace, packaging equipment, storage temperature and additives. Meat color is the single greatest appereance factor that determines whether or not meat cut will be purchased. A special problem arises with red meats such as beef with regard to color changes caused by the oxidation of the red pigment. Oxygen in the enviroment is necessary to impart the fresh meat color by formation of oxymyoglobin, which is more resistant to oxidation, compared to the deoxy form. Oxymyoglobin is formed by O_2 binding to the ferrous heme with high O_2 tension while metmyoglobinis caused by oxidation of heme pigments to a ferric state. The display life of meat is limited by the time required for oxidation of oxymioglobin to metmyoglobin, initially on the surface layers of muscle tissue. Highly pigmented meats such as beef reqiure higher oxygen concentrations than low pigmented meats such as pork. The presence of minimal concentrations of oxygen would sufice for the growth of aerobic spoilage flora of meat and they growth can be delayed by incorporation of 20 to 30% CO_2 in the gas mixtures. MAP for meat requires a barrier of both moisture and gas permeation trough packaging materials to maintain constant package environment during storage. Deteorative changes during meat storage are affected by metabolic reactions from biological membrane disruption and biochemical oxidative processes. Deterioration of quality may include discoloration, off-flavor and off-odor development, nutrient loss, texture changes, pathogenicity and progression of spoilage factors. The purpose of MAP is to maintain the desired properties of meat for the desired period of storage and display.

Packaging of fish

The shelf life of fresh fish is short. Fresh fish rapidly loses its original quality due to microbial growth and enzymatic processes. Commercially, this often makes the transport and marketing of fish a challenge. The sensitivity of fish and seafood is caused by its high water activity, neutral pH and presence of specific enzymes which rapidly undermine both taste and smell. Therefore, the fishing industry has always been willing to explore new technologies for shelf life extension. Modified atmosphere packaging, in which the composition of the atmosphere surrounding the fillet is different from the normal composition of air, can be an effective technique for delaying microbial spoilage and oxidative rancidity in fish. Temperature is of primary importance in all fresh fish storage, including MAP and vacuum packaging, as both enzymatic and microbial activity are greatly influenced by temperature. In order to maintain the high quality of fresh fish products, it is absolutely necessary to keep the temperatures as close to 0°C as possible. Some people recommend supercooling of fish i.e. cooling to -1° to -2°C. This slows down many reactions even further, but so far this has no been feasible commercially. For white fish gas mixtures containing 34-45% CO_2, 25-35% O_2 and 25-35% N_2 are recommended. Gas mixtures containing up to 60% CO_2 in combination with N_2 are recommended for oily fish. However, high levels of carbon dioxide in packaging of fresh fishery products may result in the carbon dioxide dissolving into the fish flesh causing deformation or collapse of the packaging and also affecting the product color by interfering with flesh pigments. Fillets from some species, such as herring and haddock, particularly benefit from being packaged un-

der modified atmosphere since this reduces production of chemicals such as peroxides which affect the sensory characteristics and hence shelf life of the product. The extent to which the shelf life of the product can be extended by MAP will depend on the species, fat content, initial bacterial count, gas mixture, type of packaging material and temperature of storage. Since fish can be contaminated with *C. botulinum* type E great care has to be exercised when determining the shelf life. The risks from botulism in MAP fish have been widely reviewed (Reddy et al., 1992). Growth occured more frequently from spores of type F than strains than for types B and E. Nowdays it is recognized that the growth of *C. botulinum* in foods does not depend upon the total exclusion of oxygen, nor does the inclusion of oxygen as packaging gas ensure that growth of *C. botulinum* is prevented. Depending on the storage temperature, MAP prolongs shelf life by 3 or 5 days compared with the shelf life of raw fish in a tray with film overwrap.

CONCLUSION

The use of MAP in food packaging has been practiced for about 100 years, but still the potential that can be achieved using this technology has not been realized completely. MAP, if used properly for the right commercial reasons, offers sufficient benefits to both food industry and to consumers thereby suggesting that this is one of many alternatives that the industry should consider and use as part of a high quality food marketing program. Only the highest quality food products should be used to benefit from the extended shelf life advantages of MAP. The use of this technology to make up for defects in product quality and limitations in transportation will only lead to consumer unhappiness. Its use does not eliminate the need for proper control of storage conditions, especially temperature, nor for the adequate training of food handlers at every stage of the food preparation process.

REFERENCES

1. Han, J.H. (2005). New technologies in food packaging: Overview. In J.H. Han (Ed.) Inovations in food packaging. 3-11. Elsevier Academic Press.
2. Robertson, G. (1993). Food packaging principles and practice. New York: Marcel Dekker 431-469.
3. Brody, A.L. (1989). Controlled / Modified Atmosphere / Vacuum Packaging of Foods, Food and Nutrition Press
4. Brody, L. (1998). Markets for MAP foods. In: Blakistone, B.A. (Ed.) Principles and Applications of Modified Atmosphere Packaging of Foods (pp. 14-38). Blackie Academic & Professional, London.
5. Martinez, L., Djenane, D., Cilla, I., Beltran, J.A., Roncales, P. (2006). Effect of varying oxygen concentrations on the shelf life of fresh pork sausages packaged in modified atmosphere. Food Chemistry, 94: 219 – 225.
6. Özogul, F., Taylor, K.D.A., Quantick, P., Özogul, Y., (2000). Chemical, microbiological, and sensory evaluation of Atlantic herring (*Clupea harengus*) stored in ice, modified atmosphere and vacuum pack. Food Chemistry. 71: 267 – 273.
7. Church, P.N., (1993). Meat products. Principles and application of modified atmosphere packaging of foods, 229 - 268.
8. Brody, L. (1989). Modified atmosphere/vacuum packaging of meat. In L. Brody (Ed.) Controlled/modified atmosphere/vacuum packaging of foods (pp.17-37). Trumbull, Conecticut: Food and Nutrition Press.
9. Reddy, I.M., Carpenter, C.E., (1991). Determination of metmyoglobin reductase activity in bovine skeletal muscle. Jornal of Food Science, 56, 1161-1164.
10. Reddy, N.R., Armstrong, D.J., Rhodehamel, E.J., Kauter, D.A. (1992). Shelf-life extension and safety concerns about fresh fishery products packaged under modified atmospheres: a review. Journal of Food Safety, 12, 87-118.

ЕФЕКТОТ НА ПАКУВАЊЕТО СО МОДИФИЦИРАНА АТМОСФЕРА ВРЗ ПРОДОЛЖУВАЊЕТО НА РОКОТ НА ТРАЕЊЕ НА ХРАНАТА

Милијашевиќ Милан[1], Матекало-Сверак Весна[1], Бабиќ Јелена[1]

[1]*Институт за хигиена и технологија на месо, Белград, Србија*

АПСТРАКТ

Денеска пакувањето на месо е најдинамична област на технологијата на месо и продолжува да ја конфигурира иднината на оваа гранка на прехранбената индустрија. Пакувањето на месото во малопродажбата треба да исполнува одредени технолошки и хигиенски барања, како и барања како што се атрактивен изглед, соодветна боја на месото, прифатливост од страна на потрошувачот, и тн. Потрошувачите се исто така многу чувствителни во однос на употребата на прехранбените адитиви во индустријата. Побарувачката за лесно достапна свежа храна е се поприсутна, што ја прави безбедноста на храната и достапноста на сите видови на прехранбени производи од големо значење. Во текот на последните две децении пакувањето со модифицирана атмосфера (МАП) стана доминантна технологија на пакување во малопродажбата. Главните причини кои го стимулираат развој на МАП- от се континуираниот пораст на консумацијата на свежо месо, зголемувањето на урбаното население и исцрпувањето на ресурсите на природна храна. Изложувањето на месото во пластични материјали му овозможува на потрошувачот евалуација на производот во атрактивно, хигиенско и прикладно пакување. Целта на оваа технологија е да се продолжи рокот на траење на прехранбените производи преку спречување или инхибирање на биохемиските реакции (оксидацијата на мастите, формирање на метхемоглобин), раст на бактерии што ја расипуваат храната и степенот на респирација на производот. МАП техниките сега се користат за широк спектар на свежи или разладени храни, вклучувајќи ги и суровите и готвени меса и живина, риба, свежи тестенини, овошје и зеленчук, кафе, чај и пекарски производи. За некои од овие, МАП е главен метод кој се употребува за пакување. Постојат одлучувачки економски предности за одредени компании кои што користат МАП. Оваа технологија отвора нови пазари и нуди можност за успешно воспоставување на нови производи и на тој начин за проширување на асортиманот на производи. Овој труд ги разгледува од критичен аспект најважните аспекти на пакување на различни прехранбени производи во модифицирана атмосфера.

Клучни зборови : пакување на месо во модифицирана атмосфера, прехрамбени производи, гасови

UDC: 637.1:614.31

RAW MILK FROM VENDING MILK MACHINE IN SLOVENIA: FOOD SAFETY CONTROL

Kirbiš A.[1], Biasizzo M.[1], Vadnjal S.[1], Torkar K.[2], Bauer M.[2], Jevšnik M.[2]

[1] University of Ljubljana, Veterinary Faculty, Institute for Food Hygiene, 1000 Ljubljana, Slovenia
[2]University of Ljubljana, College of Health Studies, Department of Sanitary Engineering, 1000 Ljubljana, Slovenia

ABSTRACT
Milk and milk products of dairy cows can be harbor a variety of microorganisms and can be important sources of foodborne pathogens. Most milk on the market is pasteurized but milk from vending machine is raw and this can be risk for Consumers health. The safety of raw milk sold from milk vending machine in Slovenia was investigated in relation to hygiene quality parameters according Commission Regulation (EC) No 853/2004. During the year 2011 and until May of 2012 67 samples were examined for total count and antibiotic residues. Examinations for presence of somatic cells were performed at 17 samples. Reference method ISO 4833:2003 was used for total plate count, enumeration of somatic cells was performed by instrumental method (Fosomatic) and Delvo ®SP-NT was used for detection of antibiotics. For total count 32 % of samples exceed the level of 100.000 $CFUml^{-1}$ and 6 % the level of 400.000 $SCCml^{-1}$. All samples were negative for presence of antibiotic residue. Results indicate the potential hygienic risk when the raw milk is sold by vending milk machine.
Key words: raw milk, vending milk machine, total plate count, somatic cells, antibiotic residues

INTRODUCTION

Many people in Slovenia are consuming raw unpasteurized milk due to increasing number of vending milk machines all over the country. Nutritional qualities, taste, and health benefits has all been advocated as reasons for increased interest in raw milk consumption.

Unpasteurized milk has been a known vehicle of food-borne disease. With the gradual implementation of pasteurization, the risk of microbe infection was greatly reduced (1). People continue to consume raw milk even though numerous epidemiological studies have shown clearly that raw milk can be contaminated by a variety of pathogens, some of which are associated with human illness (2).

Hygiene criteria for raw cow milk in the Commission Regulation (EC) No 853/2004 determined that the results of geometric average over a two-month period, with at least two samples per month for plate count at 30 °C should not exceed 100.000 $CFUml^{-1}$. For somatic cell count an average over a three-month period, with at least one sample per month should not exceed 400.000 (3). Maximal residual levels (MRLs) of antibiotics in foods are stated in Commission Regulation (EC) No 37/2010 (4).

Screening test for Delvotest SP-NT is a simple and fast test to detect β-lactames in milk samples in concentrations at or lower than MRL (5, 6).

Hygiene quality and antibiotic residua of raw milk sold from vending milk machine in Slovenia were investigated.

MATERIALS AND METHODS

During the year 2011 and until May of 2012 67 samples of raw milk were taken from different vending milk machines in Slovenia.

For all 67 samples total bacterial count were determined according to ISO 4833:2033. Plate count agar with skim milk powder was used (Milk plate count agar, Oxoid, United Kingdom). Plates were incubated at 30°C for 72 hours.

Delvo ®SP-NT test (DSM, Netherlands) was used us screening test for detection of antibiotics at all 67 samples. It is a simple and fast agar diffusion test based on the inhibition of growth of Bacillus stereothermophilus. It is especially used to detect β-lactames in milk samples in concentrations at or lower than MRL stated in Commission regulation No 37/2010.

Enumeration of somatic cells was performed by instrumental method (Foss, Denmark).

RESULTS

A total of 67 samples from different vending milk machines were tested for total count and antibiotic residua.

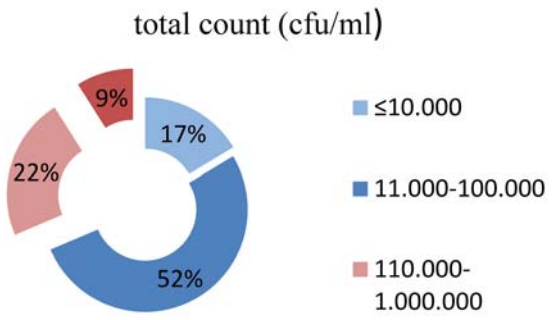


Figure 1. *Rate of the samples classified into contamination levels of total bacterial count*

All samples tested for antibiotic residua were negative.

We classified the results of total count enumeration into four contamination levels: > 10^6, 10^5- 10^6, 10^4- 10^5 and < 10^4 CFUml^{-1}. Proportions of the samples that were classified in each level are presented in Picture 1. At 69% of the samples a total number of bacteria were below and at 31% above the limit of 100.000 CFUml^{-1}.

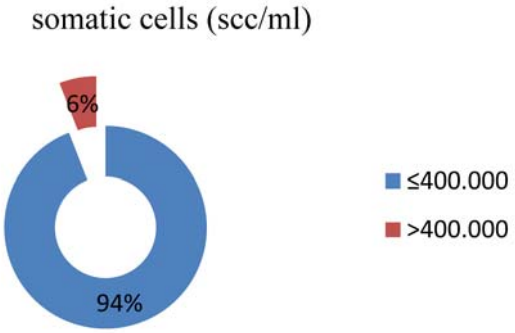


Figure 2. *Classification of samples regarding the number of somatic cells*

Results of 17 tested samples for somatic cells were classified in to levels (Figure 2). At 94% (16/17) of the samples the number of somatic cells was below or equal to 400.000 and at 6% (1/17) above that limit.

CONCLUSIONS

In this study hygienic situation regarding raw milk on the market distributed by vending milk machine in Slovenia was investigated. Results revealed that almost third of the milk samples exceed the level 100.000 CFUml-1. We could not conduct, that they are inadequate with the legislation due to the criteria of ≤100.000 CFUml-1 (results of geometric average over a two-month period). But we can conclude that at 9% of the samples where total bacterial counts exceed also the level of 1.000.000 CFUml-1 the hygienic quality is inadequate for milk sailing raw on the market. Higher number of bacteria in the samples could be a result of inadequate hygiene of milk or milk handling or also as a result of not appropriate temperature regime in vending milk machine.

Only at one of 17 samples of the number of somatic cells were above the level of 400.000 SCCml-1and all 67 samples tested for antibiotics residua were negative.

We can classified a raw milk on the market as a food with higher risk regarding contamination with pathogens such as salmonella, *Listeria monocytogenes*, thermo tolerant *Campylobacter* and verotoxin producing *Echerichia coli* (2). Special concern such as periodic control of hygiene situation an also checking for presence of pathogens at such types of foods intended to be eaten raw would be needed.

REFERENCES

1. D´Amico D.J., Groves E., Donnelly C. W. Low Incidence of Foodborne Pathogens of Concern in Raw Milk Utilized for Farmstead Cheese Production; J Food Prot 2008, 1580-9.
2. Giacometti F., Serraino A, Finazzi G., Daminelli P., Losio M. N., Arrigoni N., Piva S., Florio D., Riu R, and Zanoni R. G. Foodborne Pathogens and Disease. April 2012, 9(4): 293-297.
3. Commission Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin *OJ L 139, 30.4.2004, p. 55–205*
4. Commission Regulation No 37/2010, of 22. December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs on animal origin. Off J Eur Commun 2010; L 15(20. 1. 2010): 72 str.
5. Perme, T., Križman, M., Šinigoj-Gačnik, Ksenija, Kirbiš, A. Validation of Twinsensor BT, screening test for the detection of beta-lactams and tetracyclines in milk, and comparison to DelvotestR SP-NT. *Slov. vet. res.*. [English ed.], 2010, vol. 47, no. 3, str. 97-106.
6. Pengov, A., Kirbiš, A. Risks of antibiotic residues in milk following intramammary and intramuscular treatments in dairy sheep. *Anal. chim. acta.* [Print ed.], 2009, vol. 637, no. 1/2, str. 13-17.

КОНТРОЛА НА БЕЗБЕДНОСТА НА ХРАНАТА: СИРОВО МЛЕКО ОД АВТОМАТИ ЗА ПИЈАЛОЦИ

Кирбиш А.[1], Бјасицо М.[1], Вадњал С.[1], Торкар К.[1], Бауер М.[2], Јевшник М.[2]

[1] *Институт за хигиена на храна, Ветеринарен факултет, Универзитет во Љубљана, 1000, Љубљана, Словенија*
[2]*Катедра за санитарен инженеринг, Факултет за здравствени науки, Универзитет во Љубљана, 1000, Љубљана, Словенија*

АПСТРАКТ

Млекото и млечните производи од кравјо млеко може да претставуваат место на живеење на различни микроорганизми и може да бидат извор за патогени микроорганизми. Млекото кое се продава на пазарот е воглавно пастеризирано, но млекото од апаратите за пијалоци е сирово и може да претставува ризик за здравјето на потрошувачите. Во овој труд е испитана безбедноста на сировото млеко од автоматите за пијалоци во Словенија во соглсаност со параметрите дадени во Регулативата на ЕК бр. 853/2004. Во периодот од 2011-2012 испитани се 67 примероци за вкупен број на бакетрии, број на соматски клетки и присуство на антибиотици. За испситување на вкупниот број на бактерии беше искористен референтен метод ISO 4833:2003, за одредување на бројот на соматски клетки беше користен инструментален метод (Fossomatic) и за детекција на присуство на антибиотици беше користен Delvo SP-NT тестот. По завршените испитувања 32% од примероците ја преминуваа границата за дозволен вкупен број на бактерии која изнесува 100.000 CFU/ml, додека пак границата од 400.000 соматски клетки во ml ја преминуваа 6% од примероците. Во ниеден од испитаните примероци не беше утврдено присуство на антибиотици. Генерално добиените резултати индицираат потенцијален ризик од сировото млеко кое се користи во апаратите за пијалоци.

Клучни зборови: сирово млеко, апарати за пијалоци, вкупен број на бактерии, соматски клетки, резидуи на антибиотици

UDC: 637.514.9.064:546.48

ASSESSMENT OF CADMIUM INTAKE ASSOCIATED WITH LIVER AND KIDNEY CONSUMPTION IN SERBIA

Janković Saša[1], Antonijević Biljana[2], Ćurčić Marijana[2], Radičević Tatjana[1], Stefanović Srđan[1], Nikolić Dragica[1], Petrović Zoran[1]

[1]*Institute of Meat Hygiene and Technology, Belgrade, Serbia*
[2]*Department of Toxicology „Akademik Danilo Soldatovic", Faculty of Pharmacy, Belgrade University, Belgrade, Serbia*

corresponding author: sasa@inmesbgd.com

ABSTRACT
Cadmium (Cd) is a toxic heavy metal, well known as environmental contaminant. Cadmium occurs on agricultural land as a contaminant of phosphorous fertilizer and in sewage sludge and may enter the human food chain. It is toxic, teratogenic, mutagenic and carcinogenic to most organisms. Of all the animal tissues, kidney and liver possess highest ability to bioaccumulate Cd. Metals accumulated in livestock can be passed on to humans who consume the meat and can become a health hazard to the public.
Concentrations of Cd were measured in liver and kidney of cattle (22 samples), pigs (65 samples), lambs (12 samples) and horses (30 samples) within the Serbian National monitoring program in 2011 and 2012. Samples for Cd analysis were prepared by microwave digestion (ETHOS Milestone). Analyses were carried out on atomic absorption spectrometer Varian "SpectrAA 220" with GTA 110 graphite furnace. The limit of quantification (LOQ) for Cd was 5 ng g-1. Analytical quality control was achieved by using certified reference material BCR 186. Replicate analyses were in the range of certified values.
For the purpose of intake assessment, data obtained from the FAO/WHO GEMS/Food Consumption Cluster Diets database were used. According to this source, estimated average weekly consumption of offal of cattle, pigs, lambs and horses are 44.1, 25.9, 7.0 and 0.7 g/week, respectively. Since GEMS does not provide separate consumption data for liver and kidney, cadmium concentrations are expressed as mean of liver and kidney results, as follows: 0.150 μg g^{-1} in cattle, 0.081 μg g^{-1} in pigs, 0.040 μg g^{-1} in lambs and 9.790 μg g^{-1} in horses.
All analysed samples contained cadmium below the maximum level fixed by the European Commission Decision and Serbian national regulation, excluding liver and kidney of horses where all samples except one contained Cd above maximum residue limit. The estimated weekly intake for Cd, based on mean cadmium value in analysed organs and average body weight of 70 kg, was 0.227 mg/kg b.w./week.
Based on EFSA (European food safety authority) recommended safe limit of 2.5 mg/kg b.w./week and on obtained results, we can conclude that the intake of cadmium in the case of consuming edible offal in Serbia is lower than 10% of the safe limit and does not pose a risk to human health.
Key words: intake, offal, cadmium

This work was supported by grant from the Ministry of Education and Science, Republic Serbia (project No III 46009).

INTRODUCTION

Cadmium (Cd) is a toxic heavy metal, well known as environmental contaminant. Industrial and agricultural processes have been largely responsible for environmental pollution with Cd. Cadmium is mainly used in smelting, refineries of ores, batteries, and it was found, as an impurity, in fertilizers with rising concern for human and animal health. Cadmium occurs on agricultural land as a contaminant of phosphorous fertilizer and in sewage sludge and may enter the human food chain. It is toxic , teratogenic, mutagenic and carcinogenic to most organisms. Of all the animal tissues, kidney and liver possess highest ability to bioaccumulate Cd. Metals accumulated in livestock can be passed on to humans who consume the meat and can become a health hazard to the public.

Food is most important source of cadmium contamination – about 90% (1) . Biggest concentration of cadmium is in offal, shells, mushrooms and some plants which accumulated cadmium from soil like cocoa and rise. Depended of diet in various countries, different food items have responsibility for great intake of Cd. In European country that is fish and marine products although in Asia for instance, Cd mainly ingested from rice.

After Cd enters in organism, it is deposited in liver, kidney and bones with half life of 20 years (2).

From early 50th, when danger from professional exposition to Cd was recognized, many scientific work considering toxic effects of cadmium have been done. After oral exposition, cadmium primarily affected kidney. Cadmium damaged glomerular filtration and tubular reabsorption, aminoacidura and appearance of low mass proteins in the urine as a consequence. Bone disorder, problems with vitamin D metabolism, anemia, liver and nervous system damages are also associated with cadmium exposure. International agency for research on cancer (IARC) declared cadmium and cadmium compounds carcinogenic to humans (3), based on sufficient scientific evidence.

Having in mind that animal offal have high levels of

cadmium and at the same time have small part in total diet, aim of this study was to assess the risk due to Cd intake associated with offal consumption by integration of empirical Cd concentrations measured in liver and kidney and data on their consumption.

MATERIALS AND METHODS

Concentrations of Cd were measured in liver and kidney of cattle (22 samples), pigs (65 samples), lambs (12 samples) and horses (30 samples) within the Serbian National monitoring program in 2011 and 2012. Samples for Cd analysis were prepared by microwave digestion (ETHOS Milestone). Aliquots of approximately 0.75 g were transferred into Teflon vessels and added with 8ml nitric acid (p.a. SIGMA) and 1.5ml hydrogen peroxide (30%, p.a., MERCK). The microwave program consisted of three steps as follows: 5 min from room temperature to 180°C, 10 min hold 180°C, 20 min vent. After cooling at room temperature, the digested sample solutions were quantitatively transferred into disposable flasks and diluted to 50ml with deionized water (ELGA).

Analyses were carried out on atomic absorption spectrometer Varian "SpectrAA 220" with GTA 110 graphite furnace. The limit of quantification (LOQ) for Cd was 5 ngg-1. Analytical quality control was achieved by using certified reference material BCR 186. Replicate analyses were in the range of certified values.

Total diet study has not been undertaken in Serbia, so far. Instead of such comprehensive data base, for the purpose of intake assessment, we used the only available surrogate taken from the FAO/WHO GEMS/Food Consumption Cluster Diets database (4). According to this source, estimated average weekly consumption of offal of cattle, pigs, lambs and horses are 44.1, 25.9, 7.0 and 0.7 g/week, respectively.

The following formula was used for calculation of intake assessment expressed as weekly intake (WI) in µg/kg b.w.:

$$WI = \frac{\text{Weekly consumption (g) x Concentration of contaminant } (\mu g/g)}{\text{Body weight (kg)}}$$

For different diet scenarios hazard index (HI) was calculated based on formulas given below:

$$HI = \frac{\text{calculated weekly intake for Cd}}{\text{provisional tolerable weekly intake}}$$

RESULTS

Minimum, mean and maximum values of cadmium content in liver and kidney of cattle, pigs, lambs and horses are presented in table 1.

Table 1. *Cadmium content in animal's liver and kidney*

	Cd content (µgg-1)							
	Cattle		Pigs		Lambs		Horses	
	liver	kidney	liver	kidney	liver	kidney	liver	kidney
min	0.037	0.154	0.005	0.025	0.023	0.027	0.252	3.135
max	0.099	0.347	0.127	0.360	0.095	0.101	4.730	55.770
mean	0.064	0.236	0.033	0.129	0.032	0.047	2.454	17.126

All analysed samples of cattle, pigs and lambs contained cadmium below the maximum level fixed by the European Commission Decision (5) and Serbian national regulation (6) – 0.5 µgg-1 for liver and 1 µgg-1 for kidney. However, only one sample of horse liver contained Cd below the maximum level. After horses, higher content of Cd was in the cattle offal – 0.064 µgg-1and 0.236 µgg-1 in liver and kidney respectively. Smallest concentration of Cd was found in liver of lambs – average 0.032 µgg-1. Our results are similar to the results of previous studies in European countries (7-10).

Since FAO/WHO database does not provide separate consumption data for liver and kidney, cadmium concentrations are expressed as mean of liver and kidney results, as follows: 0.150 µgg-1 in cattle, 0.081 µgg-1 in pigs, 0.040 µgg-1 in lambs and 9.790 µgg-1 in horses. Average intake of cadmium by offal of different animals are presented in figures 1.

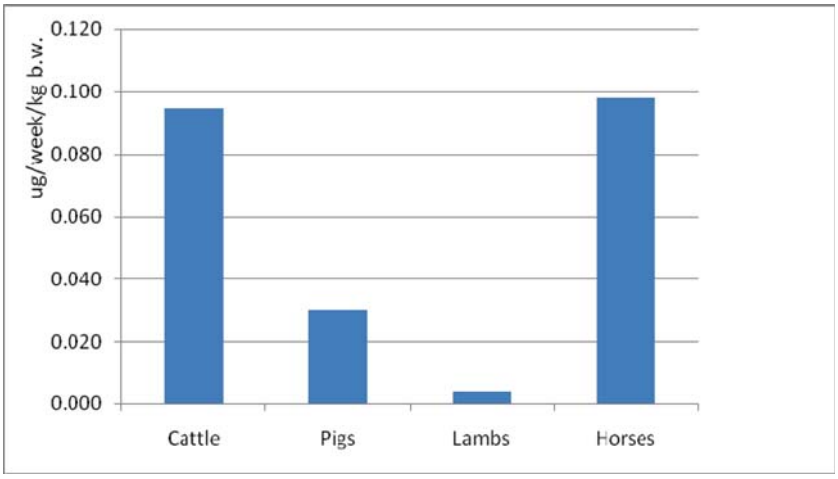


Figure 1. *Weekly intake of Cd associated with average contamination of animal's offal and supposing consumption*

Although hors offal has minimum part in diet (0.7 g/ week), the highest intake of Cd is from liver and kidney of horses, because the concentration of Cd in horse offal is much higher than Cd content in offal in other animal species. The estimated weekly intake for Cd, based on mean cadmium value in analysed organs and average body weight of 70 kg, was 0.227 mg/kg b.w./week. This result we compare with EFSA (European food safety authority) recommended intake on the weekly basis - 2.5 mg/kg b.w./week in order to calculation hazard index (HI). HI in this diet scenario was 0.09. In worst case scenario with maximum Cd concentration in all of animal species, weekly intake was 0.568 mg/kg b.w./week and still is about 5 times smaller than recommended value. HI in worst case scenario was 0.23 namely 23% of maximum safety limit.

CONCLUSION

Based on EFSA recommended safe limit of 2.5 mg/kg b.w./week and on obtained results, we can conclude that the mean intake of cadmium in the case of consuming edible offal in Serbia is lower than 10% of the safe limit and does not pose a risk to human health.

REFERENCES

1. Vahter M, Berglund M, Lind B, Jorhem L, Slorach S and Friberg L, 1991. Personal monitoring of lead and cadmium exposure--a Swedish study with special reference to methodological aspects. Scand J Work Environ Health 17 (1), 65-74.
2. ATSDR, Draft Toxicological profile for Cadmium, Public Health Service, Agency for Toxic Substances and Disease Registry, USA, 2008.
3. International Agency on Research of Cancer (IARC). IARC Cancer Databases. Available from: http://www.iarc.fr
4. FAO/WHO (Food and Agriculture Organization/ World Health Organization), 2006. The GEMS/Food Consumption Cluster Diets database, Geneva, Switzerland.
5. EC 1881/2006, 2006. Commission regulation No 1881/2006 EC of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, Official journal of European Union, L 364, 5-24.
6. Official Journal of RS 28/2011, 2011. Rulebook on maximum residue limits residues of pesticides in food and feed and food and feed which establishes maximum permissible residual quantities of pesticides.
7. Falandysz J. Manganese, copper, zinc, iron, cadmium, mercury and lead in muscle meat, liver and kidney of poultry, rabbit and sheep slaughtered in the northern part of Poland 1987. Food Addit Contam 1991;8:71]83.
8. Falandysz J. Some toxic and essential trace metals in cattle from the northern part of Poland. Sci Total Environ 1993;136:177]191.
9. Schulzschroeder G. Lead and cadmium contents in meat, liver and kidney samples from lambs and sheep. Fleischwirtschaft 1991;71:1435-1438.
10. Niemi A, Venalainen E, Hirvi J, Kappanen E. The lead, cadmium and mercury concentrations in muscle, liver and kidney from Finnish pigs and cattle during 1987]1988. Z Lebensm-Unters Forsh 1991;192:427]429.

ПРОЦЕНКА НА ВНОСОТ НА КАДМИУМ ВО СРБИЈА ПРИ КОНСУМАЦИЈА НА ЦРН ДРОБ И БУБРЕЗИ

Јанковиќ Саша[1], Антонијевиќ Билјана[2], Ќурчиќ Маријана[2], Радичевиќ Татјана[1], Стефановиќ Срѓан[1], Николиќ Драгица[1], Петровиќ Зоран[1]

[1]Институт за хигиена и технологија на месо, Београд, Србија
[2]Оддел за токсикологија "Академик Данило Солдатовиќ", Фармацевтски факултет, Универзитет во Београд, Београд, Србија

Автор за коресподенција: sasa@inmesbgd.com

АПСТРАКТ

Кадмиумот (Cd) е токсичен тежок метал, добро познат како контаминент наживотната средина. Кадмиумот се јавува кај земјоделското земјиште како контаминент на фосфатното ѓубриво или во отпадната тиња и можно е да влезе во синџирот на исхраната на човекот. Тој е токсичен, тетратоген, мутаген и канцероген за повеќето организми. Од сите животински ткива, бубрегот и црниот дроб имаат најголема способност за биоакумулација на Cd. Металите кои се акумулирани во добитокот може да преминат кај луѓето кои го косумираат месото и да станат потенцијална здравствена опасност за јавноста.

Концентрациите на Cd беа измерени во црн дроб и бубрег на говеда (22 примерока), свињи (65 примероци), јагнишa (12 примероци) и коњи (30 примероци), во рамките на Националната мониторинг програма во 2011 и 2012. Примероците за анализа на Cd беа подготвени со микробранова дигестија (ETHOS Milestone). Анализите беа изведени на атомски апсорпционен спектрометар Varian "SpectrAA 220" со GTA 110 Графитна печка. Лимитот на квантификација (LOQ) за кадмиум беше 5 ng g^{-1}. Аналитичката контрола на квалитетот беше обезбедена со примена на сертифициран референтен материјал BCR 186. Повторените анализи беа во рамките на сертифицираните вредности.

Со цел да се изврши проценка на вносот користени се податоци добиени од базите на податоци на FAO/WHO GEMS/ Food Consumption Cluster Diets. Според овој извор, проценетата просечна неделна косумација на внатрешни органи од говеда, свињи, јагнишa и коњи се соодветно 44,1, 25,9, 7,0 и 0,7 g/неделно. Бидејќи GEMS не дава одделни податоци за консумирање на црн дроб и бубрег, концентрациите на Cd се изразени како следните средни вредности од добиените резултати за црн дроб и бубрег:0,150 μg g^{-1} кај говеда, 0,081 μg g^{-1} кај свињи, 0,040 μg g^{-1} кај јагнишa и 9,790 μg g^{-1} кај коњи. Сите анализирани примероци содржеа кадмиум под максималното ниво определено со Одлуката на Европската Комисија и националната регулатива на Србија, со исклучок на црн дроб и бубрег кај коњите, каде сите примероци освен еден содржеа Cd над максималната граница на резидуи. Проценетиот неделен внос за Cd, пресметан врз основа на определената средна вредност за кадмиум во анализираните органи и просечна телесна маса од 70 kg, беше 0,227 μg/kg т.м./недела.

Врз основа на препорачаниот безбеден лимит на EFSA (European food safety authority) од 2,5 μg/kg т.м./недела и добиените резултати, може да заклучиме дека во Србија, при консумација на внатрешни органи кои се консумираат, вносот на кадмиум е под 10 % од безбедниот лимит и не претставува ризик за здравјето на човекот.

Клучни зборови: внес, намирници, кадмиум

Ова истражување беше финансиски подржано од Министерството за образование и наука на Република Србија (проект бр. III 46009).

UDC: 639.3:[577.175.8:543.544.5

HPLC/FL METHOD FOR HISTAMINE TESTING IN FISH

Stoilova Nadezhda[1], Peycheva M.[1], Yankovska T.[1]

[1]*VMP-Analysis Department, Central Laboratory of Veterinary Control & Ecology (CLVCE), Bulgarian Agency of Food Safety, Sofia, Bulgaria*

*Corresponding author: stoilova@clvce.eu, yankovska@clvce.eu

ABSTRACT

Histamine is a biogenic amine produced by decarboxilation of histidine due by decarboxylase enzyme reaction. Biogenic amines as Histamine in high concentration can affect the quality of the food: the level of histamine in fish serves as an indicator of the state of freshness. The European Council has published maximum residue limits and criteria for safety of the fish rich in histidine: Regulation (EC) 2073/2005 "Particularly fish species of the families: *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae*". Nine samples must be taken from each batch. These must fulfill the following requirements:
"1.26. Fishery products from fish species associated with a high amount of histidine:
-the mean value must not exceed 100ppm;
-two samples may have value more than 100ppm but less than 200ppm;
- no sample may have a value exceeding 200ppm".
The limits are 200 and 400ppm respectively for the case described in to the Regulation: "1.26. Fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine."(1).
We present an analytical method for determination of histamine in fish by HPLC with fluorescence detection after extraction, cleaning of the extract with SPE-procedure, derivatization of the analyte with o-phtalaldehyde. The analyte was separated on Zorbax Eclipse XDB C18 chromatographic column with easy eluting program using aqueous formic acid and methanol as a mobile phase. The wavelengths were λex 350nm and λem 450nm. Histamine was quantified down to 25ppm with LOD and 50ppm as LOQ respectively. The recovery at corresponding concentration to MRL-level is 91% and 94.5% respectively. Decision limit, Detection capability, linear range, repeatability, reproducibility were also determinate. The mass-spectrometry detection was used to ensure the qualitatively identification of histamine. The method was validated according Commission Decision 2002/657/EC criteria and was approved as confirmatory method at the Bulgarian Reference Laboratory.

Key words: histamine, histidine, biogenic amine, extraction, SPE, derivatization, HPLC-FL, development, validation

INTRODUCTION

Histamine is related to biogenic amines (tyramine, tryptamine): aliphatic, alicyclic or heterocyclic organic bases, having a low molecular weight. The amount of these amines in the body increases mainly due to enzymatic decarboxylation of the amino acid: in the case of histidine is decarboxylated to histamine: C6H9N3O2→C5H9N3+CO2.

For the formation of biogenic amines levels help some bacteria under the action of bacteria affect the final amount of biogenic amines in food product. Histamin can be found in fish, cheese and wine. Histamine and other biogenic amines contained in different foods in quantities that do not cause intoxication (2, 3)

Toxic effects occur in decarboxylation of histidine. Histamine is an indicator of the suitability of the product and proper canning.

Histamine has hormonal action, mediators perform functions. The body is released in inflammatory and allergic reactions.

According to FDA:

- Amount of histamine in the 10mg/kg is an indicator of product quality;

- Amount of histamine in the 30mg/kg is an indicator of product began with a process of deterioration;

- The amount of histamine is 50mg/kg evidence for decomposition of the product.

According to the Regulation (EO) № 1441/2007 concerning the amendment of Regulation (EO) № 2073/2005 on microbiological criteria permissible levels of histamine are 100 ÷ 200mg/kg (1).

Quantification of histamine depends on extraction (solvent, time, conditions) and the stability of the fluorescent product.

Isolation and purification of the analyte (extraction):

In the extraction of biogenic amines (with acid or alcohol) is made destruction of cell structure and precipitation of proteins. In carrying out extraction of so-called acid extraction (with perchloric acid, trichloroacetic acid) or extraction with methanol, amines can be "transferred" in the organic layer or mixture of solvents with different polarity, such as low pH (acidic), amine are dissolved in a hydrophilic phase and at high pH (alkaline conditions) change polarity and migration observed in the lipophilic phase.

Derivatization of amines:

In derivization acid halides with optimal activity is achieved when the reaction pH of the medium between

8 and 10, the products are relatively stable, but not specific.

For derivatization with specific fluorogeni by OPA (o-phthalic aldehyde), the components are specific, but are not stable. (4)

Methods for determination of biogenic aminis high performance liquid chromatography (HPLC) provide good specificity, especially when using a sensitive detector. Detection of the analyte can be performed with various instrumental methods of analysis. Suitable methods for qualitative determination of analytes are high-performance liquid chromatography with fluorescence detection (HPLC / FL), and liquid chromatography mass spectrometry.

MATERIALS AND METHODS

Histamine dihydrochloride (99% purity, Fluka) was used as analytical standards. O-phtalaldehyde (Pickering), β-merkaptoethanol (Merck), borax, trichloroacetic acid, sodium hidroxyde, formic acid, ammonium hydroxide were of pure for analysis grade reagents (Sigma-Aldrich Co.). Solvents (acetonitrile, methanol) were LC grade (LiChrosolv, Darmstad Germany). Deionized water was obtained from a ELGA water purification system (Millipore Ltd., Watford, Hertfordshire, UK).

Derivatization mixture:

Solvent A was prepared by dissolving of 0.33g borax in to 10ml deionizied water. Solvent B was prepared by dissolving of 20mg o-phtalaldehyde, 1ml methanol, 20 µl β-mercaptoethanol in to solvent A. The final volume of the mixture is 10ml. The derivatization solution is prepared in volumetric flask from dark glass and is stored in to the refrigerator for no more than week.

Standard Solutions:

Histamine stock solution with concentration of 10 g/l: Dissolve 100.0 mg of standard substance in 10.0 ml methanol. This solution is stored in a refrigerator (4 ± 2)°C for 3 months.

From the stock solution pipette respectively 0.0025, 0.005, 0.010, 0.015, 0.020, 0.030, 0.040 ml, and transferred in a test tube. There are treated for liquid chromatography: towards a standard that quantity before adding 0.5 ml 10% trichloroacetic acid and 0.6 ml of 1M sodium hydroxide, then homogenized the mixture using a vortex. 0.4 ml of each solution is transferred to vial, 0.1 ml of derivatization solution was added; the mixture was homogenized and allowed to stand at ambient conditions for 30min.

Biological Materials

Samples of fish were obtained from the market.

All samples were stored at < −18°C when not in use.

Instruments

Chromatographic analysis was carried out in a LC System Agilent 1100 (Agilent Technologies, Morges, Suisse). The system consists of a quaternary pump, an automatic injector and a fluorescence detector with a 150 W xenon lamp. The evaluated chromatographic columns was Zorbax Eclipse XDB C18 (150mm x 3mm, 5µm particle size) equipped with the same pre-columns. A centrifuge Sigma 3K15 (Osterode am Harz, Germany) with max 9500 rpm speed and a vortex mixer (Heidolph Instruments, Schwabach, Germany) were also used during sample retreatment procedure. A nitrogen evaporation system and a vacuum evaporation system (Buechi Labortechnik, Flawil, Switzerland) were used for the evaporation of the extracts. The solid phase extraction (SPE) was carried out on solid phase extraction manifold SUPELCO with OASIS MCX cartridge (200 mg, 3ml) with 3 µm particle size from Waters Corporation (Mrilend, USA). The pH was measured with an Inolab Level 1 pH meter WTW (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) equipped with a combined glass electrode. Instrumental control and data analysis were performed by Chemstation application software from Agilent Technologies. LC/tandem mass spectrometry analysis (LC/MS–MS) was performed on a TSQ mass spectrometer (ThermoFinnigan, USA). The histamine is detecting quality with LC/MS/MS with molecule ion (derivatized) m/z 288 and fragment m/z 95 (fig. 1).

Sample treatment:

Samples of fish were homogenized. A subsample (1 g) was extracted with 10% trichloracetic acid with shaking for a 15 min in room conditions, centrifuged for a 10min by 6500rpm at 00C. The extract was filtered and purified by SPE MCX cartridges: activation with a volume methanol, equilibration with deionizied water, the sample extract was loaded; the next step is washing with a volume 0.2% aqueous HCOOH and methanol. The analyte is eluting from the cartridge with 5% ammonium hydroxide in methanol and is evaporating under stream of nitrogen. The evaporated extract is dissolve in to 500µl of 10 % Trichloracetic solution. 500µl of solution of histamine was alkaline with 600 µl 1M NAOH to obtain pH=6 of the medium.

Derivatization technique:

Preparation of Derivatized Standards/Samples

To 400 µl of the alkalinity mixture of histamine 100 µl of derivatization solution were added. The mixture is allowed to stand for approx. 30min at room temperature. Chromatographic analysis is follow.

Method validation

The method was validated according to Commission Decision 2002/657/EC [5]. The validation parameters evaluated were specificity, linear range, recovery, precision (repeatability and intra-laboratory reproducibility), and decision limit and detection capability. For test of stability we use literature data (6, 7).

RESULTS

Optimization of the extraction and derivatization procedure

Sample extraction protocol was optimized in terms

of the extraction reagents, type of the extraction solvent, derivatization technique. The efficiency of two types of solvents and their mixtures was studied: water-miscible (acetonitrile (ACN), acetone (Ac), methanol (MeOH)) and water-immiscible (ethyl acetate (EtAc)). Additionally, the extraction solvents, like trichloracetic acid, were selected according to literature data (8, 9, 10). The effectiveness of the extraction and derivatization procedure was estimated by spiking fish samples by histamine at high concentration levels: 100; 200 and 400 ppm of histamine. Extract clearness and recoveries were used as estimation criteria.

Preliminary results showed that the ethyl acetate solvent do not extracted a studied analyte from fish tissues. In order to improve the efficiency of the extraction different solvents were tested. The obtained results with a single step extraction with ACN , methanol and acetone were negative. The efficiency of extraction with trichloracetic acid was with acceptable results. Recoveries ranged between 85 and 101 % related to the studied concentration level with good repeatability. The results presented here do not match with the observation made by some of the cited scientific publication which showed higher recoveries using above mentioned solvents (6, 7, 8).

Derivatization optimisation

In general the resultant product of the derivatization reaction between histamine and OPA is unstable (3). For achieving optimum derivatization efficiency, different types of derivatization agents mixing were tested. The results showed that the highest response was achieved by described derivatization agents context, i.e. under the conditions set by us and chemical concentration ratio of solutions. This can be attributed to two aspects. From the one hand, volume ratio between the samples and OPA reagent improved the efficiency and the analytical responses. From the other hand the reaction between OPA and histamine in the formed media highly depend from pH of the medium. The degradation of histamine-derivative occurred when all of the drawing processes were not observed. This caused the decrease of analytical responses. In general, the derivatization efficiency is improved with the increase of derivatization reagent concentration at the derivatization solutions A and B, described above, the pH of medium (when the dried extract is restored) and the time for reaction. The OPA freshness has an import role for the analysis determination too.

Validation

The method was validated in fish matrices according to Decision 2002/657/EC. This decision establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results obtained by laboratories. For analytes with established MRL, validation parameters were determined at concentrations equal to 0.5 MRL, 1 MRL, 1.5 MRL and 2 MRL. Here we study two concentration levels additionally because of the Regulation (EO) 2073/2005 requirements. Six samples at each concentration level were prepared by spiking with histamine. All spiked samples were submitted to liquid extraction, SPE, derivatization and HPLC procedure in triplicate. The results were representing in table 1.

CONCLUSION

An HPLC/FLD method with pre-column derivatization was proposed for the determination of histamine in fish samples. The matrix effects are eliminated effectively by employing the appropriate SPE cleanup procedure, while the sensitivity, reproducibility and detection throughput are improved greatly with the optimization of the derivatization reaction conditions between OPA and histamine. The proposed method was validated with the successful analysis of standard substances, as well as fish samples. The method is suitable for food quality and food safety control as confirmatory reference method of the screening TLC-method.

REFERENCES

1. Regulation (EO)№1441/2007
2. Karovicova J., J.Kohajdova, “Biogenic amine in food”, Review, Chem Pap. 59 (1)70-79-2005
3. Mrs Laurence Coppex, Diploma Thesis, University of Genf, Faculty of Chemistry and Pharmacy, “Derivatives for HPLC-Analysis”
4. P. Lawrance “Evaluation and Development of method for the determination of histamine in food”, march 2004/ FA30/2002/149
5. Commission Decision 657/2002/EC
6. Kerr M., Lawicki P., Aquire S., Rayner C., State Chemistry Laboratory Warribee, Public Health Division Victorian Government Department of Human Services “Effect of Storage Conditions on Histamine Formation in Fresh and Canned Tuna” www.foodsafety.vic.gov.au
7. Jin-Feng Peng, Fang K., Xie D., Ding B., Yin J., Cui X., Zhang Y., Liu J., “Development of an automated on-line pre-column derivatization procedure for sensitive determination of histamine in food with high-performance liquid chromatography-fluorescence detection”, Journal of Chromatography A., 1209, 70-75, 2008
8. G. Bontchev, “Histamine and other biogenic amines: the role in food quality control and methods for their determination” Review, Scripta Scientifica Medica, Vol. 37, pp 11-16, 2005
9. Elak Bolygo, Cooper P.A., Jessop K.M., Moffat F. “Determination of histamine in tomatoes by liquid chromatography/mass spectrometry” Journal of AOAC International, Vol.83, N3,2000
10. S. Brillantes, S. Paknoi, Totakien A. “Histamine formation in fish sauce production”, Food Chemistry and Technology

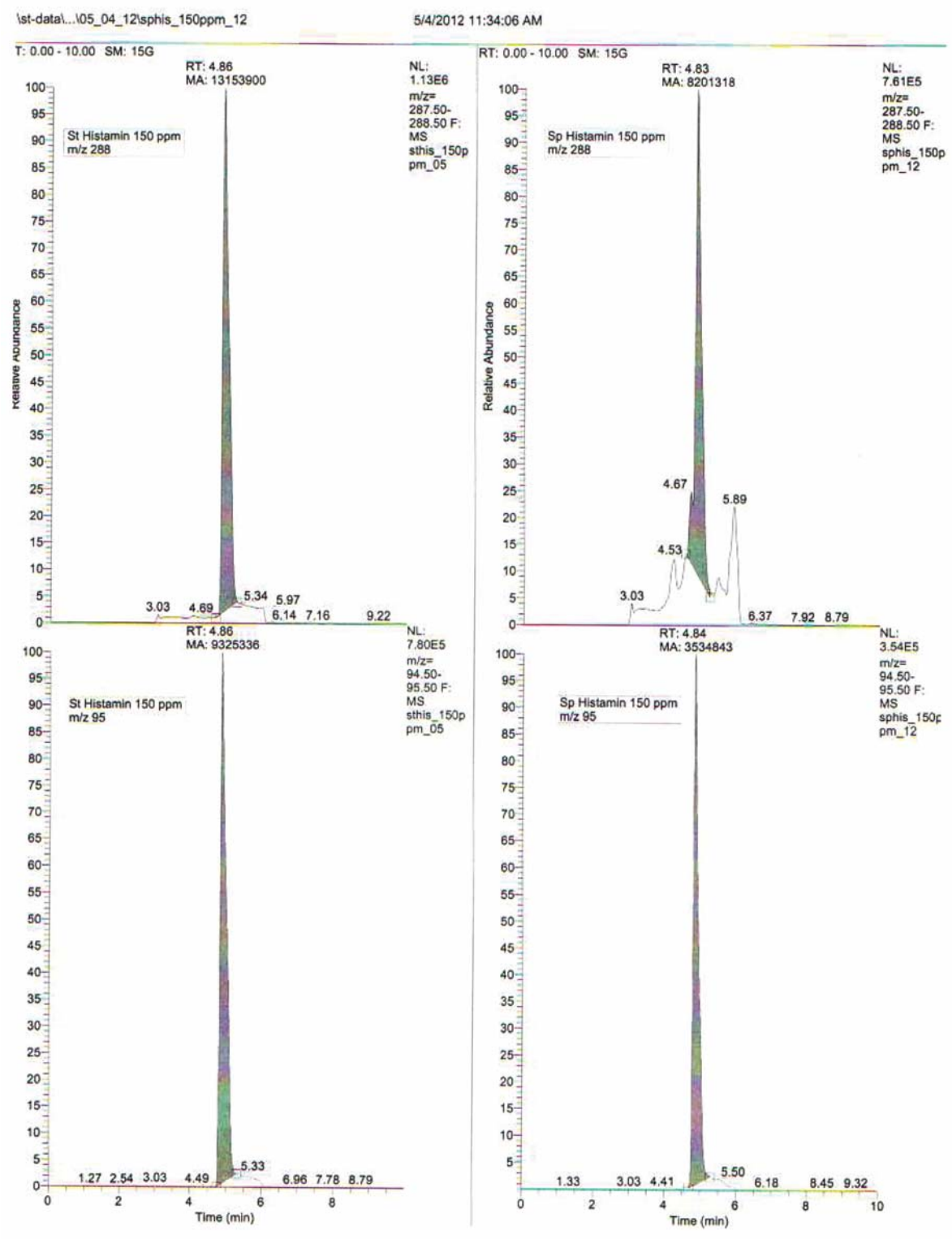


Figure 1. *LC-MS/MS chromatograms of derivatisated Histamin - standard solutions (on the left) and spiked fish (on the right) in concentration of 150 ppm*

Table 1. *Validation parameters of the LC/Fl method for histamine determination in fish tissue*

	fish
MRL, mg/kg	100/200*
liner range, mg/kg	25-400
recovery at MRL	91
RSD, % at MRL	31
CC_{α} mg/kg	137.23
CC_{β} mg/kg	174.46

**Regulation (EO) №1441/2007 concerning the amendment of Regulation (EO) № 2073/2005 on microbiological criteria*

HPLC/FL МЕТОД ЗА АНАЛИЗА НА ХИСТАМИН ВО РИБА

Стоилова Надежда[1], Пејчева М.[1], Јанковска Т.[1]

[1]Одделение за анализа на ВМП, Централна лабораторија за Ветеринарна контрола&екологија, Бугарска агенција за безбедност на храна, Софија, Бугарија

*Автор за коресподенција: stoilova@clvce.eu, yankovska@clvce.eu

АПСТРАКТ

Хистаминот е биоген амин добиен со декарбоксилација на хистидинот преку рекција во присуство на ензимот декарбоксилаза. Висока концентарција на биогените амини, како што е хистаминот, влијаат на квалитетот на храната: нивото на хистамин во рибите служи како индикатор за нивната свежост. Во Регулативата (ЕС) 2073/2005 на Советот на Европа за одредени видови риби од фамилиите *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae*, пропишани се максималните лимити на резидуи и критериумите за безбедност на риби богати со хистидин. Од секоја серија на производство мора да се земат по девет примероци, кои мора да ги исполнат следните барања:

“1.26. Производи од риба добиени од видови кои содржат големо количество на хистидин:

-средната вредност не треба да надмине 100 ppm;

- два примерока може да соджат количество поголемо од 100 ppm, но помалку од 200 ppm;

-ниту еден примерок не треба да содржи количество поголемо од 200 ppm”

Претставен е аналитички метод за определување на хистамин во риба со HPLC со флуоресцентна детекција по извршена екстракција, пречистување на екстрактот со SPE процедура, дериватизација на аналитот со *о*-фталалдехид. Аналитот беше разделен на Zorbax Eclipse XDB C18 хроматографска колона со едноставен програм за елуирање кој како мобилна фаза користи воден раствор на мравска киселина и метанол. Брановите должини за детекција беа λex 350 nm и λem 450 nm. Хистаминот беше квантифициран до 25 ppm како LOD и до 50 ppm како LOQ. Аналитичките приноси за соодветните концентрации до MRL нивото се 91 % и 94,5 %. Исто така, определени се лимитот на детекција, можноста за детекција, линеарниот опсег, повторливоста, репродуцибилноста. Детекцијата со масена спектрометрија беше применета за овозможување на квалитативна идентификација на хистаминот. Методот е валидиран согласно критериумите на Одлуката на Комисијата 2002/657/ЕС, и потврден е како конфирматорен метод на Бугарската Референтна Лабораторија.

Клучни зборови: хистамин, хистидин, биогени амини, екстракција, SPE, дериватизација, HPLC-FL, разработка, валидација

UDC: 579.67

PFGE TYPING OF MAJOR FOODBORNE PATHOGENS AS TOOL FOR TRACKING THE SOURCES OF CONTAMINATION ALONG THE FOOD CHAIN

Jankuloski Dean[1], Sekulovski Pavle[1], Mojsova Sandra[1], Angelovski Ljupco[1], Prodanov Mirko[1], Ratkova Marija[1]

[1]*Food Institute, Faculty of Veterinary Medicine, University "Ss. Cyril and Methodius,, in Skopje, Republic of Macedonia*

ABSTRACT
For the food industry, tracking of major foodborne pathogens can give scientific evidence about where food poisoning bacteria are entering a process, where cross contamination may be occurring, whether a particular strain is endemic in a factory environment and, most importantly, where controls should be directed. There are a several methods currently in use for typing of bacteria based on DNA. However, molecular subtyping based on genomic DNA macrorestriction fragment-length polymorphisms using pulsed-field gel electrophoresis (PFGE) has become an indispensable tool for unraveling the epidemiology of foodborne diseases and for tracking sources of food contamination. There are a several existing protocols for molecular subtyping of foodborne pathogens by PFGE developed such as C*ampylobacter jejuni, Clostridium botulinum, Escherichia coli* (O157:H7 and non O157), *Salmonella*, *Shigella sonnei* and *Shigella flexneri*, *Listeria monocytogenes*, *Vibrio cholerae, Vibrio parahemolyticus* and *Yersinia pestis* that are internationally accepted.
Key words: PFGE, foodborne pathogens, tracking of pathogens

INTRODUCTION

Foodborne illness is defined by the World Health Organization as „diseases,,, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food (WHO, Fact sheet N°237, Reviewed March 2007). Foodborne diseases are a serious and global problem. The WHO estimates that worldwide foodborne and waterborne diarrhoeal diseases taken together kill about 2.2 million people annually. Foodborne diseases can originate from a wide variety of different foods and could be caused by many different pathogenic organisms (e.g. bacteria or viruses) that have contaminated them at some part of the food chain, between farm and fork. Foods that are most frequently associated with foodborne illness include meat, fish and poultry. CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. In recent years, the incidence of infections caused by Shiga toxin–producing *Escherichia coli* (STEC) O157, thermotolerant *Campylobacter,* and *Listeria monocytogenes* has declined, whereas infections caused by non-typhoid *Salmonella* or *Vibrio* spp. seem to occur with unchanged or higher incidence (CDC, 2005). According to data published for 2011, top five pathogens causing domestically acquired foodborne illnesses resulting in hospitalization in estimated annual number in US, are as follows, *Salmonella* nontyphoidal 19,333 (35%), Norovirus 14,663 (26%), *Campylobacter spp.* 8,433 (15%), *Toxoplasma gondii* 4,428 (8%), *E. coli* (STEC) O157 2,138 (4%). However this order of pathogens is changed in the estimated annual number of deaths, as follows, *Salmonella* nontyphoidal 378 (28%), *Toxoplasma gondii* 327 (24%), *Listeria monocytogenes* 225 (19%), Norovirus 149 (11%), *Campylobacter spp.* 76 (6%) (CDC Estimates of Foodborne Illness in the United States, Findings, 2011). *Listeria monocytogenes* causes more deaths each year than *Salmonella* and *E. coli* O157 combined (FSA, UK, 2011). In the EU in 2009, list of confirmed cases of the important pathogens causing acquired foodborne illnesses is as follows: *Campylobacter spp.* with a total 198,252 confirmed cases, *Salmonella spp*. (*S. enteritidis* with 52.3 % and *S. typhimurium* with 23.3 % of serovars respectively) with 108,614 cases and 0.08% fatality, *Listeria monocytogenes* with 645 confirmed cases of listeriosis, with a highest fatality rate of 16.6%, VTEC with 3,573 confirmed human cases and *Yersinia enterocolitica* with 7,595 confirmed human cases. Pulsed-field gel electrophoresis (PFGE) has been used effectively as a molecular subtyping tool in outbreak investigations (Bell, B. P., et al.; 1994, Gautom, R. K.; 1997) and surveillance (Swaminathan, B.,et al.; 2001). Most foodborne diseases seem to be caused by only a few of the serotypes described. Furthermore, some isolates are fully susceptible to antimicrobial drugs or cannot be phage-typed. New, rapid and efficient molecular subtyping tools are therefore currently used. Pulsed-field gel electrophoresis (PFGE) (Ridley et al., 1998), amplified fragment length polymorphism (Aarts et al., 1998; Torpdahl and Ahrens, 2004) and sequencing-based methods, such as the multilocus sequence typing method (Kotetishvili et al., 2002; Sukhnanand et al., 2005) and multiple locus variable number tandem repeat (VNTR) analysis (Lindstedt et al., 2004; Torpdahl et al., 2006) have been used for tracing and differentiating between isolates of the same serotype. PFGE remains the ''gold standard'' method and has been adopted by many reference laboratories for the surveillance and investigation of foodborne diseases (Swaminathan et al., 2001).

PFGE-technology and principles

Pulsed field gel electrophoresis was first been utilized in 1982, and since then several apparatuses have been developed for separating large molecules of DNA, all using multiple electric fields. All systems separate DNA molecules within the same size range but differ in the speed of separation and the resolution. Below are the schematic diagrams of the various apparatuses: pulsed field gel systems as follows; PFGE-pulsed field gradient gel electrophoresis, OFAGE-orthogonal field alternation gel electrophoresis, TAFE- transverse alternating field electrophoresis, FIGE- field inversion gel electrophoresis, CHEF- contour clamped homogeneous electric field, crossed field gel electrophoresis (Waltzer), and ST/RIDE- simultaneous tangential/rectangular inversion decussate electrophoresis.

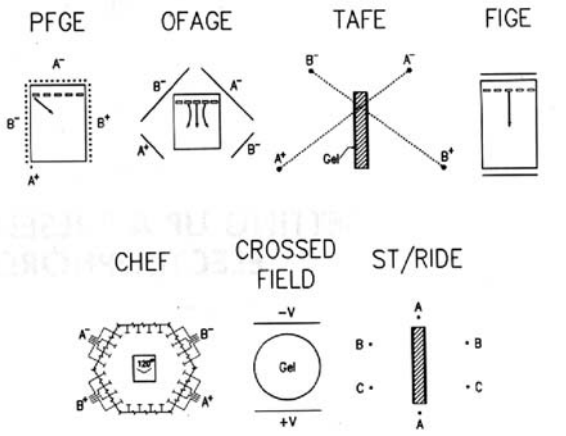


Figure 1. *Schematic diagrams of the various apparatuses of pulsed field gel electrophoresis systems*

Protocols

The PFGE method starts with the extraction of the bacterial chromosomes without damaging of the DNA, by means of a very gentle extraction procedure. The chromosomes are then restricted using a rare cutting enzyme. For example, for the *L. monocytogenes*, the enzymes are *Apa*I or *Asc*I (Carriere et al., 1991), for the *Salmonella, E. coli* O 157, *Xba*I. These restriction enzymes generate respectively between 10 to 17 fragments in the range of separation of the PFGE. The combinations of the profiles generated by the two enzymes are used to characterize the strains. A third profile generated by *Sma*I can be added to reinforce the analysis (Carriere et al. 1991). The restricted DNA fragments are commonly separated in a PFGE CHEF (Contour-clamp homogeneous electric field) system (Chu et al., 1986). For example, range of separation for *L. monocytogenes* is between 33 and 1135 kb. The migration parameters applied depend on the bacteria species. For *L. monocytogenes* and non-typhoiodal *Salmonella* the established parameters are a pulse angle of 120° and a linear switch-time ramp of 4 to 40 and 2,38 to 63,08 s respectively. These migration parameters have been standardized in the PulseNet USA protocol. The difference in the restriction profiles enables genetic comparisons among *L. monocytogenes* and non-typhoiodal *Salmonella* strains. Profiles are specific to each strain and are used as characterization data to identify them (Graves & Swaminathan, 2001). However the restriction profiles are merely an image of the genome structure and must be interpreted as such (Tenover et al. 1995).

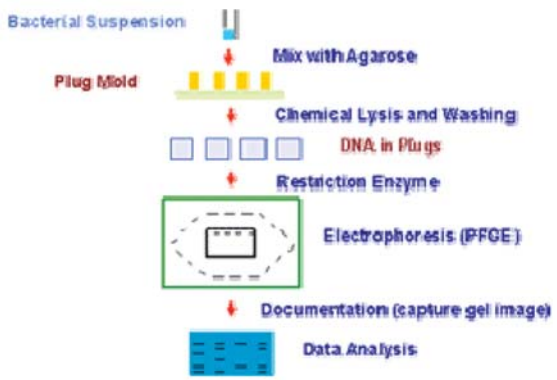


Figure 2. *Schematic diagrams of the PFGE protocol*

After staining of the gel, picture is taken into tif. format and it is imported in to FPQuest (Bio-rad, USA) for further processing.

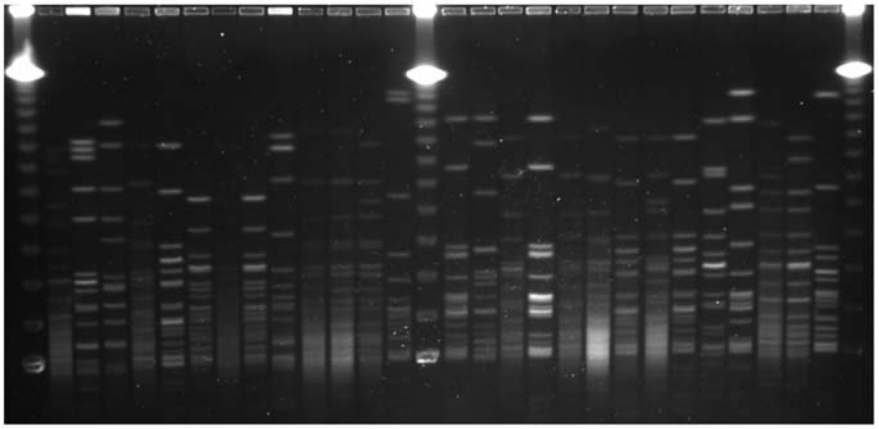


Figure 1. *Stained gel with ApaI restriction profile of L. monocytogenes. Lines no 1, 15 and 30 are CHEF DNA Size standard, Lambda Ladder (10-1000 Kbp).*

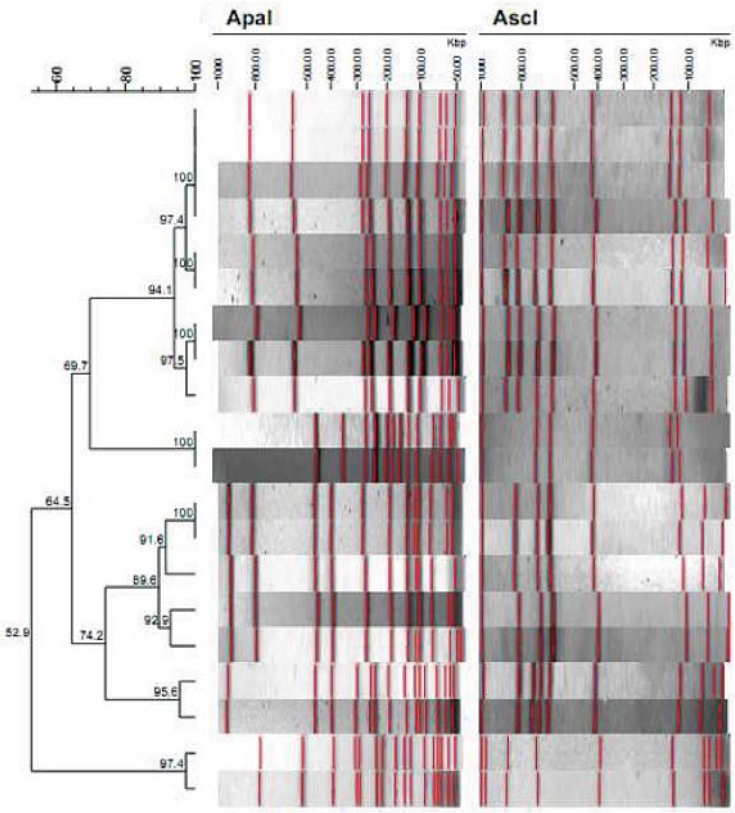


Figure 4. *Dendrogram (UPGMA clustering based on Dice correlation coefficient) of Listeria monocytogenes ApaI and AscI PFGE patterns .*

Advantages of using PFGE

PFGE subtyping has been successfully applied to the subtyping of many pathogenic bacteria. PFGE has been repeatedly shown to be more discriminating than methods such as ribotyping for many bacteria. PFGE in the same basic format can be applied as a universal generic method for subtyping of bacteria. Only the choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species. DNA restriction patterns generated by PFGE are stable and reproducible at the intra- and inter-laboratory levels. In summary, PFGE is the method of choice for epidemiologic subtyping of pathogenic bacteria at the present time.

Limitations of the PFGE Method

It`s time consuming, requires a high-level of skill, it does not work for everything (i.e. clonal patterns). Pattern results vary from person to person, can't optimize the separation in every part of the gel at the same time, bands are bands, not sequences, don't really know if the bands of same size are same pieces of DNA and the bands are not independent.

CONCLUSIONS

Genomic comparison of bacterial pathogens from human illness and food animals will also establish whether there are particular animal host reservoirs for strains of the pathogen which are more virulent for humans. These data will enable focusing of resources most effectively on particular vectors and vehicles to reduce the overall risk posed by that pathogen. A total chain approach based on tracking and genomically comparing food borne bacteria recovered from animals, food and humans has enormous potential to direct and focus efforts to improve microbial food safety. However, much of the potential of this molecular subtyping method remains unutilized due to the inability to compare DNA restriction fragment patterns between laboratories and none-existing universal nomenclature for the RFLP patterns of each foodborne pathogen, so pattern designations used in one laboratory have little meaning elsewhere. Despite all, PFGE is currently the method of choice for epidemiologic subtyping of pathogenic bacteria.

REFERENCES

1. Aarts, H.J., L.A. van Lith, and J. Keijer. 1998. Highresolution genotyping of Salmonella strains by AFLP-fingerprinting. Lett Appl Microbiol. 26:131–135
2. Bell, B. P., M. Goldoft, P. M. Griffin, M. A. Davis, D. C. Gordon, P. I. Tarr, C. A. Bartleson, J. H. Lewis, T. J. Barrett, J. G. Wells, R. Baron, and J. Kobayashi. 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers: the Washington experience. JAMA 272**:**1349-1353.
3. Bruce Birren and Eric Lai, Pulsed Field Gel Electrophoresis: A Practical Guide; 1993

4. CDC Manual on Standardized Molecular Subtyping of Escherichia coli O157:H7 by Pulsed-Field Gel Electrophoresis 1996)
5. Graves, L. M., and Swaminathan, B. 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 65: 55–62.
6. Kotetishvili, M., O.C. Stine, A. Kreger, et al. 2002. Multilocus sequence typing for characterization of clinical and environme
7. Lindstedt, B.A., T. Vardund, L. Aas, et al. 2004. Multiplelocus variable-number tandem-repeats analysis of Salmonella enterica subsp. enterica serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. J Microbiol Methods 59:163–172.
8. Sukhnanand, S., S. Alcaine, L.D. Warnick, et al. 2005. DNA sequence-based subtyping and evolutionary analysis of selected Salmonella enterica serotypes. J Clin Microbiol. 43:3688–3698.
9. Swaminathan, B., T. J. Barrett, S. B. Hunter, R. V. Tauxe, and the CDC PulseNet Task Force. 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg. Infect. Dis. 7**:**382-389.
10. Torpdahl, M. and P. Ahrens. 2004. Population structure of Salmonella investigated by amplified fragment length polymorphism. J Appl Microbiol. 97:566–573

PFGE ТИПИЗАЦИЈА НА ГЛАВНИТЕ ПАТОГЕНИ ОД ХРАНАТА КАКО АЛАТКА ЗА СЛЕДЕЊЕ НА ИЗВОРИТЕ НА КОНТАМИНАЦИЈА ПО ДОЛЖИНА НА СИНЦИРОТ НА ХРАНАТА

Јанкулоски Деан, Секуловски Павле, Мојсова Сандра, Ангеловски Љупчо, Проданов Мирко, Раткова Марија

Институт за Храна, Факултет за Ветеринарна Медицина,
Универзитет „Св. Кирил и Методиј“, Скопје, Република Македонија

АПСТРАКТ
За индустријата за храна, следењето на главните патогени во храната може да обезбеди научна основа за тоа каде бактериите кои се причина за труења влегуваат во процесот на производство, каде настанува вкрстена контаминација, дали дадениот сој е ендемски во погонот и најважното, во која насока контролата треба да биде имплементирана за да се ерадицираат патогените микроорганизми во храната. Постојат неколу методи кои во моментов се користат за типизација на бактериите кои се базираат на ДНК. Молекуларната метода за субтипизација која се базира на макрорестрикција на геномот на ДНК со полиморфизам на должината на фрагментите и примена на гел електрофореза во пулсирачко поле (PFGE) е незаменлива алатка за утврдување на епидемиологијата на болестите од храната и следење на изворите на контаминација. Постојат неколку протоколи развиени за молекуларна субтипизација на патогените со PFGE како C*ampylobacter jejuni, Clostridium botulinum, Escherichia coli* (O157:H7 и non O157), *Salmonella*, *Shigella sonnei* и *Shigella flexneri*, *Listeria monocytogenes*, *Vibrio cholerae, Vibrio parahemolyticus* и *Yersinia pestis* кои се меѓународно прифатени.
Клучни зборови: PFGE, патогени микроорганизми со потекло од храната, следење на патогените микроорганизми

UDC: 579.67:579.869.1]:006.73

EFFECT OF IMPLEMENTATION OF HACCP SYSTEM ON DISTRIBUTION OF *LISTERIA MONOCYTOGENES* IN BULGARIAN FOODS

Daskalov Hristo[1], Daskalova Alexandra[2]

[1]*National Diagnostic and Research Veterinary Institute, 1606 Sofia, Bulgaria*
[2]*Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria*

ABSTRACT
The aim of the study was to receive actual data for the level of contamination with *Listeria monocytogenes* of ready-to-eat (RTE) foods, produced in Bulgaria in period of implementation of HACCP system and introductory stage of applying in food business operators. Studies were carried out from October 2004 to December 2005 before official implementation of HACCP system and from January 2006 to May 2007 after official applying of HACCP. Data were collected from very popular cooked perishable cured meats and products, shelf-stable cooked cured meats and raw-dried cured meat products; different types of cheeses typical for Bulgarian taste (yellow and white cheese from cow and sheep milk) and some fish products (cured, smoked and dried) and fish salads. Development of individual HACCP plans and implementation of HACCP system in food producing plants didn't significantly influence the level of contamination of *L.monocytogenes* after first 1,5 year of official application. The most significant contamination with *L.monocytogenes* was found in raw dried cured meat products, roasted chicken foods and some heat treated meat commodities. Percentage of determination of *L.monocytogenes* was 4,2% before official implementation and 6,9% after obligatory applying of HACCP. *L.monocytogenes* isolates from different foodstuffs showed close biochemical characteristics and profiles.
Key words: HACCP, RTE foods, *L.monocytogenes*, distribution

Acknowledgement
To Faculty of Veterinary Medicine, Trakia University, Stara Zagora for sponsoring of Project 12/2004 and food processors for help to done research activities.

INTRODUCTION

EFSA (2007) concludes that after a general decline in the 1990s, the number of cases of listeriosis has increased since 2000 in Europe. The foods which could be associated with transmission of listeriosis were mostly ready-to-eat foods (RTE) that support growth of *L. monocytogenes*. Becker et all. 2001 noted that in defiance of new hygiene regulations, HACCP and strict control measures, the incidence of food borne infections and intoxications has been increasing in Germany, especially in cooked sausages. Lake et al. 2002, pointed that universal adoption and successful implementation of HACCP and Risk assessment systems should result in a reduction in exposure to *L.monocytogenes*. Zhu et al. 2005 concluded it their review that *L. monocytogenes* is a major safety concern for RTE meat products, which are frequently contaminated with this pathogen. Anonymous. 2005 set presence of *L.monocytogenes* as a food safety criterion for RTE products, especially for these which support bacterial growth.

The aim of the study was collecting of the data for the level of contamination with *Listeria monocytogenes* of RTE products in period of implementation of HACCP system and introductory stage of applying in food business operators in Bulgaria.

MATERIALS AND METHODS

Sampling

Studies were carried out from October 2004 to end of December 2005 (I stage - before official implementation of HACCP system) and from January 2006 to May 2007 (II stage - after official applying of HACCP system). Examinations were done on some very popular cooked perishable cured meats and products, shelf-stable cooked cured meats and raw-dried cured meat products; different types of cheeses typical for Bulgarian market (yellow and white cheese from cow and sheep milk); and some fish products (cured, smoked and dried) and fish salads. All samples of RTE foods were wrapped up in boxes or vacuum-packed and received up to 72 hours after production, kept in refrigerator conditions - $\leq 4^0$C. For the period of study were investigated samples of 90 milk, 163 meat and 21 fish RTE products.

Microbiological analysis

The samples were analyzed according to FDA method for milk products and USDA method for meat foods, described by Ryser and Donnelly (2001). From all positive petri dishes were taken 5 suspected colonies and reinoculated on TSAYE agar. Further examination comprised Gram staining, motility at 20-25^{0}C, growth at 35^{0}C, catalase activity, oxidase reaction and b-hemolysis. Additionally, biochemical identification with API Listeria ID strip (bioMerieux, Inc., Hazelwood, Mo.) was done to all *L.monocytogenes* isolates.

RESULTS AND DISCUSSION

During I stage of the study were examined 50 milk (yellow and white cheeses) - 50 samples, 82 meat (cooked perishable cured meats and products, shelf-stable cooked

cured meats and raw-dried cured meat products) and 11 fish food samples. Positive results for presence of *L.monocytoges* were found only in 6 meat specimens, level of contamination was 4.2% of all samples and respectively 7.3% of meat specimens. During II stage of investigations were analyzed 40 milk, 81 meat and 10 fish food samples. Presence of *L.monocytogenes* was obtained in 1 milk, 7 meat and 1 fish RTE products, level of contamination was 6.9% of all samples, respectively 2,5% of milk, 8.6% of meat and 10% of fish specimens.

Results of some characteristics of all *L.monocytogenes* isolates from examined food samples are presented in Table 1.

Table 1. Morphological and biochemical characteristics of Listeria monocytogenes isolates in food samples

Stage	Year, Product	Gram staining	β-hemolysis	Catalase activity	Motility at 20-25°C	Growth at 35°C	Oxidase reaction
I	2004,cured smoked pork neck (vacuumed)	+	+	+	+	+	–
I	2004,Gornooriahovitza sudjuk (vacuumed)	+	+	+	+	+	–
I	2004,Macedonian style sausage (vacuumed)	+	+	+	+	+	–
I	2005,Ambaritza (raw dried cured), (vacuumed)	+	+	+	+	+	–
I	2005,Gornooriahovitza sudjuk (vacuumed)	+	+	+	+	+	–
I	2005, Sushenitza (raw dried cured) (vacuumed)	+	+	+	+	+	–
II	2006, Sushenitza (raw dried cured) (vacuumed)	+	+	+	+	+	–
II	2006, roast chicken fillet (vacuumed)	+	+	+	+	+	–
II	2006, roast chicken leg (vacuumed)	+	+	+	+	+	–
II	2006,roast chicken sausage (vacuumed)	+	+	+	+	+	–
II	2006, marinated herring (box)	+	+	+	+	+	–
II	2006, yellow cheese (vacuumed)	+	+	+	+	+	–
II	2006, Chiprovski sudjuk (vacuumed)	+	+	+	+	+	–
II	2006, Shumenski sudjuk (vacuumed)	+	+	+	+	+	–
II	2007, Ambaritza (raw dried cured) (vacuumed)	+	+	+	+	+	–
II	2004, Gornooriahovitza sudjuk (vacuumed)	+	+	+	+	+	–

Data about API Listreia biochemical profile of *L.monocytogenes* isolates from all examined food samples are showed in Table 2.

***Table 2.** API Listeria biochemical profile of L.monocytogenes isolates from studied food samples*

Stage	Year, Product	DIM	ESC	αMAN	DARL	XYL	RHA	MDG	RIB	G1P	TAG	βHEM	Profile
I	2004,cured smoked pork neck (vacuumed)	-	+	-	+	-	+	+	-	-	-	+	**2 510**
I and II	All other 14 positive food samples (see Table 1)	-	+	+	+	-	+	+	-	-	-	+	**6 510**

**Legend: DIM – enzymatic substrate; ESC – esculin; αMAN – 4-nitrophenyl-αD-manopyranoside; DARL – D-Arabitol; XYL – D-Xylose; RHA – L-Rhamnose; MDG – methyl-αD-glucopiranoside; RIB – D-Ribose; G1P – Glucose-1-phosphate; TAG – D-tagatose; βHEM – β-haemolytic activity*

Data presented in Table 1 demonstrated that *L.monocytgenes* can be found in different RTE products, mainly in meat foods, especially in raw dried cured meat products, roasted chicken foods and some heat treated meat commodities. Results in Table 2 showed presence only of two biochemical profiles – 1 isolate with Profile 2510 and all other with 6510. Comparing our results about presence of L.monocytogenes at 4.2% before and 6.9% after implementation of HACCP with reported by Becker et all. (2001) 10.5 % in 287 tested meat samples, we can conclude lower level of contamination at the stage of implementation of HACCP. According to EU requirements set in EU Regulation 2073/2005 *L.monocytogenes* must be absent in 25g in food immediately after producing. Our results show the role of hygienic practice and effect of implementation of HACCP, especially in the stage of packaging in vacuum cover. Little et al. (2009) reported that food types with the highest prevalence of *Listeria monocytogenes* were sliced meats (3.7% within shelf life, 4.2% end of shelf life). The results of these authors are very close to our findings. The same scientists noted also that prepackaging play important role for contamination of RTE products with *L. monocytogenes*. Satisfactory microbiological quality was associated with premises on which the management was trained in food hygiene and those that complied with hazard analysis and critical control point principles. Based on our data we are agree with conclusion of Dufour, (2011) that products with a shelf life of less than 5 days or with intrinsic factors justifying the absence of growth, the criterion is 100 *L. monocytogenes* cfu/g, on the assumption this number will not increase during that time period. In contrast, for products where growth is likely under normal storage conditions or products are stored for longer than 5 days, a zero tolerance is required, especially for roasted chicken foods and some heat treated meat commodities, produced in Bulgaria.

CONCLUSIONS

Development of individual HACCP plans and implementation of HACCP system in food producing plants don't significantly influence the level of contamination of *L.monocytogenes* after first 1,5 year of official application;

The most significant contamination with *L. monocytogenes* is determined in raw dried cured meat products, roasted chicken foods and some heat treated meat commodities;

Percentage of determination of *L.monocytogenes* was 4,2% in first stage of experiment and 6,9% for second stage. *L.monocytogenes* isolates from different foodstuffs showed close biochemical characteristics and profiles;

REFERENCES

1. Anonymous. 2005. EU Regulation 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, 338 (2005), pp. 1–29
2. Becker, B., B. Trierweiler, J. Fechler, W. H. Holzapfel. 2001. Presence of *Listeria monocytogenes* in samples of cooked sausages. *Proceedings ISOPOL XIV International Symposium on Problems of Listeriosis*, May 13 – 16, 2001 Mannheim / FRG, pp. 132-135
3. Dufour, Ch.. 2011. Application of EC regulation no. 2073/2005 regarding Listeria monocytogenes in ready-to-eat foods in retail and catering sectors in Europe. *Food Control,* 22, 1491-1494
4. Lake R., A. Hudson, P. Cressey, G. Nortje. 2002. Risk Profile: *Listeria Monocytogenes* in processed ready-to-eat meats. Scientific Report of Institute of Environmental Science & Research Limited, *October 2002*, New Zealand Food Safety Authority, pp 62, www.esr.cri.nz
5. Little, C.L.; Sagoo, S.K.; Gillespie, I.A.; Grant, K.; McLauchlin, J. 2009. Prevalence and Level of *Listeria monocytogenes* and Other*Listeria* Species in Selected Retail Ready-to-Eat Foods in the United Kingdom. *Journal of Food Protection*, 72 (9), 1869-1877
6. Scientific Opinion of the Panel on Biological Hazards on a request from the European Commission on Request for updating the former SCVPH opinion on *Listeria monocytogenes* risk related to ready-to-eat foods and scientific advice on different levels of *Listeria monocytogenes* in ready-to-eat foods and the related risk for human illness. *The EFSA Journal* (2007) 599, 1-42
7. Ryser, E.T., Donnelly, C.W. Listeria. 2001. In *Compendium of Methods for The Microbiological Examination of Foods*, Fourth Edition., Eds. F.P. Downes and K. Ito, APHA, 2001, pp. 343-357
8. Zhu, M., M. Du, J. Cordray, D.U. Ahn. 2005. Control of *Listeria monocytogenes* Contamination in Ready-to-Eat Meat Products. *Comprehensive Reviews in Food Science and Food Safety*, 4, 34-42

ЕФЕКТОТ НА ИМПЛЕМЕНТАЦИЈА НА НАССР СИСТЕМ ВРЗ ДИСТРИБУЦИЈА НА *LISTERIA MONOCYTOGENES*ВО ХРАНА ОД БУГАРИЈА

Даскалов Христо[1], Даскалова Александра[2]

[1]*Ветеринарен институт за национална дијагностија и истражување, 1606 Софија, Бугарија*
[2]*Факултет за Ветеринарна Медицина, Тракиски Универзитет, 6000 Стара Загора, Бугарија*

АПСТРАКТ

Целта на студијата беше да се добијат податоци за нивото на контаминација со *Listeriamonocytogenes*во храни готови за јадење (RTE)произведенивоБугаријавопериодотнаимплементацијанаНАССР систем и во воведната фаза на примената на операторите на храна. Испитувањата се изведуваа од Октомври 2004 до Декември 2005 пред официјалната имплементација на НАССР систем и од Јануари 2006 до Мај 2007 по официјаната примена на НАССР. Податоците се земаа од многу популатните варени леснорасипливи саламурени меса и нивни производи, трајни варени саламурени меса и сушени саламурени месни производи; различни видови на сирење типични за Бугарија (жолто и бело сирење од кравјо и овчо млеко) и неколку рибини производи (саламурени, пушени и сушени) и рибини салати. Развивањето на индивируални НАССР планови и и имплементација на НАССР систем во погоните за производство на храна не покажаа значително влијание на нивото на контаминација на *L.monocytogenes*по првите 1,5 година од официјаланта примена. Најзначајна контаминација со *L. monocytogenes*беше најдена кај сирови сушени и саламурени месни производи, храна со печени пилиња и некои производи со термички обработено месо. Процентот на утврдена *L. monocytogenes*беше 4,2% пред официјалната примена и 6,9% по задолжителното воведување на НАССР. Изолатите од*L. monocytogenes* од разлилни храни покажаа слични биохемиски карактеристики и профили.

Клучни зборови:НАССР, RTE храна, *L. monocytogenes*, дистрибуција

Благодарност

До Факултетот за Ветеринарна Медицина, Тракиски Универзитет, Стара Загора за спонзорирање на Проектот 12/2004 и на производителите на храна кои помогнаа во активностите за испитувањето.

UDC: 636.2.09:616.63-097

DETERMINATION OF ZERANOL RESIDUES LEVELS IN BOVINE URINE WITH ELISA METHOD

Hajrulai-Musliu Zehra[1], Uzunov Risto[1], Dimitrieska-Stojkovik Elizabeta[1], Stojanovska-Dimzoska Biljana[1], Sekulovski Pavle[1], Stojkovski Velimir[2], Todorovic Aleksandra[1]

[1]Food Institute, [2]Institute for Biomedicine and Reproduction, Faculty of Veterinary Medicine, University Ss. "Cyril and Methodius", Skopje, Macedonia

*Corresponding author: zhajrulai@fvm.ukim.edu.mk

ABSTRACT

Zeranol is a synthetic derivative of zearalenone which has been used as an anabolic substance in sheep and cattle to increase growth at food producing animals. The usage of zeranol is prohibited in most countries of the European Union and in Macedonia. In the illegal use of zeranol it is difficult to determine its presence because the amount of zeranol given and period is not known. A high affinity polyclonal antibody-based enzyme linked immunosorbent assay (ELISA) was developed for the quantification of zeranol in bovine urine. In the improved ELISA, the linear response range was between 0.025 and 3 ng/ml , and the detection limit was 0.22 ng/ml for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 87.9%~92.3% and 2.4%~5.6%, respectively. Thirty-six bovine urine samples spiked with zeranol (ranging from 0.2 to 10 ng/ml) were detected by the ELISA, and good correlations was obtained (R^2=0.9929). We conclude that this improved ELISA is suitable tool for a mass zeranol screening method for zeranol in bovine urine. A total of 87 bovine urine samples were screened for the presence of zeranol as part of national monitoring residues where these documented cases presented zeranol concentrations were much lower than the MRPL set by the according the guidance letter from Community Reference Laboratories' (7 December 2007).

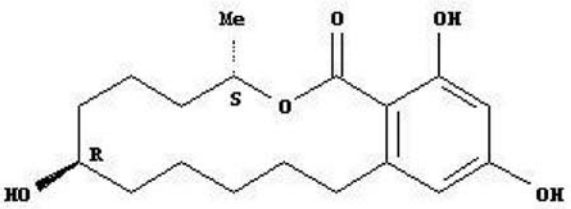
Key words: Rezorcylic acid, urine, ELISA, zeranol

INTRODUCTION

Zeranol is a non-steroidal oestrogenic growth promoter that increases the live weight gain in food animals. Fig 1. It is a semi-synthetic product derived from the naturally occurring mycotoxin zearalenone. Its administration has been banned within the European Union (EU) (Council Directive 96/22/EEC) and Member States are required to monitor food-producing animals for possible abuse (Council Directive 96/23-EC). Zearalenone is also known as the fusarium spp. toxin (F2-toxin) and is commonly found in animal feed. Zeranol and zearalenone are known to give identical metabolites, including zeranol itself, can also occur naturally in bovine urine after metabolism of Fusarium spp. toxin. The traditional method for the analysis of zeranol and other rezorcylic acid in the present is gas chromatography (GC) with liquid chromatography (Tobioka and Kawashima 1978, 1981) and mass spectrometry (Bagnati et al., 1990; Sawaya etal., 1998; Talat et al., 1999; Leslie et al., 2003). Large-scale surveillance programsrequire a rapid analysis of zeranol therefore an enzymelinked immunosorbent assay (ELISA) appeared suitable. Such assays have been developed in our laboratories since 2006.

Finally, the optimized ELISA was applied to determine zeranol in bovine urine samples. The use of zeranol in food producing animals is prohibited in most countries of the EU and in Macedonia. The aim of the present study was to determine the levels zeranol in cattle urine of various sex and age using validated ELISA methods, to get an insight into the residual levels that might indicate to the illegal use of zeranol on farm animals in this region.

Zeranol [CASRN 26538-44-3]



MATERIALS AND METHODS

A total of 87 bovine urine samples were screened for the presence of zeranol as part of national monitoring residue plan. The samples were collected within period of 12 months as they were delivered by the authorised veterinary inspectors. Samples were kept frozen at -20 ºC until analysis. A I' screen zeranol kit for ELISA was provided by Tecna (R&Diagnostics- Biotechnology, Italy). Each kit contained a microtiter plate with 96 wells coated with antibodies to rabbit IgG, zeranol standard solutions (0; 0,025; 0, 1; 0, 3; 1 and 3 ng/mL), enzyme-conjugate zeranol, anti-zeranol antibody, substrate/chromogen solution, stop reagent, conjugate and antibody dilution buffer, and washing buffer. The extraction and clean-up procedures were those described by the ELISA kit manufacturer (R&Diagnostics- Biotechnology, Italy). Urine samples (0, 5 ml) were diluted with 2.5 ml of sodium acetate buffer 50 mM pH 4.8, then was added 10 µl of β glucoronidase aril-sulfatase of Helix pomatia, the pH was controlled and in case was adjusted it at

4.8-5. The entire supernatant was allowed to reach room temperature (20-25°C) an overnight and then underwent clean-up with RIDA C18 column. Subsequently, 1 ml of the elute was pippeted into a glass tube and evaporated at 50/60ºC under a stream of nitrogen, and the residue was redisolved in 0.5 ml of kit dilution buffer. Fifty microliters of standards and control were pippeted into the standard/ sample wells in duplicate and 50 microliters of conjugate was added. The microtiter plate was covered with adhesive film, gently tapped from side to side, and incubated for 90 min at room temperature. The plate was inverted and the liquid was tapped out. The microtiter plate was washed 4 times with working wash solution diluted diluent over a 10-15 minute period. After the final wash, it was tapped onto a tissue paper. Immediately after washing, 100 µl of of developing solution was pippeted into each well. The microtiter plate was gently tapped and incubated for 15 minutes at room temperature in the dark. The color reaction was stopped by addition of 50µl of stop solution per well. A color change of blue to yellow was evident, and the optical density was measured at 450 nm within 10 minutes. Data were analyzed using a special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany). The mean absorbance values obtained for the standards and the samples divided by the absorbance value of the first standard (zero standard) and multiplied by 100 was the % absorbance. The zero standard was thus made equal to 100 % and the absorbance values were quoted in percentages. The method recovery was evaluated by fortifying negative urine samples with zeranol standards (1, 2 and 3 µg/kg).

Method validation.

The limit of detection (LOD) was obtained by spiking with 0, 5 from MRPL which is 2 ppb according the guidance letter from Community Reference Laboratories' (7 December 2007). The method recovery was determined at three level by spiking urine samples with 0,5; 1 and 1,5 times the MRPL level. For determination of repeatability, the same steps were repeated on two occasions in the same analytical conditions. Detection capabilities (CCβ) was evaluated by analyzing 20 spiked samples at 0,5 MRPL level. A typical ELISA standard curve is presented in Figure 1. Final zeranol concentrations in urine were calculated by taking the average recoveries into account.

RESULTS AND DISCUSSION

Validation results of quantitative ELISA methods include determination of the recovery, repeatability and detection capability (CCβ) of the test methods.

The estimated LOD for urine samples for zeranol was 0,22 ng/ml. The CCβ for urine sample for zeranol was 1,21 ng/ml The results of method recovery (n=18) and repeatability (n=54) are presented in Table 1.

Table 1. *Recovery and repeatability of zeranol*

Validation parameter	No. of replicates	Spiked concentration µg/kg	Determinated concentration µg/kg	Mean recovery %	Coefficient of variation %
	6	1	0.923	92.3	2,4
	6	2	1.759	87.9	4,7
Recovery	6	3	2.689	89.6	5,6
	18	1	0.901	90.0	4,9
	18	2	1.782	89.1	6,8
Repeatability	18	3	2.801	93.3	8,6

Validation of the method used in zeranol determination resulted in the mean recovery of 87.9%-92,3% and repeatability of 89.1%-93,3% with coefficient of variation (CV) of 2,4%-5,6% and 4,9%-8,6% respectively.

As can be seen in Fig.1, the zeranol calibration curve was found to be virtually linear in the 0.025 to 3 ng/ml.

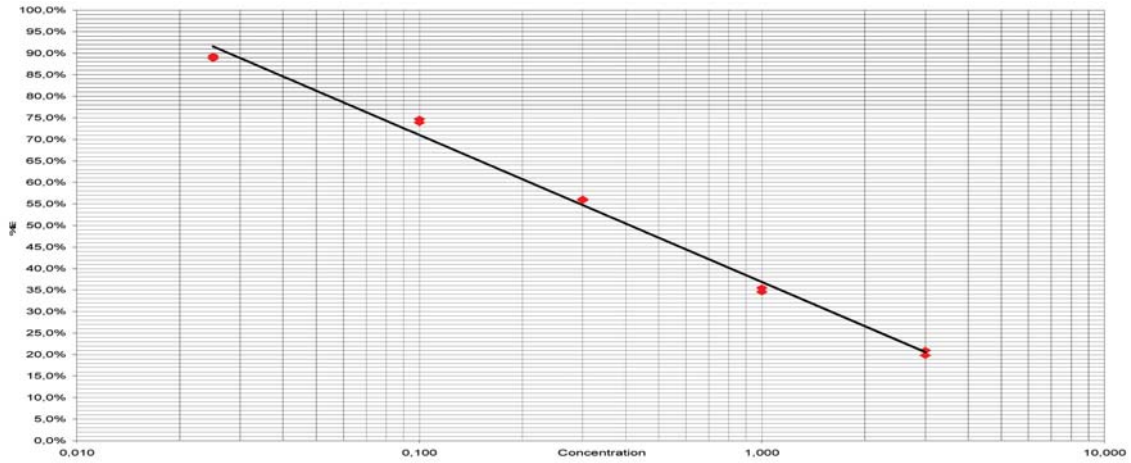


Figure 1. *Linearity of calibration curve for zeranol standards*

In Fig.2 the correlation between the absorbance ratio and zeranol concentration was evaluated over the range 0 – 3 ng/ml, R^2=0.9929.

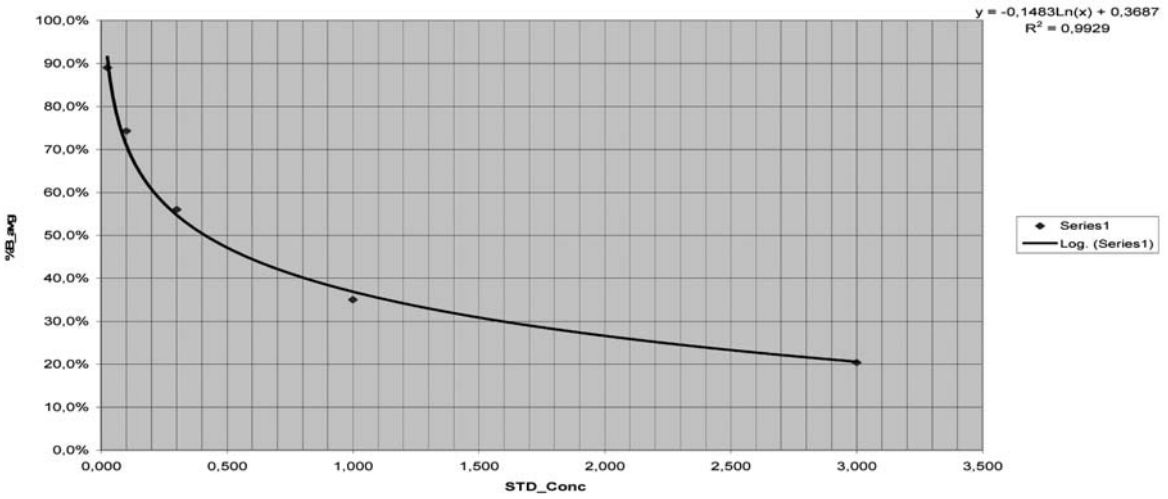


Figure 2. *Calibration curve for zeranol standards (0.025-3 ng/ml)*

Zeranol and its metabolites act as estrogen receptor agonists and exert typical estrogenic effects on animals (Lamming 1987, Le Guevel and Pakdel 2001, Leffers et al. 2001, Nagel et al. 1998, Nikaido et al. 2005). The presence of mycotoxins and serum levels of zearalanone were associated with early thelarche and mastopathy in Hungarian girls (Szuets et al. 1997). Furthermore, the presence of mycotoxins was strongly correlated with precocious puberty, and exposure to the mycoestrogenic zearalenone is thought to trigger central precocious puberty in young girls (Massart et al. 2008). Natural estrogen is a known cause of human breast and uterine cancer and increased exposure to zeranol may similarly increase risk from the existing burden of the natural compound. Most, but not all, of the short-term assays to assess mutagenicity of zeranol and some metabolites (zearalanone and taleranol) were negative (Metzler and Pfeiffer 2001). Because of the potential toxicity of these compounds, whether from natural or synthetic sources, it is important to prevent human exposure. Identifying zeranol in biospecimens is not sufficient to prove exposure to the synthetic hormone, as ingestion of *Fusarium* contaminated corn can produce similar results. However, laboratory methods exist to distinguish between metabolites resulting from exposure to zeranol and those resulting from exposure to the *Fusarium* mycotoxin. Elevated levels of zeranol or its metabolites could indicate that measures aimed at keeping the synthetic hormone out of the food supply are not adequate or that efforts to prevent *Fusarium* contamination of the food supply are not adequate. Zearalanone is stored in adipose tissue (Pillay et al. 2002). In one study (Nagel et al. 1998), an oral dose of zearalanone had a half-life of 22 hours in human blood. Both the presence of mycotoxins and serum levels of zearalanone in the range of 18.9-103µg/L were associated with early thelarche and mastopathy in Hungarian girls (Szuets et al. 1997). Since zeranol use has been banned in the European Union, the interest in detecting illegal zeranol use has resulted in the development of GC-MS (Blokland et al. 2006) and immunoassay (Tuomola et al. 2002) techniques that can be used to distinguish between exposure to zeranol and exposure to *Fusarium* toxins in biological specimens. LC/MS methods exist to detect zeranol in sub-ppb quantities in animal urine (Launay et al. 2004, Schmidt et al. 2008, Rúbies et al. 2007). In the illegal use of zeranol it is difficult to determine its presence because the amount of zeranol given and period is not known. In the improved ELISA, the linear response range was between 0.025 and 3 ng/ml , and the detection limit was 0.22 ng/ml for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 87,9%~92,3% and 2.4%~5.6%, respectively. The levels of zeranol in bovine urine, which require due measures to be taken for suspect abuse are defined according the Council Directive 1996/22/EC. The borderline urine level of zeranol demanding due measures has been set at 1,21 ng/ml to obviate the possibility of a great number of false-positive results. In comparison with physiological values reported in the literature, the results obtained in the present study and data on study animals indicated that illegal use ofzeranol residues could not be suspected in none of the studied animals. As data on urine zeranol concentrations have not yet been precisely determined, are quite inadequate for different animal species and categories, and depend on numerous factors, additional studies are definitely necessary. On the other hand, because there is possibility to occur high rates of false positive zeranol samples in urine, confirmation of the ELISA test should always be carried out by chromatographic methods coupled to spectrometric methods.

REFERENCES

1. Council directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stock framing of certain substances having hormonal or thyrostatic action and of beta-agonists and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC
2. Council directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EECand 86/469/EEC and Decisions 89/187EEC and 91/664/EEC
3. Ch.L. Xu, Ch.F. Peng, L. Liu, L. Wang, Z.Y. Jin and X.G. Chu (2006) Development of an enzyme-linked immunosorbent assay for the determination of hexoestrol. Journal of Animal and Feed Sciences, 15, 2006, 159–171
4. Bagnati R., Castelli M.G., Airoldi L., Oriundi M.P., Ubaldi A., Fanelli R., 1990. Analysis of diethylstilbestrol, dienes-

trol and hexestrol in biological samples by immunoaffinity extraction and gas chromatography-negative-ion chemical ionization mass spectrometry. J. Chromatogr. 527, 267-278
5. Sawaya W.N., Lone K.P., Hasain A., Dashti B., Al-Zenki S., 1998. Screening for estrogenic steroids in sheep and chicken by the application of enzyme- linked immunosorbent assay and a comparison with analysis by gas chromatography–mass spectrometry. J. Agr. Food Chem. 63, 563-569
6. Talat S., Esmael N., Nisar A., 1999. Assessment of the levels of anabolic compoumds in Kiwait meat industry: optimization of a multiresidue method and the results of a priliminery survey. J. Food Control 10, 169-174
7. Leslie C.D., MacNeil J.D., Reid J.A., Fesser A.C.E., 2003. Validation of screening method for residues of diethylstilbestrol, dienestrol, hexestrol, and zeranol in bovine urine using immunoaffinity chromatography and gas chromatography/mass spectrometry. J. AOAC Int. 86, 631-639
8. Lamming GE. 1987. Scientific report on anabolic agents in animal production. Veterinary Record. 121:389–392.
9. Le Guevel R., and Pakdel F. 2001. Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods. Hum Reprod. 16:1030–1036.
10. Leffers H., Naesby M., Vendelbo B., Skakkebae NE., Jorgensen A. 2001. Oestrogenic potencies of Zeranol, oestradiol, diethylstilboestrol, Bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. Hum Reprod. 16:1037–1045.
11. Nagel SC, vom Saal FS, Welshons WV. 1998. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. Proc Soc Exp Biol Med. Mar;217(3):300-9.
12. Nikaido Y, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N, Tsubura A. 2005. Effects of prepubertal exposure to xenoestrogen on development of estrogen target organs in female CD-1 mice. In Vivo. May-Jun;19(3):487-94.
13. Szuets P., Mesterhazy A., Falkay GY., Bartok T. 1997. Early telarche symptoms in children and their relations to zearalenone contamination in foodstuffs. Cereal Research Communications 25(3I):429-436.
14. Massart F, Meucci V, Saggese G, Soldani G. 2008. High growth rate of girls with precocious puberty exposed to estrogenic mycotoxins. J Pediatr. May;152(5):690-5.
15. Metzler M. and Pfeiffer E. 2001. Genotoxic potential of xenobiotic growth promoters and their metabolites. Acta Pathologica, Microbiologica et Immunologica Scandinavica 109:89-95.
16. Pillay D., Chuturgoon AA., Nevines E., Manickum T., Deppe W., Dutton MF. 2002. The quantitative analysis of zearalenone and its derivatives in plasma of patients with breast and cervical cancer. Clin Chem Lab Med. Sep;40(9):946-51.
17. Schmidt K., Stachel C., Gowik P. 2008. Development and in-house validation of an LC-MS/MS method for the determination of stilbenes and resorcylic acid lactones in bovine urine. Anal Bioanal Chem. Jun;391(4):1199-210.
18. Launay FM., Young PB., Sterk S., Blokland M., Kennedy D. 2004. Confirmatory assay for zeranol, taleranol and the Fusarium spp. toxins in bovine urine using liquid chromatography-tandem mass spectrometry. Food Addit Contam. 21:52–62.

ОПРЕДЕЛУВАЊЕ НА РЕЗИДУАЛНИ НИВОА НА ЗЕРАНОЛ ВО УРИНА СО ELISA МЕТОД

Хајрулаи-Муслиу Зехра[1]*, Узунов Ристо[1], Димитриеска-Стојковиќ Елизабета[1], Стојановска-Димзоска Билјана[1], Секуловски Павле[1], Стојковски Велимир[2], Тодоровиќ Александра[1]

[1]Институт за храна, [2]Институт за биомедицина и репродукција, Факултет за ветеринарна медицина, Универзитет "Св. Кирил и Методиј", Скопје, Македонија

*Автор за коресподенција: zhajrulai@fvm.ukim.edu.mk

АПСТРАКТ

Зеранолот е синтетички производ на зеараленонот кој се користи како анаболичка супстанца кај овците и говедата за зголемување на растот на животните кои се одгледуваат за производство на храна. Користењето на зеранолот е забрането во сите земји на Европската Унија и во Македонија. При нелегалната употреба на зеранол, утврдувањето на неговото присуство претставува потешкотија, бидејќи не се познати количината на аплицираниот зеранол и периодот на апликација. Беше разработен високо афинитетен ензимски имуносорбентен метод (ELISA) на основа на поликлонални антитела, за квантификација на зеранол во урина од говеда. Кај подобрената ELISA линеарниот одговор беше во опсегот помеѓу 0,025 и 3 ng/mL, со лимит на детекција на методот од 0,22 ng/mL. Вкупниот аналитички принос и коефициент на варијанца (CV) беше во опсегот од 97,9-92,3 % и 2,4-5,6 %, соодветно. 36 примероци од говедска урина спикувани со зеранол (од 0,2 до 10 ng/mL) беа детектирани со ELISA и постигната е добра корелација (R^2=0,9929). Може да се заклучи дека оваа подобрена ELISA претставува соодветна алатка како масовен метод за скрининг на зеранол во говедска урина. Вкупно 87 примероци од говедска урина, како дел од националниот мониторинг на резидуи, анализирани се за утврдување на присуство на зеранол. Во овие документирани случаи утврденото присуство на зеранол беше значително пониско отколку MRPL определен со циркуларното упатство на Референтните Лаборатории на ЕУ (7 декември 2007).

Клучни зборови: резорцинска киселина, урина, ELISA, зеранол.

UDC: 637.5`62.07(497.7)

DETERMINATION OF TRENBOLONE IN CATTLE MEAT WITH ELISA METHOD

Uzunov Risto[1], Hajrulai-Muslu Zehra[1], Dimitrieska-Stojkovik Elizabeta[1], Stojanovska-Dimzoska Biljana[1], Sekulovski Pavle[1], Stojkovski Velimir[2], Todorovic Aleksandra[1]

[1]Food Institute, [2]Institute for Biomedicine and Reproduction Faculty of Veterinary Medicine, Ss. "Cyril and Methodius, Skopje, Macedonia

*Corresponding author: risteuzunov@fvm.ukim.edu.mk

ABSTRACT

In recent years, hormones and hormone like substances have been recently used in livestock production to obtain a high yield performance in a shorter period of time.

These anabolic agents are used to increase the weight gain, to improve the food efficiency, storing protein and to decrease fatness. But, depending on the use of anabolic in animal feed, anabolic residues that may occur in meat and meat products present risks to human health. The present study was undertaken to detect and quantify the levels of trenbolone residues (a potent synthetic analog of testosterone) in meat in Republic of Macedonia. Cattle meat samples were collected within period of 12 months as they were delivered by the authorised veterinary inspectors. A total of 82 samples of cattle meat were analyzed for level of trenbolone by Enzyme-Linked Immunosorbant Assay method. The detection limit was 91.5 ppt for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 83.7%-99.6% and 4.1%-8.2%, respectively, a working range between 25 to 400 ppt, and the regression equation of the final inhibition curve was: $y = -0.2451x + 1.6221$, $R^2 = 0.9928$. The average experimental value of trenbolone in meat was 152.2 ppt. This value gave no evidence for the illegal use of hormones in Republic of Macedonia, but these results do not exclude the possibility of misuse of these potentially harmful chemicals in future. Therefore it is necessary to conduct permanent control of these hormones as a food quality and health safety measure.

Key words: Trenbolone, ELISA, validation, residues, cattle meat.

INTRODUCTION

In recent years, hormones and hormone like substances have been recently used in livestock production to obtain a high yield performance in a shorter period of time.

These anabolic agents are used to increase the weight gain, to improve the food efficiency, storing protein and to decrease fatness. But, depending on the use of anabolic in animal feed, anabolic residues that may occur in meat and meat products present risks to human health (1-4). Trenbolone acetate is a powerful synthetic steroidal androgen, which is used as a growth promoter in cattle. It is rapidly hydrolyzed to its metabolite 17 β- trenbolone after administration to cattle. It is thought to act on skeletal muscle, either through androgen receptors to increase protein synthesis or through glucocorticoid receptors to reduce the catabolic effects of glucocorticoids. Trenbolone acetate decreases the rate of both protein synthesis and degradation, and when the rate of degradation is less than the rate of synthesis, muscle protein rate increases (5). In many countries outside the EU trenbolone acetate is licensed as growth promoter for steers and heifers. In addition, its tremendous effectiveness in dry and lactating cull cows (38 % increased weight gain accompanied by lower fat deposition) was reported (6). The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals while the United States Food and Drug Administration (USFDA) permitted the limited use of some hormones with natural origins (such as estradiol and testosterone) and some synthetic hormones such as trenbolone in animal husbandry (7-8). The permitted limit values for trenbolone is 1 ppb in muscle (CRL GUIDANCE PAPER (7 December 2007)). In Republic of Macedonia, the use of hormones as growth promoters has been made illegal too. The aim of this study was to detect the levels of trenbolone residues in the meat in Republic of Macedonia with ELISA methods.

MATERIALS AND METHODS

Meat samples were collected from March 2011 to March 2012. The samples were kept frozen until use. We analyzed a total of 82 cattle meat samples.

Reagents. Most of the reagents that we used were contained in the RIDASCREEN Trenbolone test kit from R-Biopharm AG, Darmstadt, Germany. Kit contain: Microtiter plate with 96 wells (12 strips with 8 removable wells each) coated with capture antibodies, 6 standard solutions, 0 ppt (zero standard), 25 ppt, 50 ppt, 100 ppt, 200 ppt and 400 ppt trenbolon in 40 % methanol, conjugate (peroxidase conjugated trenbolone), anti-trenbolone antibody, substrate (containing urea peroxide, chromogen (containing tetramethylbenzidine), stop solution (containing 1 N sulfuric acid), conjugate and antibody dilution buffer.

Methanol and tertiary butyl methyl ether were of analytical grade and purchased from Merck. PBS (Phosphate buffer solution) 67 mM, pH 7.2, was prepared

by mixing 1.79 g sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O) with 9.61 g disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and 8.7 g sodium chloride (NaCl) and filling up to 1000 ml disstilled water. 20 mM PBS buffer, pH 7.2, was prepared by mixing 0.55 g sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O) with 2.85 g disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and 9 g sodium chloride (NaCl) and filling up to 1000 ml disstilled water. From fortified samples and calculation of recovery we used external standard trenbolone (Sigma- Aldrich).

Apparatus. Microtiter, plate spectrophotometer (BIO RAD model 680) (450 nm), evaporator, mixer, shaker, vortex, centrifuge, balance, RIDA® C18 column, micropipette (20 µl, 50 µl, 100 µl, 200-1000µl).

Extraction procedure. Fat and connective tissue were removed from the muscle and 10g of the ground muscle was homogenized with 10mL of 67mM PBS buffer by mixer for 5min. 2g of homogenized sample were mixed with 5mL of tertiary butyl methyl ether (TBME) in a centrifugal screw cap vial and shaken vigorously by vortex for 30-60min. The contents were centrifuged at 3000rpm for 10min. The supernatant was kept and the extraction with TBME was repeated. The supernatants were combined and evaporated then the dried extract was dissolved in 1mL of 80% methanol. The methanolic solution was diluted with 2mL of 20mM PBS-buffer and applied to a RIDA C18 column (solid phase extraction column with C18 end-capped sorbent of an average particle size of 50µm**)** in the following manner:

- Column was rinsed by flowing of 3mL methanol (100%); Column was equilibrated by injection of 2mL PBS – Buffer; 3mL of sample was loaded on column; Column was rinsed by injection of 2mL methanol (40%); Column was dried by pressing nitrogen through it for 3min; Sample was eluted slowly by injection of 1mL methanol (80 %). An aliquot of the eluate was diluted with water (1+1, v/v), then 20µL per well of resulting solution was used in the test.

Test procedure. Ridascreen ELISA kit was obtained from R Biopharm GmbH, Germany. Trenbolone standard solution used for the calibration curve were at levels of 0, 25, 50, 100, 200, and 400 ppt trenbolone in 40 % methanol,, whereas the antibody used had cross reactivity with other related compounds, as indicated by the manufacturer's literature and shown in Table 1. The standard and samples were analyzed in duplicate. To the marked microwells, 50µL of the diluted enzyme conjugate (peroxidase conjugated testosterone) was added. Then 20µL of standards or samples were added. After that 50µL of the diluted antibody solution was added and after mixing gently by rocking the plate manually, the contents were incubated at room temperature for 2h. The liquid poured out of the wells and after removal of liquid completely, all wells were filled with distilled water (250µL). After rinsing, the water was also discarded; washing was repeated two more times. Then, 50µL of substrate (urea peroxide) and 50µL of chromogen (tetramethylbenzidine) were added. After mixing thoroughly and incubating for 30min at room temperature and dark, 100µL of stop solution (1M sulphuric acid) was added. After mixing, the absorbance was read at 450nm. Colour was stable for 60min. Data were analyzed using a special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany). The mean absorbance values obtained for the standards and the samples divided by the absorbance value of the first standard (zero standard) and multiplied by 100 was the % absorbance. The zero standard was thus made equal to 100 % and the absorbance values were quoted in percentages.

Table 1. *Cross reactivity of trenbolone antibody with various compounds*

Compound	Cross reactivity (%)
17β-Trenbolone	100
Trendione	100
17α-trenbolone	82
19 nortestosterone	0,06
Testosterone	<0,01
Estradiol	<0,01
Zeranol	<0,01
DES	<0,01
Chloramphenicol	<0,01

Method validation. The limit of detection (LOD) was obtained by spiking with 0, 5 times from MRPL which 1 ppb is according the guidance letter from Community Reference Laboratories' (7 December 2007). The method recovery was determined at three level by spiking meat samples with 0,5; 1 and 1,5 times from MRPL level. For determination of repeatability, the same steps were repeated on two occasions in the same analytical conditions. Detection capabilities (CCβ) was evaluated by analyzing 20 spiked samples at 0,5 MRPL level. A typical ELISA standard curve is presented in Figure 1. Final trenbolone concentrations in meat were calculated by taking the average recoveries into account.

RESULTS AND DISCUSSION

The calculation of the gained results was made by RIDAWIN Software. For construction of the calibration curve the mean of the absorbance values obtained for the standards was divided by the absorbance value of the first standard (zero standards) and multiplied by 100. The absorption is inversely proportional to the concentration of trenbolone. As can be seen in Fig.1, the trenbolone calibration curve was found to be virtually linear in the 20 to 400 ppt.

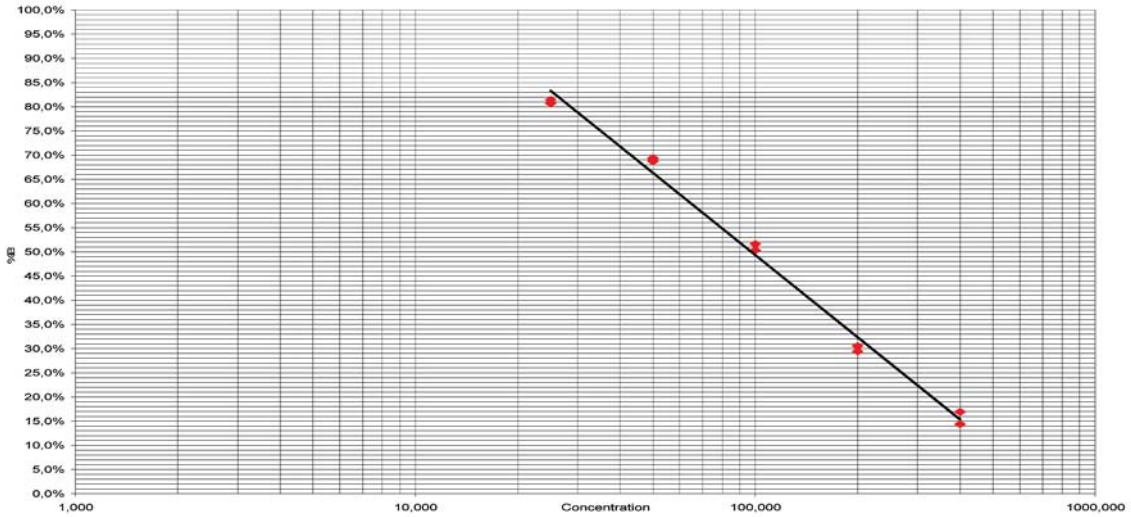


Figure.1 *Linearity of calibration curve for trenbolone standards (25-400 ppt)*

The estimated LOD for meat samples for trenbolone was 91.5 ppt. The CCβ for meat samples for trenbolone was 492.9 ppt. In Fig.2 the correlation between the absorbance ratio and trenbolone concentration was evaluated over the range 0 – 400 ppt, R^2=0.9928.

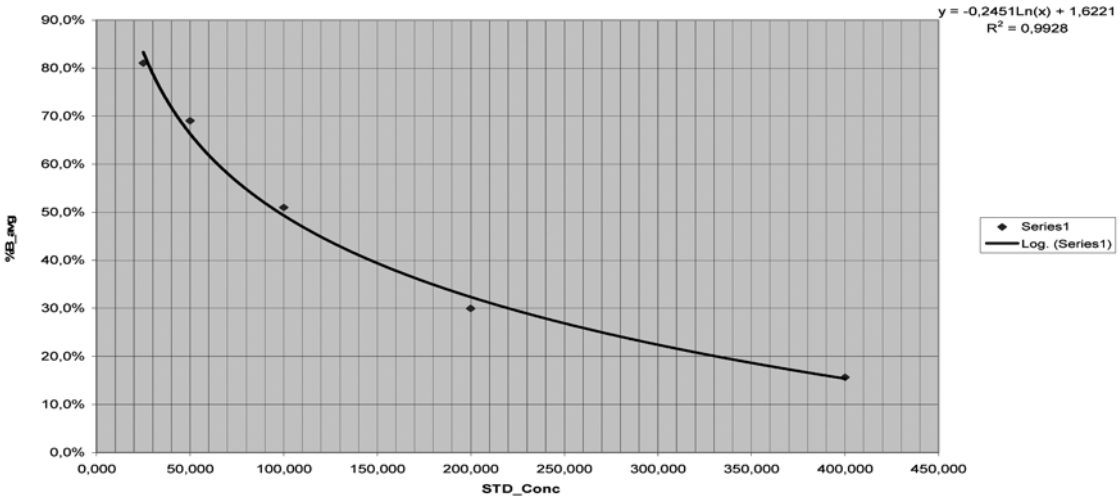


Figure.2. *Calibration curve for trenbolone standards (0-400 ppt)*

The precision of the method was calculated by measured CV%. The precision dates are shown in Table 2.

Table 2. *Precision of the method (% CV)*

	Concentration (ppt)	CV%
Standards	0.0	3.0
	25.0	0.6
	50.0	1.8
	100.0	1.5
	200.0	0.7
	400.0	4.7
CV for fortified samples	500.0	8.2
	1000.0	6.5
	1500.0	4.1

The results of method recovery (n=18) and repeatability (n=54) are presented in table 3.

Table 3. *Recovery and repeatability*

Validation parameter	No. of replicates	Spiked concentration ng/kg	Determinated concentration ng/kg	Mean recovery %	Coefficient of variation %
Recovery	6	500	418.6	83.7	8.2
	6	1000	962,1	96.2	6.5
	6	1500	1494,8	99.6	4.1
Repeatability	18	500	442.4	88.4	10.2
	18	1000	911.4	91.1	3.6
	18	1500	1432.7	95.5	4.7

Validation of the method used in on trenbolone determination resulted in the mean recovery of 83.7%-99.6% and repeatability of 88.4%-95,5% with coefficient of variation (CV) of 4.1%-8.2% and 3.6%-10.2%.

The analyses of the meat samples showed concentrations from 66.7 ppt to 258.8 ppt (mean concentration of trenbolone was 152.2 ppt).

Our test results are of importance as they give information about the use of trenbolone preparations in national animal husbandry and in the food industry. The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals while the United States Food and Drug Administration (USFDA) permitted the limited use of some hormones with natural origins (such as estradiol and testosterone) and some synthetic hormones such as trenbolone in animal husbandry (1-4). The permitted limit values for trenbolone is 1 ppb in muscle (CRL GUIDANCE PAPER (7 December 2007)). A survey carried out in R. Macedonia from March 2011 to March 2012 demonstrated that the incidence of residues of trenbolone in tissues of slaughter animals is not a problem, which means that trenbolone gave no evidence of illegal use of these hormones in R. Macedonia. The National Residues Control Plan guarantees fulfilment of the requirements which are of importance to health of both humans and animals as well as marketing of animals, food and other products of animal origin.

REFERENCES

1. The Presence of Some Anabolic Residues in Meat and Meat Products Sold in Üstanbul, B.lent NAZLI**, Hilal .OLAK, Ali AYDIN, Hamparsun HAMPÜKYAN Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Üstanbul University, 34320, AvcÝlar, Üstanbul – TURKEY, Received: 27.06.2003
2. Cecave, M.J., Hancock, D.L. : Effects of anabolic steroids on nitrogen metabolism and growth of steers fed corn silage and cornbased dietssupplemented with urea or combinations ofsoybean meal and feathermeal. J. Anim. Sci., 1994; 72: 515-522.
3. Moran, C., Ourke, J.F., Prendiville, D.J., Bourke, S., Roche, J.F.: The effect of estradiol trenbolonee acetate, or zeranol on growth rate, mammary development, carcass traits, and plasma estradiol concentrations of beef heifers. J. Anim. Sci., 1991; 69: 4249- 4258.
4. Sawaya, W., Lone, K.P., Hasain, A., Dashti, B., Al-Zenki, S.: Screening for estrogenic steroids in sheep and chicken by the application of enzyme-linked immunosorbent assay and a comparison with analysis by gas chromatography-mass spectrometry. Food Chem., 1998; 63: 563-569.
5. Human helt and trenbolone residue in bovine meat, 1B. Jannat, 2M. R. Oveisi, *2N. Sadeghi, 2M. Hajimahmoodi, Iran. J. Environ. Health. Sci. Eng., 2007, Vol. 4, No. 4, pp. 203-206
6. Screening of trenbolone-17βin milk samples after application of trenbolone acetate to a cull cow, Iris G. Lange, Andreas Daxenberger, Heinrich H. D. Meyer Institut für Physiologie, Forschungszentrum für Milch und Lebensmittel, Technische Universität München Weihenstephaner Berg 5, D- 85350 Freising-Weihenstephan, Germany
7. Council directive 96/22/EC of 29 april 1996 concerning the prohibition on the use in stock framing of certain substances having hormonal or thyrostatic action and of beta-agonists and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC
8. Council directive 96/23/EC of 29 april 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EECand 86/469/EEC and Decisions 89/187EEC and 91/664/EEC

ОПРЕДЕЛУВАЊЕ НА ТРЕНБОЛОН ВО МЕСО ОД ГОВЕДА СО ELISA МЕТОД

Узунов Ристо[1], Хајрулаи-Муслиу Зехра[1], Димитриеска-Стојковиќ Елизабета[1], Стојановска-Димзоска Билјана[1], Секуловски Павле[1], Стојковски Велимир[2], Тодоровиќ Александра[1]

[1]Институт за храна, [2]Институт за биомедицина и репродукција, Факултет за ветеринарна медицина, Универзитет "Св. Кирил и Методиј", Скопје, Македонија

*Автор за коресподенција: risteuzunov@fvm.ukim.edu.mk

АПСТРАКТ

Во последниве години хромоните и сипстанците слични на хормони се користат во одгледувањето на добитокот за добивање на високо приносни карактеристики за пократок временски период.

Овие анаболици се употребуваат за зголемување на масата, подобрување на екфектот на искористување на храната, депонирање на протеините и намалување на масното ткиво. Но во зависност од примената на анаболиците во добиточната храна, нивни резидуи кои може да се јават во месото и производите од месо претставуваат ризици за здравјето на луѓето. Оваа студија беше спроведена за детектирање и квантификување на нивоата на резидуи од тренболон (синтетички аналог на тестостеронот) во примероци од месо од Република Македонија. Примероците од говедско месо беа собирани во текот на 12 месеци, а беа доставувани од овластените ветеринарни инспектори. Вкупно 82 примерока говедско месо беа анализирани за утврдување на нивото на тренболон со Ензимски врзан имуносорбентен метод. Лимитот на детекција при определувањето беше 91,5 ppt. Вкупниот аналитички принос и коефициент на варијанца (CV) беше во опсегот од 83,7-99,6 % и 4,1-8,2 %, соодветно, со работен опсег од 25 до 400 ppt. Равенка на регресија на конечната инхибиција беше : y= -0,2451x + 1,6221, R^2 = 0,9928. Вкупната експериментално определена вредност на тренболонот во месо беше 152,2 ppt. Оваа вредност укажува дека нема нелегална употреба на хормони во Република Македонија, но овие резултати не ја исклучуваат можноста за злоупотреба на овие потенцијално штетни супстанции во иднина. Заради тоа, неопходно е да се спроведува постојана контрола на овие хормони како мерка за контрола на квалитетот на храната и безбедност за здравјето.

Клучни зборови: тренболон, ELISA, валидација, резидуи, говедско месо.

UDC: 635.8:[546.36:543.422.3-78

ESTIMATION OF TIME OF SEMI-DECAYOF ^{137}Cs IN MUSHROOMS

Todorovik Aleksandra, Sekulovski Pavle, Dimitrieska Stojkovik Elizabeta, Hajrulai-Musliu Zehra, Stojanovska Dimzoska Biljana, Uzunov Risto

Food Institute, Faculty of Veterinary Medicine Skopje
University of "Ss. Cyril and Methodius" Skopje
mizasandra@fvm.ukim.edu.mk

ABSTRACT
The mushrooms are particularly interesting type for examination because they absorb minerals, and with that they can not avoid contamination. Because of their specific content they are a trap for the contaminators. The purpose of this work is to determine the time of semi-decay of ^{137}Cs, that is, to conceive if there is difference between the measured and the calculated time of semi-decay of this radionuclide. Different species of mushrooms are takes as samples for analysis due to their great accumulation of this radionuclide. There were 5 types of mushrooms examined, and for each type 12 samples were analyzed.
After the performed analyses, the difference between the calculated and the measured activity of ^{137}Cs at all examined types of mushrooms is determined.
Key words: radioactivity, radionuclides, mushrooms, gamma spectrometry

INTRODUCTION

The radio nuclides, natural or artificial, with the process of migration come into the soil or the water, and through them in the products of animal or herbal origin and contribute foe overall radiation with humans. [2-3]

In order to protect the ecosystem i.e. the population of the environment of one area, it is necessary to make estimation of individualnoto nivo na kontaminacija. But, because the diversity of the organism is enormous even in small areas, this estimation is difficult and sometimes impossible.

The answer of the increase of contamination of the plants in the environment is modified with other factors of the environment and with the physiological status of the plants.

The adventage of the biomonitoring over the classical method of analysis is that it takes into consideration the integral effect of all tha factors and the contamination.

The content of the radionuclides in bioindicator types provides an insight into the level of radioactive contamination of the given ecosystem. For these needs, among the vegetation types the fungus and the lichens. [4]

After the Chernobile accident in 1986 the concentracion of ^{137}Cs and ^{90}Sr significantly was increased in a lot of European countries, and the need to research of these plants how they function as biologic indicators of radioactive pollution. [5]]

The contamination of the mushrooms depends on more factors: altitude above sea level, physical and chemical composition of the soil, meterorological conditions, the amount of rains etc.

The era of application of nuclear weapons began in 1945 (Hiroshima, Nagasaki), and the first trial of this weapon was performed by USA. The radionuclides, released in the environment with the process of translocation and elimination, come on the ground and the water, and through them, in the victuals with plant and animal origin. An accident which marks the 20th century is the one in the nuclear plant Lenin in Chernobyl when $12 \approx 10^{18}$ Bq radioactive material is thrown out in the environment, of which ^{137}Cs is most important [5-6] . As a fireproof radionuclide, it is especially important for the biological systems [6]. The fission yield of ^{137}Cs is 6,2% and has long time of semi-decay (30,7 years) so together with ^{90}Sr, these two are the most represented fission products in the nature.

Of ^{137}Cs which is found in the atmospheric rains, 75-99% gets in contact with the ground, and 1-25% is retained at the vegetation. The destiny of cesium which comes to the ground depends on the characteristics of the ground [7].

The accumulation of cesium on the ground depends on many factors (the type of soil), and numerous explorations have proved that it is mostly retained at the layer 5cm from the surface of the ground, since its speed of its penetration through the soil is 1-3 cm g^{-1}. Still, the speed depends on the type of soil and the quantity of athospheric rains [7].

Radioactive contamination from this radionuclide can happen at humans by:

1. Inhalation
2. Ingestion

The biological time of semi-elimination at humans is 10-110 days. This depens on the age limit, the size of the musle mass etc.

What is very important is that the specificity of ^{137}Cs is such that in the human organism is acts the same way as potassium and rubidium, which means that it is found in every cell of the organism i.e. it is distributed evenly in all organs [5].

However, the probability for break-down of this radioactive atom, in time interval D*t,* does not depend on the conditions in which the atom existed and on the condition it is found, but it depends only on the length of that time interval [8].

The number of radioactive atoms is decreasing over time exponentially (Fig.1):

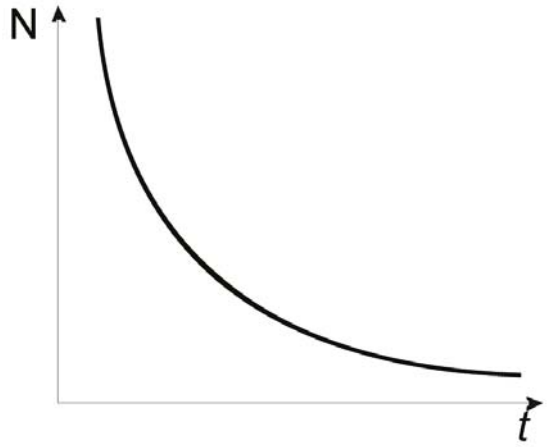


Figure 1. *The exponential law of radiactive decay*

If in a certain moment there are *N*-atoms of some radioactive element, and if from that number *dN* atoms are decayed in short time interval *dt*, the speed of the decay is: *-dN/dt.* Since the speed of decay is proportional to the total number of radiactive atoms *N*, what follows is that:

$$-\frac{dN}{dt} = \lambda N \qquad (1)$$

By integrating the equation (1) in interval of $t = 0$, when N_0 atoms were present, and the time *t*, for which the number of undecayed atoms has decreased from N_0 to *N*, the basic law for radioactive decay is formed*:*

$$\ln\left(\frac{N}{N_0}\right) = -\lambda t \quad (2)$$

$$N = N_0 e^{-\lambda t} \qquad (3)$$

Time of semi-decay ($t_{1/2}$) is the time for which half of the initially present radioactive atoms will decay, i.e. the time for which the activity of the radionuclide will be decreased for half of the initial value.

By replacing in (2) of $t = t_{1/2}$ and $N = N_0/2$:

$$N_0/2 = N_0 e^{-\lambda t_{1/2}} \qquad (4)$$

$$\log 2 = \lambda t_{1/2} \log e \qquad (5)$$

$$t_{1/2} = 0{,}693/\lambda \qquad (6)$$

The numerous values for the time of semi-decay are within millionth parts of second, up to billion years [6].

MATERIAL AND METHOD

The samples of mushrooms are taken from different localities in the Republic of Macedonia (Kicevo, Kocani, Veles, Bitola, Radovis). The activity of the mushrooms in 2011 is determined by semi-conductive gamma spectrometer Canberra Packard which provides identification of radionuclides and estimation of their activity. The efficiency of the detector is 30% measured of ^{60}Co.

Data for the activities of the same types of mushrooms are taken, together with analyses made in 2006 for the level of the radionuclide ^{137}Cs. In this paper the activity of the articfical CEZIUM is determined by gammaspectrometry.

As a standard for calibration of the efficency the calibration standard (mixture

241-Am, 203-Hg, 137-Cs, 60-Co, 113-Sn, 85-Sr, 109-Cd, 139-Ce, 57-Co, 88-Y) is used.

The testing was in accordance with the method IAEA Technical Report 295. The time for measurement of each speciment is 10800s, and the relative error is smaller than 10%.

After termination of the measurement, the software of the instrument gives a written report with already calculated values for activity of ^{137}Cs, which values will be compared to the ones from 2006.

The results are expressed in Bq/kg fresh mass.

RESULTS AND DISCUSSION

From the results one can conclude that the activities of ^{137}Cs in 2006 have bigger value than those activities of ^{137}Cs in the period of 2009, which is expected considering the fact that the activity of every isotope decreases over time (the law of radioactive decay presented in the equation) [8].

$$A = A_o e^{-\lambda t}$$

Using the measured activities in the period from 2006, as initial we will calculate, according to the above presented law of radioactive decay, the activities which the cesium ^{137}Cs should have (for its specific time of semi-decay) for the period from 2011

The days from 01.01.2006 and 01.01.2011 are used for calculations as average times for the above presented periods, thus for **t** are taken 1825 days, that is, exactly 5 years.

Table 1. *Measured and calculated results for the activities of ^{137}Cs for different types of mushrooms.*

Types of mushrooms	Measured average activity of ^{137}Cs in Bq/kg for period 2005 – 2006	Measured average activity of ^{137}Cs in Bq/kg for period 2010 – 2011 год	Calculated average activity of ^{137}Cs in Bq/kg for period 2010 – 2011 год
Boletus edulis	5,06	2,54	4,74
Amanita caesarea	1,73	1,61	1,61
Cantharellus cibarius	12,00	4,84	11,2
Lactarius deliciosus	6,23	1,06	5,18
Morchella conika	5,88	5,25	5,49

If we compare the measured values of the activities of ^{137}Cs separately for each type of mushrooms in the period from 2011 and the calculated values of the activities for ^{137}Cs for the same mushrooms respectively, we will notice that the activities measured for the period from 2011 have quite smaller values that the calculated.

If a reason for decreasing the activities of ^{137}Cs for the three years would only be the time for semi-decay of ^{137}Cs, then the calculated and the measured activities should match at least approximately.

But according to table 1 it is visible that the measured activities ^{137}Cs for all types of mushrooms, except of the Amanita caesarea, are quite lower than the expected calculations.

The reason for the different activities of ^{137}Cs from the expected ones is due to many factors:

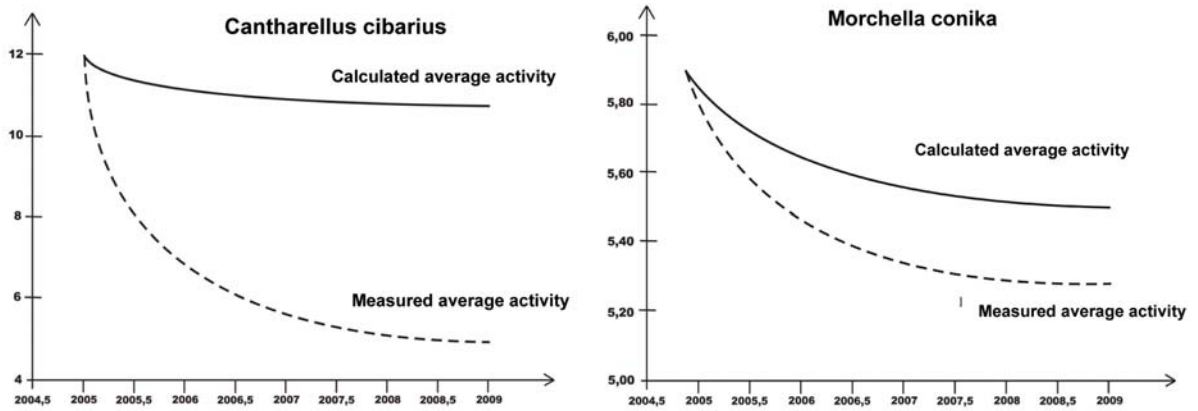
♦ Mechanical removal of ^{137}Cs, by rinsing the soil from rains;

♦ Different penetrative power of water in the soil wherewith the intensity of rinsing of ^{137}Cs is different;

♦ If at those places there was yield of mushrooms during these three years, with their picking, part of the quantity of ^{137}Cs is carried away.

The matching of the measured and the calculated activity at ^{137}Cs for the Amanita caesarea can be explained with the small penetration of the soil and the small quantities of rains. [8-9]

Graphic 1. *Comparisons between calculated and measured activity at different types of mushrooms for the time period 2006 and 2011*



From the tables, one can vividly see that there is a significant difference between the calculated and the measured values of ^{137}Cs, in both time periods.

CONCLUSION

The analyses made within this research point to the fact that the distribution of Cs ^{137} in the odserved types of mushrooms is not equal but it varies depending on the type of the mushrooms.Also,if we compare of Cs ^{137} in the same types id 2006 and 2011 we notice that ther is a decrease of the activity in the recent years which is to be expected. Also, that the real time of the activity of ^{137}Cs is shorter in relation to the physical time. These presented results at the mushrooms are a foundation for further analyses and measurements for the activity of ^{137}Cs, as well as other radionuclides in order to see whether there is a difference between the real and the physical time of activity in other samples being used in the nutrition.

REFERENCES

1. Gillet, A.G&Crout, N.M.J.2000. Areview of ^{137}Cs transfer to fungi and consequences for modelling environmental transfer. Journal of Environmental Radioaktivity, 48
2. Nivoa radioaktivne kontaminacije čovekove sredine i ozračenost stanovništva Jugoslavije 1986. godine usled havarije nuklearne elektrane u Černobilju, Savezni komitet za rad, zdravstvo i socijalnu zaštitu, Beograd, 1987
3 Anovski et al Zgolemena radioaktivna kontaminacija na zivotna sredina vo R.Makedonija uslovena od Cernobilskata nesreka, Zbornik na trudovi od 2 Savetovanje o izlaganju zracenju iz prirodne sredine i procene radiacionog rizika (Arangelovac 1986)
4. Cucolovic A, Momilovic M, XXV Simpozijum za zastitu od zracenja Srbije I Crne gore ,Zbornik radova, Kopaonik, 2009, str. 25-29.
5. I. Kryshev, R. M. Aleksakhin, I. N. Ryabov, et al., *Radioecological Consequences of the Chernobyl Accident*, Nuclear Society of the USSR, Msocow (1991).
6. R. M. Aleksakhin, L. A. Buldakov,V. A. Gubanov, et al., in: *Large Radiation Accidents: Consequences and Protective Measures*, edited by L. A. Il'in and V. A. Gubanov, IzdAT, Moscow (2001).
7. http://www.bt.cdc.gov/radiation/isotopes/cesium.asp
8. http://www.bt.cdc.gov/radiation/isotopes/cesium.asp
9. W. Schimmack. Migration of radiocesium in two forest soils as obtained from field and column investigations, *The Science of the Total Environment,* 116 (1992) 93-107 Elsevier Science Publishers B.V., Amsterdam

ПРОЦЕНКА НА ВРЕМЕ НА ПОЛУ-РАСПАД НА ^{137}Cs ВО ПЕЧУРКИ

Тодоровиќ Александра, Секуловски Павле, Димитриеска Стојковиќ Елизабета, Хакрулаи Муслиу Зехра, Стојановски Димзоска Билјана, Узунов Ристо

Институт за храна, Факултет за ветеринарна медицина,
Универзитет "Св. Кирил и Методиј", Скопје, Македонија

mizasandra@fvm.ukim.edu.mk

АПСТРАКТ

Печурките се посебно интересен тип за испитување поради нивната способноста да апсорбираат минерали и со ова тие не можат да ја избегнат контаминацијата. Поради нвниот состав тие се замка за контаминентите. Целта на овој труд е да се одреди времето на полу-распад на ^{137}Cs т.е. да се процени разликата помеѓу измерените и проценетото време на полу-распад на овој радионуклид. Како примероци за испитување се земени различни видови на печурки поради нивната способност за акумулација на овој радионуклид. Испитани се 5 типа на печурки и по 12 примероци од секој тип.

По завршените анализи разликата помеѓу пресметаните и измерените активности на ^{137}Cs кај сите типови на примероци е утврдена.

Клучни зборови: радиоактивност, радионуклиди, печурки, гама спектрометрија

UDC: 637.5`65.065:579.842.14

INACTIVATION OF SALMONELLA TYPHIMURIUM ON POULTRY MEAT BY ELECTROLYZED WATER

Çilİplikçioğlu Güzin[1], Demirel Yağmur Nil[1], Şireli Ufuk Tansel[1]

[1]*Food Hygiene and Technology Department, Ankara University Faculty of Veterinary Medicine, Ankara, Turkey*

ABSTRACT
Salmonella is the most frequent causes of food poisoning in humans. Eggs and poultry are the main common sources of such outbreaks. One of the most commonly isolated sero-types from poultry is *S.* Typhimurium. A number of interventions are used extensively by the meat and poultry industries to reduce bacterial contamination. Electrolyzed Water (EW) is currently gaining popularity as a sanitizer in the food industry. The objective of this study was to determine the efficacy of EW in the inactivation of *Salmonella* Typhimurium in chicken meat and monitor the effects during the shelf –life. Chicken wings were inoculated with two types of *Salmonella* Typhimurium, ATCC 14028 and a wild strain. Inoculated samples dipped in three different EW that include 30, 60 and 70 ppm chlorine, for 15, 30 and 60 seconds. Chicken wings were sampled at day 0 in order to determine the antimicrobial effect, the rest of the samples stored in 7 °C for 3 and 7 days to monitor the effect of shelf-life. As a result, EW reduced the *Salmonella* Typhimurium approximately 2 log CFU/ml on day 0. However, no reduction observed during the 7 days. There was no difference between 30, 60 and 70 ppm chlorine included EW's, and also 15, 30 and 60 seconds.
Key words: Electrolyzed water, *Salmonella* Typhimurium, decontamination.

INTRODUCTION

Salmonella infection is a major cause of gastroenteritis in humans (salmonellosis) worldwide and is often associated with consumption of raw or undercooked poultry (1). A large number of *Salmonella* serotypes have been associated with poultry meat and the top 4 serotypes are Enteritidis, Typhimurium, Newport and Javiana (2). Extensive experience, research and field trials have identified a diversity of management and intervention strategies for the reduction and, potentially, elimination of enteropathogens, such as *Salmonella* from poultry (3). Organic acids, chlorinated compounds, trisodium phosphate, heat, steam or hot water are generally recognized as safe (GRAS) interventions and are used extensively by the meat and poultry industries to reduce bacterial contamination on carcass surfaces (4). EW is currently gaining popularity as a sanitizer in the food industry on foods and processing surfaces (5). EW water was initially developed in Japan and it has been reported to have strong bactericidal effects on most pathogenic bacteria that are important to food safety (6). Generation of EW, in general, involves reactions in a cell containing inert positively charged (anode) and negatively charged (cathode) electrodes, respectively, separated by a membrane, and through which a dilute salt solution passes. By subjecting the electrodes to direct current voltage, negatively charged ions such as hydroxide and chloride in the salt solution move to the anode to give up electrons and become oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid and hydrochloric acid, while positively charged ions such as hydrogen and sodium move to the cathode to take up electrons and become hydrogen gas and sodium hydroxide. As a result, two types of water possessing different characteristics are generated. An electrolyzed basic solution [pH>11 and oxidation–reduction potential (ORP) < -800 mV] is produced from the cathode side, which has strong reducing potential and may be used as a cleaning solution. An electrolyzed acid solution (pH<2.7 and ORP>1100 mV and presence of hypochlorous acid) is produced from the anode side, which has strong oxidation potential and bactericidal effect and can be used as a disinfectant (7). The major advantages of using EW are less adverse environmental impacts and without the difficulties of transporting and storing potentially hazardous chemicals. It is safe for staff, non-irritating, has minimal toxicity and low cost (8). The only disadvantages are non-stable and its bacterial effect decreased in the presence of organic matter (9).

This study was designed to evaluate the effectiveness of EW for inhibiting *S.* Typhimurium and its potential application in reducing *S.* Typhimurium on chicken wings. The other objective of this study was to determine the efficacy of EW during the shelf –life.

MATERIALS AND METHODS

Bacterial cultures

Salmonella Typhimurium ATCC 14028 and a wild type *Salmonella* Typhimurium were used for this study (obtained from Veterinary Control Central Research Institute Etlik – Ankara). Each strain was grown on Brillant-green phenol-red lactose sucrose (BPLS, Merck VM331547 140) agar at 37 °C for 24 h under an aerobic condition for counting the microbial load. After counting the microbial load, cultures were incubated at 37 °C for 24 hours in Brian Heart Infusion Broth (OXOID, CM0225). Then, each culture was diluted in sterile peptone water (OXOID, CM009) to obtain inoculums containing 10^5 cfu/ml bacteria.

Electrolyzed water

Commercial products which have different chlorine concentration, pH and ORP were used in the study. The

chlorine concentration, pH and ORP of electrolyzed waters were, 30 ppm, 5.0, 925 mV; 50 ppm, 2.6, 1076 mV; 70 ppm, 2.2, 1100 mV, respectively.

Samples and inoculation

60 chicken wings were obtained from a local slaughterhouse and transported to the laboratory inside the coolers. For all experiments chinken wings surfaces were treated with UV light, surfaces were exposed evenly by turning every 10 min for up to 30 min. 0.1 ml of each culture, containing approximately 5 $\log_{10}$ CFU/ml, was inoculated onto UV-treated chicken wings external surfaces under a biological safety hood. Bacterial cultures were allowed to attached to chicken wings surfaces for 20 min at room temperature, prior to any treatments. Using this procedure approximately 3 $\log_{10}$ CFU/ml of *S.* Typhimurium ATCC 14028 and 4 $\log_{10}$ CFU/ml of wild type *S.* Typhimurium were obtained on chicken wings surfaces, respectively.

Treatment and bacteriological analysis of chicken samples

Inoculated chicken wings were placed individually in sterile bags containing 500 ml of three different electrolyzed water and shaken gently at room temperature for 15, 30 and 60 seconds. At the end of the treatment, each sample was placed immediately into 100 ml of sterile buffered peptone water (MERCK, VM323428 134) and rubbed gently with hands from the outside of the sterile bag for 2 min. Buffered peptone water were assayed through serially diluting in 9 ml of sterile peptone water and then directly plating 0.05 ml of each dilution in duplicate on BPLS and Xylose Lysine Desoxycholate Medium (XLD, OXOID, CM0469) and incubated at 37 °C for 24 h before counting (10).

Shelf-life study

Experimentally inoculated chicken wings were dipped in 500 ml of three different electrolyzed water at room temperature for 15, 30 and 60 seconds and stored at 7 °C in sterile bags and sampled at days 3 and 7. Sampling and microbiological analyses were performed as described above.

Statistical analysis

Data were analyzed by the General Linear Model procedure of the SPSS program for determine the effect of different chlorine concentration, time and days. Days were compared by ANOVA using Duncan's multiple range test to determine the significant differences.

Results

Table 1 and Table 2 shows the logarithmic microbial counts for *S.* Typhimurium ATCC 14028 and wild type *S.* Typhimurium, respectively. When numbers of bacteria recovered from control and EW dipped chicken wings were compared, only dipping with 70 ppm EW for 60 seconds reduced levels of *S.* Typhimurium ATCC 14028 1 $\log_{10}$ CFU/ml. However, for wild type *S.* Typhimurium, except dipping with 30 ppm EW for 15 seconds, in all treatments reduction level was found 2 $\log_{10}$ CFU/ml. Statistical analysis showed that there was no significant difference between chlorine concentration and time for all treatments (P □ 0.05). This study also investigated the efficacy of EW during the shelf –life. The results showed that after the day 0 all of the EW's lost their efficacy (Table 1 and Table 2).

Data collected during the present study demonstrate that on the day 0, dipping chicken wings with EW is effective for reducing the number of bacteria. Moreover, the results show that, EW is not stable and losing its efficacy on day 3 and 7.

Table 1. *The logarithmic microbial counts for S. Typhimurium ATCC 14028 on day 0, 3 and 7.*

DAYS	TREATMENT	TIME		
		15 sec	30 sec	60 sec
Day 0[A]	**EW 1**	3 $\log_{10}$	3 $\log_{10}$	3 $\log_{10}$
	EW 2	3 $\log_{10}$	3 $\log_{10}$	3 $\log_{10}$
	EW 3	3 $\log_{10}$	3 $\log_{10}$	2 $\log_{10}$
Day 3[B]	**EW 1**	4 $\log_{10}$	4 $\log_{10}$	4 $\log_{10}$
	EW 2	4 $\log_{10}$	4 $\log_{10}$	3 $\log_{10}$
	EW 3	4 $\log_{10}$	4 $\log_{10}$	5 $\log_{10}$
Day 7[C]	**EW 1**	6 $\log_{10}$	6 $\log_{10}$	6 $\log_{10}$
	EW 2	5 $\log_{10}$	6 $\log_{10}$	5 $\log_{10}$
	EW 3	5 $\log_{10}$	5 $\log_{10}$	5 $\log_{10}$

EW 1: *30 ppm chlorine concentration, pH 5.0, ORP 925 mV;* ***EW 2:*** *50 ppm chlorine concentration, p H 2.6, ORP 1076 mV;* ***EW 3:*** *70 ppm chlorine concentration, pH 2.2, ORP 1100 mV.*

A*: Microbial count for S. Typhimurium ATCC 14028 in control group is 3 $\log_{10}$ CFU/ml,* ***B:*** *Microbial count for S. Typhimurium ATCC 14028 in control group is 4 $\log_{10}$ CFU/ml,* ***C:*** *Microbial count for S. Typhimurium ATCC 14028 in control group is 6 $\log_{10}$ CFU/ml.*

Table 2. *The logarithmic microbial counts for wild type S. Typhimurium on day 0, 3 and 7.*

DAYS	TREATMENT	TIME		
		15 sec	30 sec	60 sec
Day 0[A]	EW 1	3 $\log_{10}$	2 $\log_{10}$	2 $\log_{10}$
	EW 2	2 $\log_{10}$	2 $\log_{10}$	2 $\log_{10}$
	EW 3	2 $\log_{10}$	2 $\log_{10}$	2 $\log_{10}$
Day 3[B]	EW 1	4 $\log_{10}$	4 $\log_{10}$	4 $\log_{10}$
	EW 2	4 $\log_{10}$	4 $\log_{10}$	4 $\log_{10}$
	EW 3	4 $\log_{10}$	4 $\log_{10}$	4 $\log_{10}$
Day 7[C]	EW 1	6 $\log_{10}$	6 $\log_{10}$	6 $\log_{10}$
	EW 2	5 $\log_{10}$	5 $\log_{10}$	5 $\log_{10}$
	EW 3	5 $\log_{10}$	5 $\log_{10}$	5 $\log_{10}$

EW 1: *30 ppm chlorine concentration, pH 5.0, ORP 925 mV;* ***EW 2:*** *50 ppm chlorine concentration, p H 2.6, ORP 1076 mV;* ***EW 3:*** *70 ppm chlorine concentration, pH 2.2, ORP 1100 mV.*

A: *Microbial count for wild type S. Typhimurium in control group is 4 $\log_{10}$ CFU/ml,* ***B:*** *Microbial count for wild type S. Typhimurium in control group is 4 $\log_{10}$ CFU/ml,* ***C:*** *Microbial count for wild type S. Typhimurium in control group is 6 $\log_{10}$ CFU/ml.*

REFERENCES

1. Vandeplas, S., Dubois-Dauphin, R., Beckers, Y., Thonart, P., The´Wis, A. (2010). Salmonella in chicken: current and developing strategies to reduce contamination at farm level. *J. Food Prot.*, **73** (**4**): 774–785.
2. Centers for Disease Control and Prevention (2009). Summary of notifiable diseases United States: National Enteric Disease Surveillance: *Salmonella* Annual Summary, **58** (**53**): 1-104.
3. Cox, J. M., Pavic, A. (2009). Advances in enteropathogen control in poultry production. *J. Appl. Microbiol.,* **108**: 745–755.
4. Fabrizio, K. A., Sharma, R. R., Demirci, A., Cutter, C. N. (2002). Comparison of electrolyzed oxidizing water with various antimicrobial interventions to reduce *Salmonella* species on poultry. *Poult. Sci*., **81**:1598–1605.
5. Loretz, M., Stephan, R., Zweifel, C. (2010). Antimicrobial activity of decontamination treatments for poultry carcasses: a literature survey. *Food Control*, **21**: 791–804.
6. Huang, Y. R., Hung, Y. C., Hsu, S. Y., Huang, Y. W., Hwang, D. F. (2008). Application of electrolyzed water in the food industry. *Food Control*, **19**: 329–345.
7. Hsu, S. Y. (2005). Effects of flow rate, temperature and salt concentration on chemical and physical properties of electrolyzed oxidizing water. *J. Food Eng.*, **66**: 171–176.
8. Liao, L. B., Chen, W. M., Xiao, X. M. (2007). The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. *J. Food Eng.,* **78**: 1326–1332.
9. Rey, J. F., Kruse, A. (2003). ESGE/ESGENA Technical Note on Cleaning and Disinfection. *Endosc.,* **35**: 869–877.
10. Park, H., Hung, Y. C., Bracket, R. E. (2002). Antimicrobialeffect of electrolyzed water for inactivating *Campylobacter jejuni* during poultry washing. I*nt. J. Food Microbiol., 7***2(1–2)** : 77–83.

ИНАКТИВАЦИЈА НА *SALMONELLA TYPHIMURIUM*НА МЕСО ОД ЖИВИНА СО ЕЛЕКТРОЛИЗИРАНА ВОДА

Чил Ипликчоуглу Гузин[1], Демирел Јагур Нил[1], Ширели Уфук Тансел[1]

[1]Катедра за Хигиена и Технологија на Храна, Анкарски Универзитет, Факултет за Ветеринарна Медицина, Анкара, Турција

АПСТРАКТ

*Salmonella*е најчестиот причинител на труење од храна кај луѓето. Главен извор за овие труења се јајцата и живината. Еден од најчесто изолираните серотипови од живината е *S*. Typhimurium.Од страна на месната индустрија и индустријата за живина екстензивно се користат голем број на интервенции за да се намали бактериската контаминација. Електролизираната вода (EW) вопоследновреме е многупопуларнакакосредствозасанитацијавоиндустријатазахрана. Целтанаоваастудијабешедасе одредиефикасностана EW во инактивацијата на *Salmonella*Typhimuriumво пилешко месо и да се следи ефектот за време на рокот на траење. Пилешки крилца беа инокулирани со два типа на *Salmonella*Typhimurium, ATCC 14028 и див сој. Инокулираните примероци беа потопени во EW вокои имаше три различни концентрации на хлор, 30, 60 и 70 ppm, во време од 15, 30 и 60 секунди. Пилешките крилца беа испитани на ден 0 за да се утврди антимикробниот ефект, а другите примероци беа чувани 3 и 7 дена на 7°C за да се следи ефектот врз рокот на траење. EW ја намали *Salmonella*Typhimuriumза приближно 2 log CFU/ml на ден 0. Меѓутоа, не се воочи намалување на бројотпо 7 дена. Немаше разлика меѓу 30, 60 и 70 ppm хлор во EW, и 15, 30 и 60 секунди.

Клучни зборови:електролизирана вода, *Salmonella*Typhimurium, деконтаминација

UDC: 663.2:579.64

FLUOROMETRIC VALIDATION PROCEDURE FOR DETERMINATION OF OCHRATIOXIN A IN WINE

Stojanovska-Dimzoska Biljana[1], Hajrulai-Musliu Zehra[1], Dimitrieska-Stojković- Elizabeta[1], Uzunov Risto[1], Todorovic Aleksandra[1], Sekulovski Pavle[1]

[1]*Food Institute, Faculty of Veterinary Medicine, University "Sts. Cyril and Methodius", Skopje, R. Macedonia*

*Corresponding author: bsdimzoska@fvm.ukim.edu.mk

ABSTRACT

Fluorometry with previous immunoaffinity column clean-up is a method for determination of ochratoxin A in wine which is validate in order to evaluate its performances. The linearity of the method was checked, and a good coefficient of correlation (0,9814) was found. The limit of detection was satisfactory (0,199 ng/ml). Repeatability, as measurement of the precision, estimated through RSD values showed acceptable value only for the concentration level of 0,1 ng/ml (6,47%), but too high for concentration level of 1,0 ng/ml (17,86%). This is a big deviation from true value, especially when red wine samples are analysed. It is a due to the red colour present in the final eluat, which gives high readouts and it can produce false positive results when fluorometry was applied. However, due to the factors that influence fluorometric analysis, it was found that it presents relatively accurate, precise and selective, quantitative method in the field of determination of ochratoxin A in wine. Fluorometry can be applicable only as a screening method for the prediction of ochratoxin A contamination in wine, especially in the laboratories who are dealing with a big number of samples for mycotoxins analysis.

Keywords: method validation, ochratoxin A, immunoaffinity columns, fluorometry, wine.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin mainly produced by some species of the genera *Aspergillus* and *Penicillium*. It might contaminate agriculture commodities (cereals and cereals products, wine, beer, grape juice, coffee, cocoa and cocoa products). OTA receives increasing attention as there is growing evidence that this mycotoxin might be responsible not only for intoxication in livestock after consumption of contaminated food and feed, but may also be involved in the etiology of Balkan endemic nephropathy (1). OTA is nephrotoxin to all animal species (kidney is the most sensitive target organ). It also exerts immunotoxic, teratogenic, genotoxic, mutagenic and carcinogenic effects at higher dose levels (2); therefore, presents serious risks for the human health. The International Agency for Research on Cancer (IARC) evaluated OTA as a possible carcinogen in humans (group 2B). The intake of different contaminated food and drinks might provide a total amount of OTA near 100 ng per kg body weight that presents a PTWI (provisional tolerable weekly intake) set by the World Health Organization (3). A maximum residual level (MRL) for OTA has been established by the Commission Regulation (EC) No. 123/2005 and it is in the range from 0,5 to 10 μg/ kg for different commodities. Our country has adopted the EU regulations since December 2005 (4).

Wine OTA contamination has been reported all over the world (5) considered the fact that it is the second major source of OTA intake (13 %). Wine is a product significantly important for the European economy and population and therefore it requires from each member or EU exporter country to carry out systematic surveys to assure that the wine is OTA-free and safe.

Different analytical methodologies have been established for OTA determination (6). Fluorometry is an analytical method for determination of ochratoxin A and mycotoxins in general, which has no such selectivity, accuracy and sensitivity as LC-FD, but it can be used as a screening method, especially in the laboratories who are dealing with a big number of samples for mycotoxin analysis. The use of immunoaffinity columns (IAC) for the clean-up procedure is highly recommended, allowing the isolation of the analyte from most matrix interferences, due to its selectivity.

The aim of this work was to evaluate the method performances for the fluorometry and to carry out the method validation. Those parameters would be used to set a fluorometry as a screening method for prediction of ochratoxin A in wine. Then, thirty wine samples were analysed with this method.

MATERIALS AND METHODS

Apparatus

The fluorometer (Vicam V1, series 4) was delivered from Vicam (Watertown, MA, USA). It is necessary to check purity of chemicals before employing the analysis. One (1) ml of water should give zero fluorescence (blank sample) and 1 ml OchraTest™ Eluting Solution should also give zero value for fluorescence. In this manner the quality of the cuvettes was also checked. Fluorometric calibration was done according to the manufacturer (7) with mycotoxins calibration standards: red calibration standard with maximum value of 36 ng/ml and green

calibration standard with minimum value of -3 ng/ml. The performed calibration was checked with yellow calibration standard which readings should be in the limited range.

Reagent and standard solutions

HPLC grade solvents (methanol, acetonitrile, water) benzene, NaCl, NaOH, $NaHCO_3$ (pro-analysis grade chemicals) and glacial acetic acid 100% (suprapur) were delivered from Merck (Darmstadt, Germany). PEG 8000 was purchased from Biochemica, Fluka. OchraTest™ Eluting Solution was from Vicam (Watertown, MA, USA).

Immunoaffiity OchraTest® columns (Vicam, USA) were used for clean-up procedure. OTA standard was purchased from Supelco (50 µg/ml, dissolved in benzene:acetic acid (99:1)). A stock solution (2 µg/ml) was prepared from this solution by diluting an aliquot with solvent mixture and was further kept at + 4°C. 1,5 ml of stock solution was transferred into a silanized vial and evaporated under a stream of nitrogen. The content was redissolved in a vial with 1,5 ml LC mobile phase (filtered through a 0,20 µm filter) and quantitatively transferred into a volumetric flask of 25 ml and diluted to volume with the filtered mobile phase. The final OTA concentration in this solution was 100 ng/ml. According to the official AOAC method (8), five working standards in a range from 0 to 1,0 ng OTA/ml were prepared and used for calibration.

Wine samples

The samples were originated from wine producing region in Macedonia and were purchased from a local store. The samples were kept sealed in the refrigerator at + 4°C, in their original bottles until analyses. All samples were analyzed to find OTA level and they were run in duplicate. For the recovery experiment, OTA-free red and white wine samples were spiked with known amount of OTA solutions at two levels (0,1 ng/ml and 1,0 ng/ml).

Analytical procedure

All wine samples were always degasified in ultrasonic bath for 20 min before treatment. 10 ml of wine was diluted and mixed vigorously with a solution containing 5 % $NaHCO_3$ and 1 % PEG 8000. The pH was adjusted to 8,5 with 1 M NaOH. Then, solution was filtered through glass microfiber filter and 10 ml of filtrate were applied onto an IAC (OchraTest™ column) at a flow rate of about 1 drop/sec. The washing step was performed with 5 ml of washing solution (2,5 % NaCl and 0,5 % $NaHCO_3$) and then with 5 ml water at a flow rate of 1-2 drops/sec. The column was dried by passing air through it, and afterwards, OTA was eluted with 2 ml of OchraTest™ Eluting Solution at a flow rate of about 1drop/sec in a glass cuvette. Reading of ochratoxin A concentration was after 60 sec. The OchraTest™ Eluting Solution contain 0,1 N NaOH instead of methanol, because this solution increase the signal of fluorometer in its operating range (excitation 360 nm and emission 450 nm).

RESULTS AND DISCUSSION

Method validation procedure was performed according to the manufacturer instruction (7). The range of the fluorometer was determined by spiking OTA-free (LC-FD determined) white wine samples at concentrations of 0; 0,1; 0,2; 0,5 and 1,0 ng/ml. Each sample was run in duplicate. The results are presented in the Table 1. We used lower concentration range because maximum measurement level of the fluorometer was 36 ng/ml. The choice of using white wine samples was made concerning the fact that red wine samples have complex matrix and the red colour was present in the final eluat (gives high readouts and false positive result).

Table 1. *Range of the method*

spiked concentration (ng/ml)	detected concentration (ng/ml)	mean concentration (ng/ml)
0	0 0	0
0,1	0,164 0,296	0.23
0,2	0,396 0,52	0.458
0,5	0,44 1,28	0.86
1,0	2,28 2,28	2.28

Linearity of the method was checked performing calibration curve as correlation between spiked and detected concentrations. The correlation coefficient was 0,9814 which means a good and satisfactory linearity, but as can be seen from the Table 1, there is a big deviation from a true value, probably as a result of others interfering fluorescence substances.

For this study limit of detection (LOD) was defined as follows: LOD = mean + 3 SD, where the mean is determined from readouts of ten (10) OTA-free samples (determined by LC-FD) and SD is the standard deviation of those 10 readouts. LOD using this protocol was 0,199 ng/ml.

Repeatability, as measurement of the precision, estimated through RSD values was determined using spiked OTA-free white wine samples with two concentration level (0,1 and 1,0 ng/ml). Each sample was tested six (6) times. The mean, standard deviation and RSD were determined and they are shown in the Table 2.

As can be seen from the Table 2, there is a big deviation from true values as in the case of range determination. Especially high percent of RSD was found for concentration of 1,0 ng/ml which is 17,86 %.

Thirty wine samples were examined employing fluorometry and compared with LC-FD in order to make comparison between two methods. All wine samples were with OTA concentration level bellow LOD, but there are differences between results obtained from bought methods. Those differences are coming especially at red wine samples. It is due to the red colour present in the final eluat, which gives high readouts and it can produce false positive result when fluorometry was performed (9).

CONCLUSIONS

As we can see from the results, fluorometry present relatively accurate, precise and selective quantitative method for determination of OTA in wine. The method show good coefficient of correlation (0,9814) and satisfactory limit of detection (0,199 ng/ml). The RSD value (as measurement of precision) was satisfactory only for the concentration level of 0,1 ng/ml (6,47 %), but too high for concentration level of 1,0 ng/ml (17,86 %). The other disadvantage was high readouts and false positive results when red wine samples were analysed. On the other hand the method is safe, simple, easily performed in less than 10 minutes and requires no special skills and there is no need for expensive instrumentation.

For those reasons, immunoaffinity column clean-up followed by fluorometric determination can be used only as a screening method for a prediction of ochratoxin A contamination in wine and other methods (LC-FD) should be applied as method of choice for the determination of ochratoxin A in wine.

Table 2. *Repeatability of the method*

number of samples	**0,1 ng/ml**	**1,0 ng/ml**
	detected concentration (ng/ml)	
1	0,22	2,28
2	0,208	3,36
3	0,208	2,64
4	0,216	3,0
5	0,2	3,4
6	0,24	2,28
mean	**0,215**	**2,82**
STD	**0,013**	**0,505**
RSD (%)	**6,47 %**	**17,86 %**

REFERENCES

1. Creppy E.E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 127, 19-28.
2. FAO FOOD AND NUTRITION PAPER. (1990). Manuals of food quality control. Training in mycotoxins analysis. No. 14/10. Rome
3. FAO/WHO (Food and Agriculture Organization/World Health Organization) (1996). Toxicological evaluation of certain food additives and contaminants. Joint FAO/WHO Expert Committee on Food Additives (JECFA). WHO Food Additives Series 35. Geneva, Switzerland.
4. Regulation for general requirements in food safety (2005). Official Gazette in R. of Macedonia No. 118/2005.
5. Pacin A., Rasnik, S., Vega, M., Saelzer, R., Ciancio Bovier. E., Rios, G., and Marinez, N. (2005). Occurrence of ochratoxin A in wines in the Argentinian and Chilean mar-

kets. ARKIVOC (XII) 214-223.
6. N.Ratola, P.Barros, T. Simões, A.Cerdeira, A.Venâncio, A.Aves. (2006). Worldwide interlaboratory study on the determination of ochratoxin A in different wine type samples. Talanta 70, p. 720-731.
7. OchraTest Instruction Manual, (1999). Vicam (Watertown, MA, USA).
8. AOAC Official Methods of Analysis (2005). Chapter 49, p.66. AOAC Official Method 2001.01. Ochratoxin A in wine and beer. Immunoaffinity colum clean-up/liquid chromatographic analysis (First action 2001, Final action 2005).
9. Biljana Stojanovska-Dimzoska, Zehra Hajrulai-Musliu, Elizabeta Dimitrieska-Stojkovic, Pavle Sekulovski. (2010). Comparison of two different analytical methods for determination of ochratoxin A in wine. 32nd Mycotoxin Workshop, Demark, poster presentation p.25

ФЛУОРОМЕТРИСКА ВАЛИДАЦИОНА ПРОЦЕДУРА ЗА ОПРЕДЕЛУВАЊЕ НА ОХРАТОКСИН А ВО ВИНО

Стојановска-Димзоска Билјана[1], Хајрулаи-Муслиу Зехра[1], Димитриеска-Стојковиќ Елизабета[1], Узунов Ристо[1], Тодоровиќ Александра[1], Секуловски Павле[1]

[1]Институт за храна, Факултет за ветеринарна медицина - Скопје, Универзитет "Св. Кирил и Методиј ", Скопје, Република Македонија

*Автор за коресподенција: bsdimzoska@fvm.ukim.edu.mk

АПСТРАКТ

Флуорометријата со претходно пречистување со примена на имуноафинитетни колони е користен метод за определување на охратоксин А во вино, валидиран со цел да се евалуираат неговите перформанси. Линеарноста на методот е проверена и утврдена е добра вредност на коефициентот на корелација (0,9814). Лимитот на детекција беша задоволителен (0,199 ng/mL). Повторливоста, како мерка за прецизноста, е проценета преку RSD, чии вредности беа прифатливи само за концентрациското ниво од 0,1 ng/mL (6,74 %), но беа премногу високи за ниво на концентрации од 1,0 ng/mL (17,86 %). Ова е значително отстапување од средните вредности, особено за анализа на примероци од црвено вино. Тоа е заради присутната црвена боја во конечниот елуат, кој дава високи отчитувања а со тоа и лажно позитивни резултати при примена на флуорометријата. Меѓутоа и покрај факторите кои влијаат на флуорометриското определување, утврдено е дека таа претставува релативно точен, прецизен и селективен метод за определување на охратоксин А во вино. Флуорометријата може да биде применлива само како скрининг метод за проценка на контаминацијата на вино со охратоксин А, особено за лаборатории кои имаат голем број на примероци за анализа на микотоксини.

Клучни зборови: валидација на метод, охратоксин А, имуноафинитетни колони, флуориметрија, вино.

UDC: 636.52/.58.09:[579.835.12.017.4:615.33
636.52/.58.09:[615.33.015.8:579.835.12

ANTIBIOTIC RESISTANCE OF *CAMPYLOBACTER* STRAINS ISOLATED FROM BROILERS IN MACEDONIA

Angelovski Ljupco, Sekulovski Pavle, Jankuloski Dean,
Ratkova Marija, Prodanov Mirko, Mojsova Sandra

Food Institute, Faculty of veterinary medicine
University of "Ss. Cyril and Methodius" in Skopje

angelovski@fvm.ukim.edu.mk

ABSTRACT

In this study 176 isolates of *Campylobacter jejuni* and 69 isolates of *Campylobacter coli* detected in samples from broiler flocks were tested for their antimicrobial susceptibility. The antimicrobial resistance of each isolate was determined for the following antimicrobial agents: erythromycin, ciprofloxacin, tetracycline, streptomycin and gentamicin. The parameters used for the determination of the antimicrobial resistance are given in the *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A4* (Clinical and Laboratory Standards Institute, USA).

The results of the testing showed that *C. jejuni* isolates expressed highest antimicrobial resistance towards ciprofloxacine (42,4% of resistant isolates) and tetracycline (39,77%). Much lower antimicrobial resistance was detected towards erythromycin (5,11%), streptomycin (3,4%) and gentamicin (1,7%). Similar conclusions were made after the analysis of the *C. coli* isolates. Highest resistance was detected towards ciprofloxacin (62,3%) and tetracycline (57,97%). Only 12 isolates of *C. coli* (17,39%) showed resistance to streptomycin, while the resistance detected towards erythromycin (11,59%) and gentamicin (1,44%) was similar to the resistance detected in *C. jejuni* isolates.

Overall, higher antimicrobial resistance was detected to quinolones and tetracyclines and lower resistance towards macrolides and aminoglycosides. Observing the results it can be noticed that *C. jejuni* and *C. coli* showed low resistance to erythromycin and gentamicin. The results emphasize the higher resistance of *C. coli* versus *C. jejuni* to ciprofloxacin, tetracycline, streptomycin and erythromycin.

Key words: broiler flocks, *Campylobacter jejuni, Campylobacter coli*, antimicrobial resistance

INTRODUCTION

Campylobacteriosis is a significant public health problem in many developed countries. In Europe alone more than 200,000 confirmed cases were reported to the European Food Safety Authority in 2007 (1). Inadequately cooked meat, particularly poultry, unpasteurized milk and contaminated drinking water are the most common sources for epidemic and sporadic food borne cases (2, 3). Poultry products are known as an important source of *Campylobacter* spp. At the age of two to three weeks, 50-90% of the poultry in the flock is colonized with termophilic *Campylobacter* spp. (4). Two Campylobacter species, *Campylobacter jejuni* and *Campylobacter coli*, are responsible for the majority of human infections, among which 80–90% are due to *C. jejuni* (5).

Most Campylobacter infections are self-limiting (3-5 days), but in very young individuals, the elderly persons and people with chronic diseases prolonged or severe campylobacteriosis can occur (6). For treating such Campylobacter infections, fluoroquinolones (e.g., ciprofloxacin) and macrolides (e.g., erythromycin) are the drugs of choice (Skirrow et al., 2000). There is growing concern that the use of antibiotics in food animals can lead to the development of resistant pathogenic bacteria that can affect humans through the food chain. In this study the antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli* isolated from broiler flocks in Macedonia was examined.

MATERIALS AND METHODS

Specimen isolation

All of the analyzed isolates of *C. jejuni* (n = 176) and *C. coli* (n = 69) were detected in samples taken from broiler flocks on farm and at the slaughterhouse (caecal swabs, chicken faeces, caeca, carcass swabs).

The samples were enriched in Preston selective broth (Fluka) and incubated microaerophilically on 42°C for 24 hours (7).

The enrichment was streaked on modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) and incubated under microaerobic conditions at 37°C for 48 h.

Identification of isolates

Isolates were identified using standard parameters including Gram staining, oxidase and catalase testing, temperature tolerance, morphology, indoxyl acetate test, hippurate hydrolysis, growth and production of H_2S on triple sugar iron agar.

Testing of the antimicrobial resistance

For the antimicrobial susceptibility testing of the isolates disk diffusion method (Kirby Bauer method) was used. The inoculums were prepared with density adjusted to 0,5 McFarland turbidity standard. The inoculum was delivered with sterile swabs on Columbia blood agar (Biomerieux) and the following antimicrobial

agents were used: erythromycin, ciprofloxacin, tetracycline, streptomycin, gentamicin (Oxoid). The following CLSI breakpoints (8) were used for the classification of the isolates:

Table 1. *CLSI (former NCCLS) breakpoints used for determination of the antimicrobial resistance of C. jejuni and C. coli*

	Antimicrobial agent	concentration (µg)	Zone of inhibition (mm)		
			resistant	intermediate	susceptible
1.	Ciprofloxacin	5	≤15	16-20	21≥
2.	Erythromycin	15	≤13	14-22	23≥
3.	Gentamicin	10	≤12	13-14	15≥
4.	Tetracycline	30	≤14	15-18	19≥
5.	Streptomycin	10	≤12	13-14	15≥

RESULTS

The results of antimicrobial susceptibility testing for *C. coli* and *C. jejuni* are shown in Table 2 and 3.

The results of the the tests identify that *C. jejuni* isolates expressed highest antimicrobial resistance towards ciprofloxacin (42,4% of resistant isolates) and tetracycline (39,77%). Much lower antimicrobial resistance was detected towards erythromycin (5,11%), streptomycin (3,4%) and gentamicin (1,7%). Similar conclusions were made after the analysis with the *C. coli* isolates. Highest resistance was detected towards ciprofloxacin (62,3%) and tetracycline (57,97%). Only 12 isolates of *C. coli* (17,39%) showed resistance to streptomycin, while the resistance detected towards erythromycin (11,59%) and gentamicin (1,44%) was similar to the resistance detected in *C. jejuni* isolates.

DISCUSSION

This study is the first to highlight the importance of antibiotic susceptibility of *Campylobacter* spp. isolated from broilers in Macedonia. The principal purpose of monitoring antimicrobial resistance trends in enteric pathogens is to provide clinicians with data that can be used to select appropriate treatment regimens. Surveillance should emphasize antibiotics that are being used routinely to treat diarrhea, as well as any alternatives, such as fluoroquinolones, macrolides, and gentamicin. Equally important is the accessibility of the data to those providing primary care.

However, as everywhere else in the world, the increase of Campylobacter-resistant strains seems to be related to the amounts of antibiotics used in animals (9). Thus, to prevent transfer of resistant bacteria or resistance genes from animals to humans via the food chain (10), measures that should be implemented are: reduction of the use of antibiotics, encourage narrow-spectrum specific antibiotic therapy instead of broad spectrum an-

Table 2. *Antimicrobial susceptibility of C. jejuni isolates*

Antimicrobial used	Erythromycin	Ciprofloxacin	Tetracycline	Streptomycin	Gentamicin
Susceptibility	R	R	R	R	R
Total No. of isolates = 176	9 (5,11%)	75 (42,6%)	70 (39,7%)	6 (3,4%)	3 (1,7%)

Table 3. *Antimicrobial susceptibility of C. coli isolates*

Antimicrobial used	Erythromycin	Ciprofloxacin	Tetracycline	Streptomycin	Gentamicin
Susceptibility	**R**	**R**	**R**	**R**	**R**
Total No. of isolates = 69	8 (11,59%)	43 (62,31%)	40 (57,97%)	12 (17,39%)	1 (1,44%)

timicrobials (11), and replacement of antibiotics with improvements in hygiene and flock management. Consequently, it would be useful to set up an surveillance network to monitor antimicrobial resistance in bacteria of animal origin likewise the countries in the European Union.

REFERENCES

1. EFSA (2010) Trends and sources of zoonoses and zoonotic agents and food-borne outbreaks in the European Union in 2008.
2. Butzler, J.P., Oosterom, J. (1991) Campylobacter: pathogenicity and significance in foods. Int. J. Food Microbiol., 12:1-8.
3. Altekruse, S.F., Stern, N.J., Fields, P.I., Swerdlow, D.L. (1999) Campylobacter jejuni - an emerging foodborne pathogen. Emerg. Infect. Dis.,5:28-35.
4. Newel, G.D. (2002) The ecology of Campylobacter jejuni in avian and human hosts and in the environment. Int. J. Infect. Dis., 6:3516-3521.
5. Nachamkin, I., Engberg, J. and Aarestrup, F.M. (2000) Diagnosis and antimicrobial susceptibility of Campylobacter spp. In: Campylobacter, 2nd edition. Nachamkin I and Blaser MJ (eds.). Washington, DC: ASM Press, pp. 45–66.
6. Skirrow, M.B. and Blaser, M.J. (2000) Clinical aspects of Campylobacter infection. In: Campylobacter, 2nd edition. Nachamkin I and Blaser MJ (eds.). Washington, DC: ASM Press, pp. 69–88.
7. ISO/CD 10272-1 (2006) Microbiology of food and animal feeding stuffs. Horizontal method for detection and enumeration of Campylobacter spp. part 1: Detection method. International Organisation for Standardisation (ISO), ISO Central Secretariat, 1 rue de Varembé, Case Postale 56, CH - 1211, Geneva 20, Switzerland.
8. National Committee for Clinical Laboratory Standards (NCCLS) (2000). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A4, National Committee for Clinical Laboratory Standards, Villanova, Pa.
9. Van den Bogaard, A.E., Stobberingh, E.E. (2000) Epidemiology of resistance to antibiotics. Links between animals and humans. Int. J. Antimicrob. Agents, 14:327-335.
10. Barton, M.D. (2000) Antibiotic use in animal feed and its impact on human health. Nutr. Res. Rev. 13:1-22
11. Barton, M.D. and Hart, W.S. (2001). Public health risks: antibiotic resistance. Asian-Aust. J. Anim. Sci. 14:414-422.
12. Van Looveren, M., Daube, G., De Zutter, L., Dumont, J.M., Lammens, C., Wijdooghe, M., Vandamme, P., Jouret, M., Cornelis, M., Goossens, H. (2001) Antimicrobial susceptibilities of Campylobacter strains isolated from food animals in Belgium. J. Antimicrob. Chemother. 48:235-240.
13. Padungton, P., and Kaneene, B.J. (2003) Campylobacter spp. in Human, Chickens, Pigs and Their Antimicrobial Resistance. J. Vet. Med. Sci. 65(2):161-170.

АНТИМИКРОБНА РЕЗИСТЕНЦИЈА НА *CAMPYLOBACTER* СОЕВИ ИЗОЛИРАНИ КАЈ БРОЈЛЕРИ ВО Р. МАКЕДОНИЈА

Ангеловски Љупчо, Секуловски Павле, Јанкулоски Деан, Раткова Марија, Проданов Мирко, Костова Сандра

Институт за храна, Факултет за ветеринарна медицина
Универзитет "Св. Кирил и Методиј" Скопје

angelovski@fvm.ukim.edu.mk

АПСТРАКТ

Во оваа студија беше испитувана антимикробната резистенција кај 176 изолати на *Campylobacter jejuni* и 69 изолати на *Campylobacter coli* детектирани во примероци од бројлерски јата. Кај секој изолат беше испитувана антимикробната резистенција на следните антибиотици: еритромицин, ципрофлоксацин, тетрациклин, стрептомицин и гентамицин. Параметрите кои се користеа за одредување на резистенцијата се дадени во *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A4* (Clinical and Laboratory Standards Institute, USA).

Резултатите добиени при анализите покажуваат дека изолатите на *C. jejuni* имаат највисока резистенција кон ципрофлоксацинот (42,4%) и тетрациклинот (39,77%). Многу пониска резистенција е утврдена кон еритромицинот (5,11%), стрептомицинот (3,4%) и гентамицинот (1,7%). Слични заклучоци се изведени и при анализите со изолатите на *C. coli.* Највисока резистенција беше утврдена кон ципрофлоксацинот (62,3%) и тетрациклинот (57,97%). Само 12 изолати на *C. coli* (17,39%) покажаа резистенција кон стрептомицинот додека детектираната резистенција кон еритромицинот (11,59%) и гентамицинот (1,44%) беше во слични граници како и кај изолатите на *C. jejuni.*

Генерално, повисока резистенција е евидентирана кон кинолоните и тетрациклините и пониска кон макролидните и аминогликозидните препарати. Сумирајќи ги резултатите се забележува дека *C. jejuni* и *C. coli* покажуваат ниска резистенција кон еритромицин и гентамицин. Исто така резултатите укажуваат и на повисока резистенција на *C. coli* наспроти *C. jejuni* во однос на ципрофлоксацинот, тетрациклинот, стрептомицинот и еритромицинот.

Клучни зборови: бројлерски јата, *Campylobacter jejuni, Campylobacter coli*, антимиробна резистенција

UDC: 637.5`65.065:"579.835.12

RISK FACTORS AFFECTING PRESENCE OF *CAMPYLOBACTER* SPP. IN POULTRY AND POULTRY MEAT

Vashin Ivan, Stoyanchev Todor

Department of Food Hygiene, Technology and Control, Faculty of Veterinary Medicine, Trakia University, Stara Zagora 6000, Bulgaria

ABSTRACT
Prevalence of both pathogen species *C. jejuni* and *C. coli* in stock live populations, especially in poultry farm, respectively, survival and dissemination during the slaughter processing is well recognized factor. This paper attempts to summarize the scientific observations on the presence of *Campylobacter* spp. at the farm and through the chain of poultry meat, including processing. Birds (sparrows, pigeons, doves and quail) living around the poultry farms are possible source of *Campylobacter* spp., as in pigeons the carriage is from 30 to 100%. Poultry (broilers and waterfowl) intended for slaughter are carriers of *Campylobacter* in almost 100%. Production of poultry meat and carcass skin are contaminated in varying ratio (usually up to 26.7%)
Key words: poultry meat, birds, cross contamination, *C. jejuni, C. coli*

INTRODUCTION

Campylobacter is recognized as a leading bacterial cause of gastrointestinal infections in humans, with over 3 million annuall cases. Campylobacter infections in humans are considered to be food-borne diseases, as in outbreaks (epidemiological) are relatively rare and their infection source are consumption of raw milk or untreated water. In sporadic infections source is the consumption of raw or inadequately heat-treated poultry meat (Neimann et al., 2003). Microorganisms of the genus Campylobacter are often detected in the broiler farms. The percentage of positive for *Campylobacter* spp. flocks varies considerably, with reports of 90% in Great Britain, 76% in England, 27% in Sweden, Germany - 27,9% (Stern et al., 1994; Jacobs-Reitsma, 1997; Ridsdale et al., 1999; Nachamkin et al., 2000). So far a wide range of different phenotypic and genotypic methods have been used for epidemiological study of *Campylobacter* infections. Typing by *fla-A* gene of *Campylobacter* species, is with better results than serotyping (Stern et al.,1997).

The aim of this paper is to summarize factors affecting the presence of *Campylobacter* spp. at the farm and through the chain of poultry meat, including processing, storage and marketing to the consumer.

MATERIAL AND METHODS

Test samples were collected from: wild birds living around the farm (pigeons, sparrows, pruning knives, quail), poultry intended for slaughter (poultry and ducks), technological equipment and chilled carcasses directly in the processing plant. Isolation and differentiation of microorganisms of the genus *Campylobacter* was carried out by horizontal method for detection and identification of *Campylobacter* spp. Incubation of samples was performed in enrichment broth (Merck, 1.08190) with antibiotic selective supplement (Merck, 1.02249) and on selective Campylobacter agar (Merck, 1.02248) containing antibiotic selective supplement (Merck, 1.02249). Samples were incubated in a microaerophilic conditions at 37°C and 42°C for 48 h. Obtained pure cultures were tested further for the production of oxidase, catalase, hippurat hydrolysis, indoxylacetat hydrolysis. Bacteria cell with specific colonial and biochemical characteristics of *Campylobacter* species were differentiated by API Campy® (BioMérieux,20800).

Molecular-biological analysis by PCR was performed with DNA extracted directly from intestinal contents (cecum content). DNA was isolated by Quant-iT ™ Assay Kit (InvitroGen, USA) for samples with high and low DNA concentration. For conventional PCR universal primers for *Campylobacter* spp. (Eurogentec, Belgium) and species-specific primers for *C. jejuni* and *C. coli* were used.

RESULTS AND DISCUSSION

Tested wild birds living in the region around the poultry farms are intestinal carriers of *Campylobacter* spp. (Table 1). Using the agar culture techniques we identified positive samples from Turtle Dove (10%) and pigeons (30%), whereas by PCR method we found carriers in Turtle Dove (23.1%) and additional positive samples in pigeons (100%). Species specific DNA for *C. jejuni* were detected in 22 samples and 2 for *C. coli*. There were no samples with presence of both *Campylobacter* species. *C. jejuni* dominate over all samples, while only one was positive for *C. coli*. Obtained results demonstrate that *fla-A* PCR technique is able to prove existence of *C. jejuni* and *C. coli* directly in fecal samples without preliminary incubation and cultivation. In similar studies in broiler chickens Whyte et al., (2001) found levels of contamination 6.04 - 6.61 log10CFU/g before transport to the slaughterhouse and 6.83-7.32 log10 after transport, respectively. Authors pointed out the advantage of PCR with universal primers for *Campylobacter* spp. and agarose gel electrophoresis and significantly higher sensitivity of the PCR reaction (3.55-3.96 log CFU/ml). Detected carriers of campylobacteria in wild birds indicates that they are possible vector and source of this microorganism in the broiler farms. Close contacts between wild birds and poultry can lead to cross-infection.

Table 1. *Carriers of Campylobacter spp. in intestine of wild birds living around poultry farms*

Samples	n	*Campylobacter* positive						
		Cultural method		PCR method				
		n	%	n / n_0	%	*C.jejuni*	*C.coli*	Other species
Wild quail	10	0	-	0/10	-	-	-	-
Sparrows	17	0	-	0/2	-	-	-	-
Turtles	13	0	-	3/13	23,1	3	-	-
Turtle Dove	10	1	10,0	0/2	-	-	-	-
Pigeons	10	3	30,0	10/10	100	8	1	1
Total		**4**	**6,7**	**13**	**35,1**	-	-	-

Poultry in the farms and after transport to the slaughterhouses are asymptomatic carriers of *Campylobacter* both in their digestive tract and on their feathers and skin surfaces (Table 2). Percentage of positive samples in poultry is 61,5% and 97,4% on the skin surface and in intestine, respectively. In contrast, ducks are with really lower initial contamination, and processing is not responsible for their additional contamination. Species *C. jejuni* (usually over 75%) is predominating *C. coli*, instead of poultry types.

Table 2*. Presence of Campylobacter spp. in broilers Poultry and ducks, before slaughter and after processing and chilling in the plant*

Samples	n	Poultry			Ducks		
		***Campylobacter* positive, (%)**	***C. jejuni* (%)**	***C. coli* (%)**	***Campylobacter* positive, (%)**	***C. jejuni* (%)**	***C. coli* (%)**
Skin surface (live birds)	85	61,5%	66,7%	33,3%	12,5%	100%	-
Cecum intestine	85	97,4%	84,2%	15,8%	72,5%	72,4%	27,6%
Carcass surface (after chilling)	85	71,8%	75,0%	18,4%	7,5%	100%	-
Total		**76,9%**	**75,3%**	**22,5%**	**30,8%**	**78,3%**	**21,6%**

Our data shows that 68.7% of analyzed samples in the slaughterhouse were Campylobacter positive (Table 3). Positive samples were detected in all technological points of slaughter process, and also in refrigerated poultry carcasses. Some processes can reduce the number of bacteria, including those of Campylobacter spp. (20,5% in scalding) but elimination can not be achieved completely. According to research by Slavik et al. (1994) scalding at 56°C water temperature is with greatest reduction effect, but the number of survived Campylobacter on the skin surface remains high (log10 3.39). Defeathering and evisceration are points with the highest contamination and can be considered as critical points for Campylobacter cross-contamination. Chilling resulted in partial reduction of bacterial contamination. Other researchers have shown that samples collected from the carcass internal cavity and from the skin after evisceration, as well from the working surfaces of evisceration equipment can have up to 100% positive samples (Berndtson et al., 1996).

Table 3*. Prevalence of Campylobacter spp. on carcasses during processing*

Point of slaughter process	Samples, (n)	*Campylobacter* positive (%)
Exanguination	90	64,1%
Scalding	90	20,5%
Defeathering	90	89,7%
Exenteration	90	97,4%
Chilling	90	71,8%
Total		68,7%

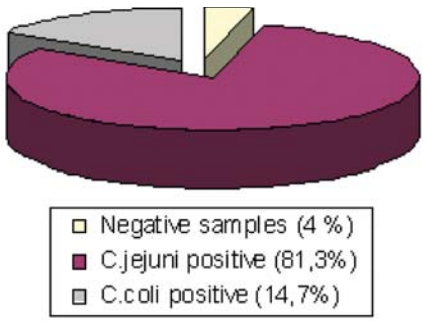


Figure.1. *Prevalence of Campylobacter spp. on hands of workers supporting evisceration*

Available manual handling and participation of workers in the slaughtering process are also a factor leading to cross contamination from carcass to carcass. In the present study the swab samples from the hands of workers were in 96% *Campylobacter* positive. As the most critical remains manual support in the process of evisceration (Figure 1).

In conclusion, we consider that wild birds, mostly pigeons are possible source for the microorganisms of the genus *Campylobacter* in the region of poultry farms. PCR method for molecular diagnosis is leading to shortening of diagnostic time and is suitable for fast assay without sample cultivation. *Campylobacter* cross-contamination occurs during slaughter processing and its prevention seems to be impossible. Major critical points are defeathering, and presence manual handling during evisceration and after carcass chilling. Slaughterhouses ensure conditions suitable for existence and survival of *Campylobacter* spp. through processing and in raw meat and carcasses. Set of measures related to finial product (poultry meat) should be introduced, focusing on the chain after slaughterhouse to the consumer.

REFERENCES

1. Neimann, J., Engberg, J., Molbak, K. and Wegener, H. C., 2003. A case-control study of risk factors for sporadic campylobacter infections in Denmark. Epidemiol. Infect., 130, 353 – 356.
2. Stern, N. J., M. R. S. Clavero, J. S. Bailey, N. A. Cox and M. C. Robach, 1995. *Campylobacter* spp. in broilers on the farm and after transport. *Poultry Sci*., **74**, 937-941.
3. Jacobs-Reitsma, W. F., N. M. Bolder and R. W. Mulder, 1994. Caecal carriage of *Campylobacter* and *Salmonella* in Dutch broiler flocks at slaughter. *Poultry Sci*., **73**, 1260-1266.
4. Ridsdale, J. A., H. I. Atabay and J. E. L. Corry, 1998. Prevalence of *Campylobacters* and *Arcobacters* in ducks at the abattoir. *J. Appl. Microbiol*., **85**, 567-573.
5. Nachamkin, I., Engberg, J. and Aarestrup, F.M., 2000 Diagnosis and antimicrobial susceptibility of *Campylobacter* spp. In Nachamkin,I., and Blaser, M.J. *Campylobacter*, 2nd ed. Am.Soc. Microbiol. Washington, D.C., pp. 211-138.
6. Stern, N.J. 1997. Flagellin a gene restriction fragment length polymorphism patterns of *Campylobacter spp*. Isolates from broiler production sources. *Avian Dis*. **41**,899-905.
7. Whyte, P., Collins, J.D. McGill, K., Monahan, C., O"Mahony, H. 2001. The effect of transportation stress on excretion rates of *Campylobacters* in market-age broilers. *Poult. Sci.,* 80, 817-820.
8. Slavic, M. F., J. W. Kim, M. D. Pharr, D. P. Raben, S. Tsai and C. M. Lobsinger, 1994. Effect of trisodium phospgate on *Campylobacter* attached to post-chill chicken carcasses. *J. Appl. Microbiol*., **57**, 324-326.
9. Brendtson, E., M. L. Danielsson-Tham, and A. Engvall, 1996. *Campylobacter* incidence on a chicken farm and the spreads of *Campylobacter* during the slaughter process. *Int. J. Food Microbiol*. **32**, 35-47.

РИЗИК ФАКТОРИ КОИ ВЛИЈААТ НА ПРИСУСТВО НА *CAMPYLOBACTER* SPP. КАЈ ЖИВИНАТА И ВО ПИЛЕШКОТО МЕСО

Вашин Иван, Стојанчев Тодор

Катедра за хигиена, технологија и контрола на храна,Факултет за ветеринарна медицина, Тракиски Универзитет, Стара Загора, 6000, Бугарија

АПСТРАКТ

Преваленцата на *C. jejuni* и *C. coli* во популацијата на домашни животни, посебно на живинарските фарми, како и нивното преживување и ширење за време на колењето на животните се добро познати факти. Овој труд ги сумира научните согледувања за присуството на *Campylobacter* spp. На фармите и низ синџирот на добивање и преработка на пилешкото месо. Дивите птици (врапците, гулабите и препелиците) кои живеат околу живинарските фарми се можен извор на *Campylobacter* spp., бидејќи гулабите се носители со преваленца од 30-100%. Живината (бројлерите и патките) наменети за колење се носители на *Campylobacter* spp. во скоро 100%. Производството на пилешко месо и пилешката кожа се контаминирани во различни размери (вообичаено до 26.7%).

Клучни зборови: пилешко месо, птици, вкрстена контаминација, *C. jejuni*, *C. coli*

UDC: 637.12`632/`638.06:[636.09:615.33.074

OCCURANCE OF RESIDUES OF TETRACYCLINES IN RAW EWE'S MILK

Dimitrieska-Stojković Elizabeta[1], Stojanovska-Dimzoska Biljana[1], Hajrulai-Musliu Zehra[1], Stojković Goran[2], Prodanov Risto[1]

[1]*Institute for food, Faculty for Veterinary Medicine, Skopje, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia*
[2]*Institute for chemistry, Faculty of Natural Sciences and Mathematics Ss Cyril and Methodius University, Skopje, Republic of Macedonia*

*Corresponding author: edimitrieska@fvm.ukim.edu.mk

ABSTRACT

Tetracyclines are antimicrobial substances used in food producing animals to prevent and treat infection diseases caused by wide spectrum of bacteria. Additionally, they might be used as feed additives to improve feed efficiency and growth. The use of these drugs has become serious problem as regarding the presence of residues in milk which might cause allergic reactions in some hypersensitive individuals. A total of 259 ewe's raw milk samples were collected and subjected to examination for presence of residues of tetracyclines over a three-year period. Modified and optimized HPLC-DAD method was used as a screening and confirmatory method. In both cases the method applied has been validated according to the requirements laid down by European Commission Decision 2002/657/EC. Detection capability of the screening method was estimated to be 56.6 µg/kg, 53.8 µg/kg, 58.4 µg/kg and 53.9 µg/kg for oxytetracyline, tetracycline, chlorotetracycline and doxycycline respectively. Applying the validated screening method 20 suspicious positive samples were identified, which was equal to a frequency of 7.72 %. The HPLC-DAD method confirmed the presence of residues in 16 samples, and two of them contained residues of total tetracyclines above the decision limit values of the confirmatory method. The range of the determined compounds was between 57.7 µg/kg and 132.1 µg/kg. The identities of tetracyclines in the milk samples were justified by comparison of the UV spectra of analytes detected in milk samples and the pure compound's solutions. In total, the number of confirmed non-complied samples was equal to a frequency of 0.77 %. The estimated daily intakes (EDIs) calculated for the tetracyclines determined showed lower exposure levels than the recommended values of acceptable daily intakes.

Key words: tetracyclines, ewes, milk, screening, confirmation, HPLC-DAD

INTRODUCTION

Tetracyclines (Fig. 1) are antibiotics widely used in veterinary practices, tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) being among the most applied. These compounds are able to leave residues in food products coming from medicated animals. In ewes, tetracyclines are used when treating general, respiratory, urinary, and local infections. A specific indication for administering tetracyclines in sheep is infectious mastitis. A frequent and pervading source of milk contamination is intramammary administration [1]. The European Union has established the maximum residue level (MRL) for OTC, TC and CTC in milk at 100 µg/kg [2] and reliable analytical methodology is required for an effective control of the regulations.

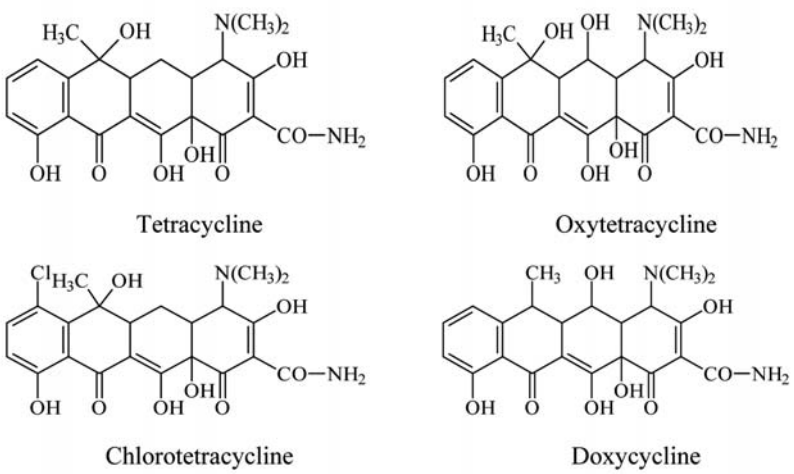


Figure 1. *Chemical structures of tetracyclines*

Microbiological, immunochemical and chromatographic methods have been described for monitoring of tetracyclines in milk and animal tissues [3-5]. The microbiological and immunochemical methods have been widely applied as screening methods because of their simplicity and low time consuming, but due to the lack of specificity, false positive results and semi-quantitative measurements they provide uncompleted data for the presence and quantity of residues detected. For overcoming this problem selective, specific, accurate and precise methods shall be employed which are fulfilling the requirements for confirmatory methods according to the European Commission Decision 657/2002/EC [6]. The purpose of the requirements proscribed in this regulative was obtaining comparable results produced by different laboratories by application of various methods. Since milk presents an emulsion with a high content of fat and proteins, an efficient clean-up should be applied before the liquid chromatography analysis.

In line with the requirements set by the Council Directive 96/23/EC [7] the Macedonian program for monitoring residues in live animals and animal products has been in place for over ten years. In 2010 and 2011 the Macedonian legislation was fully aligned with the EU legislation concerning residues of veterinary medicinal preparations in foodstuffs of animal origin in accordance with the Council Regulation 37/2010/EU [2], and the established MRLs were fully adopted.

The aim of this study was to determine the presence and quantity of residues of TC, CTC, OTC and DC in raw ewe's milk. The residues were monitored in milk samples which have been collected from the primary production and at the delivery to the milk processing plants. For screening and confirmation of the residues of tetracyclines in ewe's milk samples a rapid, accurate and precise High Performance Liquid Chromatographic - Diode Array Detector (HPLC-DAD) method was applied. Before the instrumental detection the samples were subjected to efficient clean up procedure applying polymer solid-phase extraction (SPE) cartridges. Both screening and confirmatory methods were validated to fulfil the performance criteria set in the Commission Decision 2002/657/EC [6].

MATERIALS AND METHODS

Reagents and standard solutions. Methanol, acetonitrile and water were HPLC grade and were purchased by Sigma-Aldrich (St. Louis, MO, USA). Oxalic acid dehydrate, citric acid monohydrate, disodium hydrogen phosphate dehydrate and trichloroacetic acid were with p.a. purity and purchased from Merck (Darmstadt, Germany), while ethylene diaminetetraacetic acid disodium salt was supplied from Sigma-Aldrich (St. Louis, MO, USA). SPE Hydrophilic-lipophylic balance (HLB) Oasis cartridges (60 mg, 3 mL) were products of Waters (Milford, MA, USA). Standards of OTC, TC, CTC and DC were supplied by Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions were prepared with concentration of 1 mg/mL in methanol. Intermediate standard mix of 25 µg/mL was used for preparation of spiking solution with concentration of 2500 ng/mL and six calibration standards for the HPLC method in the range from 100 ng/mL to 2500 ng/mL.

Instruments. For the HPLC-DAD screening and confirmatory application a modified and optimised reverse-phase method was used [5]. The isocratic separation was performed on a 250 mm C8 analytical column (Supelco, Belafonte, USA) at 35 °C. The compounds were detected at 365 nm, on a Perkin Elmer LC 235C DAD instrument. As a mobile phase isocratic mixture of 0.01 mol/L oxalic acid/methanol/acetonitrile (70:20:10, *V*/*V*/*V*) was used. Total run time was 27 min, and injection volume was 50 µL. UV spectra were collected in the range from 190 to 365 nm. For sample preparation Sigma 2K15 centrifuge with cooling at 4 °C, and nitrogen evaporator TECNE DriBlock DB – 3D at 35 °C were employed.

Sampling. A total of 259 ewe's raw milk samples were collected from the primary production and at the delivery to the milk processing plants in the period from 2009 until 2011. Samples were kept at 4-8 °C if they were analysed within 24 hours or at -20 °C for at most 1 month.

Sample preparation. 5 g of milk samples were deproteinized with 2 mL of 20 % trichloroacetc acid and 20 mL of McIlvaine buffer [5] were added. After the shaking the samples were centrifuged at 4 °C, and the supernatant was subjected to SPE on Oasis HPLB cartridges. The eluting solvent was evaporated in the stream of nitrogen at 35 °C. For the HPLC-DAD analysis the residues were dissolved in oxalic acid solution and filtered through 0.45 µm cartridge filters into autosampler vial. Dilution factor was 0.2.

Method validation. Performance characteristics of the screening and confirmatory HPLC-DAD method have been evaluated as foreseen in Commission Decision 2002/657/EC [6]. Limits of detection (LODs) and limits of quantification (LOQs) were obtained though the standard deviation (SD) of the signal values for 20 blank samples. Recovery and precision were assessed by analysis of the spiked blank samples at 0.5, 1 and 1.5 times the MRL level (n=6). Detection capabilities (CCβ) were estimated analysing 20 spiked blank samples at 0.5 of MRL. The decision limits (CCα) of the confirmatory method were determined by analysing 20 spiked samples at MRL level. Additionally, the confirmatory HPLC-DAD method shall fulfil the requirements and criteria for identification of the analytes detected, laid down in the legislation [6].

Calculation of estimated daily intake. The estimated daily intake (EDI) was calculated by the equation given by Juan et al. [7], through the determined concentrations of tetracyclines in ewe's milk samples, on basis of 60 kg bodyweight of an adult. The calculated values for EDIs were compared with the recommended acceptable daily intakes (ADIs) for tetracyclines [8].

RESULTS AND DISCUSSION

The main aim of our work was to apply rapid, easy and specific screening and confirmatory methods for analysis of tetracyclines residues in raw milk samples sampled for the official control programmes and self-controls of the food producers and processors. Since OTC, TC and CTC are substances with MRL, this study should provide data for the proper administration of these drugs with respect to the producer's instructions. On the other hand, DC is not approved for usage at the milk-producing animals; a possible misuse of this substance is to be detected. For obtaining reliable and comparable results the analysis of residues of tetracycline substances in raw milk samples has to be performed with screening and confirmatory

methods that fulfill the specific requirements of the Commission Decision 2002/657/EC [6]. According to this regulation, the screening methods shall be validated not to detect more than 5 % false compliant results, and confirmatory methods not more than 5 % false noncompliant results. Additionally, the confirmatory methods have to fulfill the criteria for positive UV-Vis identification of the compounds detected in the samples.

The proposed HPLC-DAD method is modification of the published one for determination of tetracycline residues in milk and meat [5]. A typical chromatograms of the matrix matched standard solution, blank and spiked sample at MRL level are presented on Figure 2. The applied hydrophilic-lypophilic solid phase extraction cartridges obtained clean extracts without a presence of interfering peaks at the retention times of the analytes, meaning that the criteria for selectivity are satisfied.

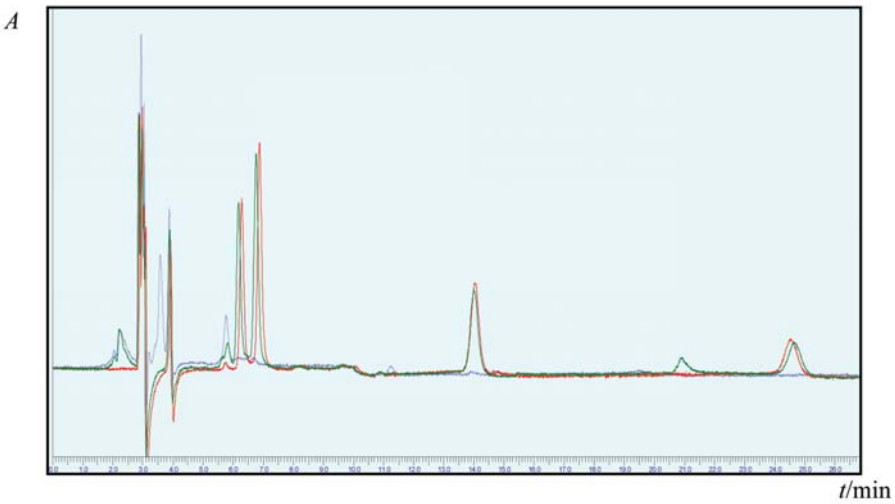


Figure 2. *Standard mixture of OTC, TC, CTC and DC (in the order of appearance) with concentration of 500 µg/L (—); blank milk sample (—); spiked milk sample at 100 µg/kg (—)*

The methods performances according to the requirements laid down in the Commission Decision 2002/657/EC (EC, 2002) were checked for the screening and confirmatory HPLC-DAD method and the validation data are presented in Table 1.

Table 1. *Validation data for the screening and the confirmatory HPLC-DAD method for residues of tetracyclines*

Analytes	LOD (µg/kg)	LOQ (µg/kg)	CCβ[1] (µg/kg)	RSD[2] (%)	CCα[3] (µg/kg)	RSD[4] (%)
Oxytetracycline	9.0	29.9	56.6	7.2	111.7	7.1
Tetracycline	13.8	46.1	53.8	8.8	112.9	7.9
Chlorotetracycline	16.7	55.7	58.4	3.4	108.3	5.1
Doxycycline	20.7	69.2	53.9	4.0	111.7	7.2

[1]CCβ – detection capability of the screening method
[2]RSD – precision of the screening method
[3]CCα – decision limit of the confirmatory method
[4]RSD – precision of the confirmatory method

For the screening method used the obtained CCβ values for all tetracyclines was lower than the established MRL, with satisfactory recoveries (80.1-99.1 %) and precision (3.4-8.8 %). The estimated detection capabilities proved that the proposed HPLC-DAD method could be successfully used as a screening method, detecting not more than 5 % false negative results. All the samples, in which higher concentrations of tetracyclines than the established CCβ values are detected, should be re-tested applying the confirmatory HPLC-DAD method. The obtained decision limits are very close to the established MRLs for tetracyclines, providing that at most 5 % false positive samples are detected. The validation data have confirmed the suitability of the applied methods for performing screening and confirmatory analysis. The additional criteria for the confirmatory methods that have to be met are the matching of the absorption maximum of the analytes in samples and the standard solutions, as well as checking for the variability in the spectra caused by the sample matrix and the detector performance [6].

A total of 259 raw milk samples were subjected to EIA screening method over a three year period. The obtained results from the screening are presented in Table2. The screening has determined an average prevalence for tetracyclines of 8.33 %, 13.44 % and 9.64 % for 2009, 2010 and 2011, respectively. In overall, the most frequently detected substance was oxytetracycline with

obtained concentration range from 22.8 μg/kg to 209.6 μg/kg. Although DC is not approved for application at milk producing animals, yet, in 2009 and 2010 in 3.13 % and 1.25 % of the samples tested a suspicious presence of DC was determined within the concentration range from 20.7 μg/kg to 64.9 μg/kg.

Table 2. *Results from the screening of the ewe's raw milk samples*

Analyzed substances	2009 (N=96)		2010 (N=80)		2011 (N=83)	
	Range (μg/kg)	Mean (μg/kg)	Range (μg/kg)	Mean (μg/kg)	Range (μg/kg)	Mean (μg/kg)
Oxytetracycline	22.8-125.3	10.8	25.4-209.6	16.2	23.6-50.3	5.6
Tetracycline	23.3-82.9	3.7	21.3-48.9	4.0	21.2-59.9	7.0
Chlorotetracycline	15.4-28.6	0.5	13.6-32.5	1.4	16.7-22.8	0.5
Doxycycline	37.6-64.9	1.6	20.7-28.2	0.4	n.d.	-
Total tetracyclines	15.4-125.3	16.5	21.3-209.6	22.0	21.2-59.9	13.1

N – number of analyzed sample
n.d. – not detected

The samples in which the determined amount of tetracyclines was over the established CCβ value of the screening method were subjected to a confirmatory analysis. The results from the confirmation are presented in Table 3. The presence and quantity of tetracyclines was determined in 16 of the samples tested. OTC was confirmed in 12 samples (63.2 %), TC in 3 samples (15.8 %) and DC in one sample (5.3 %). CTC was not confirmed in none of the samples analyzed with the confirmatory method. In two of the samples tested (10.5 %) the determined OTC values have exceeded the CCα value, i.e. they were non-complied regarding the MRL. Additionally, in one of the sample DC was confirmed indicating the unauthorised administration of this drug at ewes. In all cases the criteria for positive identification when full UV-Vis detection is applied have been fulfilled.

Table 3. *Results from the confirmation of the ewe's raw milk samples*

Analyzed substances	2009 (N=11)		2010 (N=6)		2011 (N=2)	
	Range (μg/kg)	Mean (μg/kg)	Range (μg/kg)	Mean (μg/kg)	Range (μg/kg)	Mean (μg/kg)
Oxytetracycline	57.7-123.2	59.2	58.8-132.1	40.1	93.3	46.7
Tetracycline	65.9-80.5	24.4	63.6	10.9	>LOQ	-
Chlorotetracycline	>LOD	-	>LOD	-	>LOD	-
Doxycycline	>LOD	-	64.9	10.8	>LOD	-
Total tetracyclines	57.7-123.2	72.6	LOD-132.1	68.2	LOD-93.3	46.7

N – number of analyzed sample with the confirmatory method
n.d. – not detected

This study confirms that tetracyclines are relatively frequently used antimicrobial substances at milk producing ewes. Further processing of milk may influence on lowering the concentrations of tetracycline antibiotic. The fact that milk is not heat-treated at all or over a very short time interval increases the risk for consuming processed milk products containing residues of tetracyclines. It has been proven that during the heat treatment of milk, only a partial reduction of tetracycline residues occurs [2], which depending on the substance and temperature applied varies from 16 % to 35 %. A study conducted by production of an authentic *carcioricotta* cheese from ewe's and goat milk containing residues of OTC [9] indicates that during the cheese processing the drug concentration has increased for more than two times. This is due to the chelating ability of tetracyclines with the calcium (II) ions from the milk, resulting in a high residue transfer to the milk derivative.

The estimation of EDI based on the calculated average concentrations for tetracyclines in the period 2009 – 2011 revealed value of 1.43 μg kg^{-1} BW/day. The calculations are based on determined average total tetracyclines amount of 17.2 μg/kg, and daily milk consumption for an adult of 200 mL. The European Agency for the Evaluation of Medicinal Products (EMEA) has established an ADI value of 1 for the individual substances and value of 3 for total tetracyclines [8]. ADI has not been established for doxycycline yet.

CONCLUSION

The screening and confirmatory methods used for determination of individual and total tetracyclines in ewe's raw milk samples were validated according to the Commission Decision 2002/657/EC and showed to be simple, rapid, precise and accurate with recoveries higher than 80 % and RSDs less than 10 %. Overall, the confirmed tetracyclines residues over the three year survey revealed a low frequency of positive samples (<1 %). The estimated EDI showed that the contribution of milk to dietary intake of tetracyclines was more than two times lower than the recommended ADI for tetracyclines by the World Health Organization. Having on mind the fact that during the traditional white cheese production significant portion from the residues might remain in the product, the ewe's milk farm production should be continuously subjected to official controls.

REFERENCES

1. Commission Regulation (EU) 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, OJ L 15/1, 20.01.2010, p. 1–2 (ANNEX I)
2. Navratilova P., Borkovcova I., Dračkova M., Janštova B., Vorlova L. (2009) Occurrence of tetracycline, chlortetracyclin, and oxytetracycline residues in raw cow's milk, *Chech J. Food Sci.*, 27: 379–385.
3. Gaudin V., Maris P., Fuselier R., Ribouchon J.-L., Cadieu N., Rault A. (2004) Validation of a microbiological method: the STAR protocol, a five-plate test, for the screening of antibiotic residues in milk, *Food Addit. Contam.*, 21: 422–433.
4. Pastor-Navaro N., Maquieira A., Puchades R. (2009) Immunoanalytical determination of tetracycline and sulfonamide residues in edible products: a review, *Anal. Bioanal. Chem.*, 395: 907–920.
5. Cinquina, A. L., Longo, F., Anastasi, G., Gianetti, L., Cozzaani, R. (2003): Validation of high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlorotetracycline and doxycycline in bovine milk and muscule, *J. Cromatogr. A*, 987: 227–223.
6. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results OJ L 221, 17.8.2002, p. 8–36
7. Juan, C., Moltó, J.C., Mañes, J., Font, G. (2010). Determination of macrolide and lincosamide antibiotics by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry in meat and milk, *Food Control*, 21, 1703-1709.
8. EMEA (1995). Oxytetracycline, Tetracycline, Chlortetracycline, summary report (3). Committee for veterinary medicinal products, EMEA, EMEA/MRL/023/95.
9. Cinquina A. L., Giaanetti L., Anastasi G., Draisci R., Palleschi L., Cozzani R. (2000). Residue study of oxytetracycline in goat milk and its derivatives by micro-liquid chromatography-mass spectrometry, *Proceedings of the EuroResidue IV Conference*, 1: 277–284, Veldhoven, The Netherlands 8–10 May.

ПРИСУСТВО НА РЕЗИДУИ ОД ТЕТРАЦИКЛИНИ ВО СИРОВО ОВЧО МЛЕКО

Димитриеска-Стојковиќ Елизабета[1],Стојановска-Димзоска Билјана[1], Хајрулаи-Муслиу Зехра[1], Стојковиќ Горан[2], Проданов Ристо[1]

[1]Институт за храна, Факултет за ветеринарна медицина - Скопје, Универзитет "Св. Кирил и Методиј ", Скопје, Република Македонија
[2]Институт за хемија, Природно-математички факултет, Универзитет "Св. Кирил и Методиј ", Скопје, Република Македонија

*Автор за коресподенција: edimitrieska@fvm.ukim.edu.mk

АПСТРАКТ

Тетрациклините се антимикробни супстанци кои се користат кај животните кои се одгледуваат за производство на храна за превенција и третирање на инфективни заболувања предизвикани од широк бактериски спектар. Дополнително, можно е тие да се користат и како адотиви во добиточната храна за подобрување на искористувањето на храната и растот. Употребата на овие лекови претставува проблем во однос на присуството на нивни резидуи во млекото, што може да предизвика алергиски реакции кај хиперсензитивни особи. Вкупно 259 примероци од овчо млеко, во тек на 3 години, собрани се и испитани за присуство на остатоци од тетрациклини. За скрининг и конфирмација употребен е модифициран и оптимизиран HPLC-DAD метод. Во двата случаја применетиот метод е валидиран согласно пропишаните барања во Одлуката на европската Комисија 2002/657/ЕС. Можноста за детекција на скрининг методот е проценета на 56,6 μg/kg, 53,8 μg/kg, 58,4 μg/kg и 53.9 μg/kg соодветно за окситетрациклин, тетрациклин, хлортетрациклин и доксициклин. Со примена на валидираниот скрининг метод идентификувани се 20 сомнителни позитивни примероци, што е еднакво на зашестеност од 7,72 %. HPLC-DAD методот го потврди присуството на резидуи во 16 примероци, а два од нив содржеа резидуи од вкупни тетрациклини над лимитот на одлучување на конфирмациониот метод. Опсегот на определените соединенија се движеше од 57,7 μg/kg до 132,1 μg/kg. Идентитетите на тетрациклините во примероците од млеко беа потврдени со споредба на UV спектрите на детектираните аналити во примероците и растворите на чисти супстанци. Вкупно земено, бројот на конфирмирани несообразни примероци соодветствува на зачестеност од 0,77 %. Проценетите дневни вносови (EDIs) пресметани за определените тетрациклини укажуваат на пониски нивоа на изложеност на ризик отколку препорачаните вредности за прифатливиот дневен внос.

Клучни зборови: тетрациклини, овци, млеко, скрининг, конфирмација, HPLC-DAD

UDC: 637.5.06:[636.09:615.33.074

RESULTS FROM MONITORING THE EDIBLE ANIMAL TISSUES FOR RESIDUES OF SOME VETERINARY DRUGS

Dimitrieska-Stojković Elizabeta[1], Arsova Gordana[1], Hajrulai-Musliu Zehra[1], Stojanovska-Dimzoska Biljana[1], Uzunov Risto[1]

[1]*Institute for food, Faculty for Veterinary Medicine, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia*

*corresponding author: edimitrieska@fvm.ukim.edu.mk

ABSTRACT

A total of 635 muscle and kidney samples were collected during 2010 and 2011 at slaughter houses within the monitoring plan for food from animal origin in Macedonia. The tissue samples were examined for chloramphenicol, sulfonamides, quinolones and tetracyclines. Enzyme-linked immunosorbent methods were used for the determination of chloramphenicol, sulfonamides and quinolones, and high performance liquid chromatography with Diode Array detection was applied for screening of tetracyclines. The methods were validated according to the recommendations laid down by European Commission Decision 2002/657/EC. The obtained data for the method's accuracy, precision and detection capabilities confirmed that the methods were appropriate for detection of antimicrobial substances determined, at the concentration level of interest. The measured range of concentrations was 13.9-74.5 μg/kg for sulfonamides, 9.7-28.0 μg/kg for quinolones and 18.4-80.1 μg/kg for total tetracyclines, with calculated mean values 6.6 μg/kg for sulfonamides, 3.8 μg/kg for qinolones and 1.6 μg/kg for tetracyclines. No exceeding of the established maximum residue levels has been observed. Chloramphenicol has not been detected over the minimum required performance level (MPIJI) value of the screening method in none of the samples tested. The calculated estimated daily intakes for the average daily consumption of 135 g of meat, reveals levels 20 to over 1000 times lower than the values of the acceptable daily intakes fixed by World Health Organization and European Medicines Agency.

Key words: muscle, kidney, veterinary drug residues, chlorampenicol, tetracyclines, sulfonamides quinolones, ELISA, HPLC-DAD

INTRODUCTION

Antimicrobial veterinary drugs are utilized at food producing animals not only for treatment of diseases, but also sub-therapeutically, to maintain health and promote growth. The use of unauthorized antibiotics or the failure to follow the label directions for approved antibiotics could result in unsafe antibiotic residues in food products. These residues could exhibit direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or they may cause problems indirectly through induction of resistant strains of bacteria. Due to harmful effects of veterinary products residues, surveillance systems are enforced in the European Union related to the requirements laid down in the Council Directive 96/23/EC [1]. According with these requirements, the Macedonian legislation was fully aligned with the EU legislation concerning residues of veterinary drugs in foodstuffs of animal origin in reference to the Council regulation 37/2010/EU [2].

In order to detect such residues in food and tissues, bioassay techniques are widely used as screening methods. Although these methods generally do not distinguish between members of a class of antibiotics, still they provide a semi-quantitative estimate of 'total' residues detected. Nevertheless, they continue to be used because of their simplicity and low-cost. However, before samples are declared to contain concentrations of antibiotics exceeding the tolerance levels, confirmation (and identification of the individual compounds) by sufficiently selective and sensitive instrumental methods such as LC–MS or GC–MS are required [3]. All methods used for that purpose have to detect antibiotics at or below their permissible limits or MRLs and also have to be validated in accordance with the Commission Decision 2002/657/EC [4].

The annual consumption of meat in Macedonia for year 2009 was around 50.5 kg per capita [5]. In the last decades there is a lack of data from investigations of veterinary drug residues in tissue samples collected from Macedonia. With an aim to monitor veterinary drug contamination in tissue samples from slaughter houses, residual concentrations of chloramphenicol, sulfonamides, qinolones and tetracyclines were examined. Sample analyses applying validated method according to the Commission Decision [4] provide accurate and reliable analytical results. Additionally, an estimation of the dietary intake of veterinary drugs residues derived from meat consumption was calculated.

MATERIALS AND METHODS

A total of 635 muscle and kidney tissue samples were collected during 2010 and 2011 from slaughter houses in Macedonia. 221 kidney samples were analyzed for residues of sulfonamides and quinolones, 221 muscle samples for residues of tetracyclines and 193 muscle samples for residues of chloramphenicol. The tissue samples were with origin from different animal species – bovine, ovine and porcine. Samples were stored at 4-8°C

if analyzed within 24 hours after sampling, or kept at less than -20°C for four weeks.

The Chloramphenicol ELISA kit (type AB630) and quinolones (enrofloxacin, ciprofloxacin, norfloxacin, danofloxacin, oxolinic acid, flumequine) ELISA kit (type AB630) were purchased by TECNA (Trieste, Italy). The Multi-sulfonamides (sulfadimidine, sulfamerazine, sulfachloropyridazine, sulfisoxazole, sulfadiazine, sulfachloropyridazine, sulfametoxazole) ELISA kit (type 5101SULMp) was supplied from Europroxima (Arnhem, The Netherlands). Ethyl acetate, citric acid, disodium hydrogen phosphate and oxalic acid were with p.a. purity, supplied from Merck (Darmstadt, Germany). Sodium EDTA was supplied by Sigma (St. Louis, USA). Methanol, acetonitrile and water were with HPLC grade, and were supplied from Sigma (St. Louis, USA).

The certified standards of sulfadimidine, sulfisoxazole, oxytetracycline hydrochloride, tetracycline hydrochloride, chlorotetracycline hydrochloride, doxycycline hyclate, enrofloxacin and chloramphenicol, with a purity over 97 %, were supplied from Sigma-Aldrich (St. Louis, USA). From these substances standard solutions were prepared and used for spiking blank tissue samples at different levels. Following fortification, samples were allowed to equilibrate for some time before extraction.

For sample preparation vortex model Relax Top by Heidolph (Schwabach, Germany), centrifuge model 2K15 by Sigma (St. Louis, USA), evaporator model DriBlock DB-3D by TECHNE (Staffordshire, UK) were used. The optical density at 450 nm for the EIA tests was measured by microplate reader BDSL Immunoscan (Labsystem, Switzerland). Tetracyclines were determined by HPLC-DAD, employing isocratic elution on reverse-phase C_8 Ascentis column (Supelco, Belafonte, USA), with a mixture of 0,01 mol/dm^3 oxalic acid, methanol and acetnitrile in ratio 70:20:10 (V/V/V). Detection of tetracyclines was performed on Perkin Elmer LC 235C diode array instrument (Norwalk, USA) at 365 nm.

The whole procedure for testing chloramphenicol, sulfonamides and quinolones, was according to the manufacturer's instructions. Tetracyclines (oxytetracycline, tetracycline, chlorotetracycline and doxycycline) were determined by modified and optimized screening reverse-phase High-performance liquid chromatography/diode array detector (HPLC-DAD) method [6]. Previously the samples were undergone through extraction with McIlvaine buffer and purification of the sample extracts with OASIS HLB solid-phase extraction cartridges (Waters, Milford, USA). The residues were finally dissolved in 0.01 mol/dm^3 oxalic acid buffer, filtered through 0.45 μm syringe filters and injected into the HLPC system. The dilution factor was 0.2.

The estimated daily intake (EDI) was calculated by the data for the mean detected concentrations for the analyzed residues and average daily consumption based on 60 kg body weight.

Results and discussion

The objective of this study was to screen the residual levels of chloramphenicol as a prohibited substance, and tetracyclines, quinolones and sulfonamides throughout two years monitoring of antimicrobials in Macedonia, to reveal if the abuse of prohibited substance is present, or the maximum residue limits have been exceeded. According to the literature data, this is the first study to evaluate the contamination of edible animal tissues with antimicrobial drug residues and the dietary exposure to these substances in Macedonia.

The analysis of veterinary drugs residues were performed applying in-house validated screening methods according to the criteria laid down in Commission Decision 2002/657/EC [4]. Performance characteristics of EIA methods and the screening HPLC-DAD method were determined as prescribed for qualitative screening methods in Commission Decision 2002/657/EC [4]. The limit of detection (LOD) and limit of quantification (LOQ) were obtained by adding 3 and 10 times the standard deviation of 20 blank samples to the mean blank value. The detection capability (CCβ) for tetracyclines, quinolones and sulfonamides was determined by spiking of 20 blank samples of tissues at the one half of the MRL values, while for chloramphenicol the spiking level was below the established MRPL value of 0,3 μg/kg. Recovery was assessed by performing the experiments where fortified tissue samples were analyzed in ten replicates, at the respective maximum residue level (MRL) or minimum required performance level (MRPL) values for the substances being analyzed. From the recovery experiments the method precision was obtained, as well. The calculations were performed by the formula provided in the EU Commission Decision [4].

Validation data corresponding to the screening meth-

Table 1. *Method validation data for the screening methods of the antibiotics analyzed in tissue samples*

Analyte	Target tissue	LOD (μg/kg)	LOQ (μg/kg)	CCβ (μg/kg)	Recovery %	Precision %
Sulfonamides	kidney	13.9	44.6	62.2	96.7	7.4
Quinolones (Enrofloxacine)	kidney	9.7	32.0	132.8	90.6	21.8
Oxytetracycline	muscle	6.7	22.5	70.5	94.2	12.5
Tetracycline	muscle	10.5	31.8	67.9	89.2	10.9
Chlorotetracycline	muscle	17.4	58.0	62.3	96.8	7.5
Doxycycline	muscle	18.2	60.8	63.8	92.9	8.4
Chloramphenicol	muscle	0.034	0.112	0.18	82.7	19.3

od performance (LOD, LOQ, CCβ, recovery and precision) are presented in Table 1. All screening methods used obtained CCβ values less than the fixed MRL or MRPL values and recoveries higher than 70 %, in accordance with the regulations set by Commission Decision 2002/657/EC. Moreover, the precision values were lower than the recommended maximum by the Horwitz equation [4]. The validation data have justified that the applied methods were appropriate for the detection of residues of veterinary drugs measured.

A total of 635 tissue samples were subjected to the screening methods. The determined antimicrobial's concentrations and MRLs for tetracyclines, quinolones and sulfonamides, as well as the MRPL value for chloramphenicol are summarized in Table 2. All concentrations found for chloramphenicol were lower than the critical MRPL value established by Commission Decision 2003/181/EC [7]. Residues of tetracyclines over the LODs of the method applied have been found in 23 samples (10.4 %), with an average amount of 1.6 μg/kg. Total sulfonamides and quinolones were found in 15 (6.8 %) and 8 samples (3.6 %), respectively, and the determined average concentrations were significantly lower than the LOD values. None of the samples examined contained residues of quinolones, tetracyclines and sulfonamides over the established MRL by Commission Regulation (EU) 37/2010.

Table 2. *Veterinary drug residues (range and mean) in tissue samples collected in the period 2010 and 20111 and critical values regulated by EU and Macedonian legislation*

Analytes	2010			2011			MRL (μg/kg)
	n	Range (μg/kg)	Mean (μg/kg)	n	Range (μg/kg)	Mean (μg/kg)	
Sulfonamides	118	13.9-39.3	7.4	103	14.5-74.5	5.8	100
Quinolones	118	10.5-28.0	3.7	103	9.7-22.0	3.9	200
Oxytetracycline	118	18.4-79.5	2.3	103	38.0-80.8	2.0	100
Tetracycline	118	27.5-55.6	1.2	103	43.2-59.8	1.5	100
Chlorotetracycline	118	35.9-60.6	1.3	103	30.6-77.6	1.4	100
Doxycycline	118	>18.2	-	103	>18.2	-	100
Total tetracyclines	118	18.4-79.5	1.6	103	30.6-80.8	1.6	100
Chloramphenicol*	95	0.006 -0.145	0.052	98	0.003-0.142	0.035	0.3

* Chloramphenicol is not authorized for use in food producing animals in the European Union and in Macedonia (in MRL column the indicated value is MRPL)

Sulfonamides and tetracyclines, together with beta-lactams are considered to be the most frequently utilized antimicrobial substances. Sulfonamides play important role as effective chemotherapeutics of bacterial and protozoan diseases and as growth promoters in veterinary medicine. The Commission regulation has established the MRL as a sum of all substances belonging to the sulfonamide group, which for tissues should not exceed 100 μg/kg [2]. According to World Health Organization (WHO) [8] the acceptable daily intake (ADI) for sulfadimidine was established at 3 mg for 60 kg BW. The highest determined total sulfonamide's concentration was 74.5 μg/kg. Tetracyclines are globally used as broad spectrum antibiotics in veterinary medicine against a wide range of Gram-positive and Gram-negative aerobics and anaerobic bacteria. In the present study, the highest tetracycline's level detected was below the MRL value and it was 80.80 μg/kg for oxytetracycline. The mean tetracyclines concentration was more approximately eight times lower than the assigned MRL. Quinolones are a group of relatively new antimicrobials synthesized from 3-quinolone carboxylic acid. The established MRL for these substances are 200 μg/kg [2] In the present study, the mean quinolones concentration (expressed as enrofloxacin) showed levels more than ten times below the established MRLs.

Due to the potential risk to human health, the use of chloramphenicol is prohibited in food-producing animals in the European Union [2]. The European Union introduced the concept of the minimum required performance limit (MRPL) of 0.3 μg/kg, the highest concentration level at which the screening and confirmatory method shall demonstrate satisfactory performances regarding the sensitivity, accuracy and precision [4]. In this investigation, the measured chloramphenicol mean concentration of 35 ng/kg was substantially lower than the MRPL value, and practically it was the signal obtained from the blank.

According to the Commission Decision 2002/657/EC [4] it is mandatory to confirm the obtained positive samples by the screening with confirmatory analysis, applying methods that provides unambiguous identification and quantification of the concerned analyte.

For the purpose of evaluation of dietary exposure with veterinary drugs residues through the intake of animal tissues controlled in the present study, the EDIs for consumers were estimated. Table 3 presents the EDIs of veterinary drug residues based on the concentrations

found in the present work, calculated with presumed average daily meat consumption for an adult of 135 g [5]. Residue values of all MRL drugs measured, ranged from 0.032 to 0.213 mg/kg BW/day, and were 20 to over 1000 times lower than the set values of ADIs by the European Medicinal Agency (EMEA) [9,10]. However, ADIs values have not been established for chloramphenicol.

Table 3. *Estimation of daily intakes (EDIs) of veterinary drug residues through tissues consumption based on the mean concentrations found in the period 2010-2011*

Analyte	EDI	ADI
	µg/kg BW/day	µg/kg BW/day
Sulfonamides	0.168	50
Enrofloxacin	0.213	372
Oxytetracycline	0.054	1
Tetracycline	0.032	1
Chlorotetracycline	0.035	1
Doxycycline	/	NE**
Total tetracyclines	0.121	3
Chloramphenicol*	0.92*	NE**
Total acceptable daily intake	0.503	428

*EDI for chloramphenicol was expressed as ng/kg BW/day
**NE: Not yet have been established

The highest calculation for EDI was obtained for total tetracyclines, which is approximately 5 % of the ADI value. The total EDI value obtained was 0.503 mg/kg BW/day, substantially lower than the total acceptable daily intake. Therefore, the toxicological risk associated with the consumption of analyzed tissue samples, could not be considered as a public health issue with regards to the analyzed veterinary antimicrobial substances.

CONCLUSION

The methods used for determination of veterinary drugs residues in edible animal tissue samples were validated according to Commission Decision 2002/657/EC and proved to be rapid, simple and reliable, exhibiting good accuracy and repeatability. The obtained recoveries were over 70% with precision values fulfilling the requirements of the Horwitz equation. In the two years period of monitoring, the residues measured for sulfonamides, quinolones and tetracyclines were below the maximum residue limits (MRLs) set by the legislation. None of the samples exceeded the MRPL value for chloramphenicol. The EDIs calculated indicates that the contribution of edible animal tissues to dietary intake of the investigated antibiotics was from 20 to over 1000 times below the ADIs proposed by EMEA and WHO. This indicates that the edible tissues in Macedonia, in average, contain low levels of veterinary drugs, and therefore, they could be considered as safe for human consumption.

REFERENCES

1. Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC OJ L 125, 23.5.1996, p. 10–32.
2. Commission Regulation (EU) 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, OJ L 15/1, 20.01.2010, p. 1–2 (ANNEX I).
3. A.A.M. Stolker, U.A.Th. Brinkman (2005). Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals—a review, J. of Chromatogr. A, 1067, 15-53.
4. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results OJ L 221, 17.8.2002, p. 8–36.
5. Worldwide Animal Meat Consumption per Capita (www.ChartsBin/wiew/bhy, 09.06.20120).
6. Cinquina, A. L., Longo, F., Anastasi, G., Gianetti, L., Cozzaani, R. (2003): Validation of high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlorotetracycline and doxycycline in bovine milk and muscule, J. Cromatogr. A, **987**: 227–223.
7. Commission Decision 2003/181/EC of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results OJ L 221, 17.8.2002, p. 8–36.
8. JECFA (1995): Joint FAO/WHO expert Committee on food additives. WHO technical report series 851, forty second meeting of JECFA.
9. EMEA (1995): Oxytetracycline, Tetracycline, Chlortetracycline, summary report (3) Committee for veterinary

medicinal products, EMEA, EMEA/MRL/023/95.
10. EMEA (2002): Enrofloxacin, summary report (5) Committee for veterinary medicinal products, EMEA, EMEA/MRL820/02 final, January 2002.

РЕЗУЛТАТИ ОД МОНИТОРИНГ НА ЖИВОТИНСКИ ТКИВА КОИ СЕ КОНСУМИРААТ ЗА РЕЗИДУИ ОД НЕКОИ ВЕТЕРИНАРНИ ЛЕКОВИ

Димитриеска-Стојковиќ Елизабета[1], Арсова Гордана[1], Хајрулаи-Муслиу Зехра[1], Стојановска-Димзоска Билјана[1], Узунов Ристо[1]

[1]Институт за храна, Факултет за ветеринарна медицина - Скопје, Универзитет "Св. Кирил и Методиј ", Скопје, Република Македонија

*автор за коресподенција: edimitrieska@fvm.ukim.edu.mk

АПСТРАКТ
Вкуп 635 примероци од мускул и бубрег, во рамките на мониторинг планот за храна од животинско потекло во Македонија, беа собрани од кланици во текот на 2010 и 2011. Примероците од ткива беа анализирани за присуство на хлорамфеникол, сулфонамиди, хинолони и тетрациклини. Ензимски-врзаните имуносорбентни методи беа користени за оределување на хлорамфеникол, сулфонамиди и хинолони, а високо-ефикасната течна хроматографија со детектор со низа од диоди беше применета за скрининг на тетрациклини. Методите беа валидирани согласно препораките пропишани во Одлуката на Европската Комисија 2002/657/ЕС. Добиените податоци за точноста, прецизноста и можноста за детекција потврдија дека методите се соодветни за определување на селектираните антимикробни супстанци, на нивото на концентрации кое е од интерес. Измерениот опсег на концентрации за сулфонамиди беше 13,9-74,5 µg/kg, за хинолони 9,7-28,0 µg/kg и 18,4-80,1 µg/kg за вкупни тетрациклини. Во ниту еден случај не е утврдено надминување на воспоставеното максимално ниво на резидуи. Хлорамфениколот не е детектиран над минималното потребно ниво на перформанси (MRPL) на скрининг методот во ниту еден анализиран примерок. Пресметаните проценки за дневниот внос при просечна дневна консумација од 135 g месо, утврдија нивоа кои беа за 20 до над 1000 пати помали од вредностите за прифатлив дневен внос определени од Светската здравствена организација и Европската агенција за лекови.
Клучни зборови: мускул, бубрег, резидуи на ветеринарни лекови, хлорамфеникол, тетрациклини, сулфонамиди, хинолони, ELISA, HPLC-DAD

UDC: 636.09:[615.33.074:543.422.3-76

QUALITY CONTROL OF POTENTIATED SULFONAMIDE COMMERCIAL VETERINARY FORMULATIONS BY UV-SPECTROPHOTOMETRY

Mihajlović Jelena[1], Dimitrieska - Stojković Elizabeta[2], Velev Romel[2], Stojković Goran[1*]

[1]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Skopje, Republic of Macedonia*
[2]*Institute for Food, Faculty of Veterinary Medicine – Skopje, University "Ss. Cyril and Methodius", Skopje, Republic of Macedonia*

*Corresponding author: goranst@pmf.ukim.mk

ABSTRACT

Combinations of a sulfamethoxazole with trimethoprim in a fixed ratio in commercial preparations, very often are used for chemotherapeutic practice in veterinary medicine. As well as in human medicine, veterinary formulations with this combination of active drugs ingredients, which are commonly termed "potentiated sulfonamide", must be of good quality, safe and effective.

For a reliable quality control of the active compound's content it is essential to apply selective, accurate and precise analytical procedures. Liquid chromatographic and spectrophotometric methods that include specific sample preparation are the most frequently utilized. In this investigation, a simple ultraviolet-visible (UV-Vis) spectrophometric method has been developed for determination of sulfamethoxazole/trimethoprim content.

The method has excellent linearity in the concentration range 1–30 μg⁄mL. Correlation coefficient ranges from 0.9997 to 0.9999, going from classical to derivatives UV spectroscopy. Statistical analysis confirmed that the method has satisfactory accuracy and precision according to the Horwitz criterion, where RSD < 4.00 % for commercial veterinary medicinal product Hemosul-P (oral powder) and RSD < 3.40 % for Hemosul-S (inj. sol.). Applying the suggested procedure, it was possible to perform selective analysis for SMX in the pharmaceutical preparations without removing the excipients. Since there is no need for sample preparation and sophisticated apparatus, the described method presents rather inexpensive procedure for quantitative determination of SMX in these veterinary medicinal products (VMPs). The obtained results are in a good agreement with the declared contents.

The proposed UV method is suitable to be utilized for quality control of these VMPs in terms of assuring proper and effective drug administration. Finally, this results in presence of safe levels of drug residues in the food of animal origin.

Key words: potentiated sulfonamide, sulfamethoxazole/trimethoprim, UV spectrophotometry, veterinary medicinal products

INTRODUCTION

In the practice of veterinary medicine several separate combinations of a sulfonamide with trimethoprim in a fixed ratio (5 : 1) are used clinically. These combinations are commonly termed "potentiated sulfonamide antimicrobial agents". Examples of such potentiated sulfonamide preparations include sulfamethoxazole/trimethoprim (co-trimoxazole), sulfadiazine/trimethoprim (co-trimazine) and sulfadoxine/trimethoprim (co-trimoxine). Potentiated sulfonamides have th e desirable property of reducing, by several folds, the minimum inhibitory concentration (MIC) of both the sulfonamide and the trimethoprim against a wide range of pathogenic organisms. Lowered MICs needed to control infections result in small doses of drugs used in each animal and thereby a reduction in the total dose of drug administered to the animal [1].

Veterinary drugs Hemosul-S (inj. sol.) and Hemosul-P (oral powder) are sulfamethoxazole/trimethoprim products, in parenteral and oral dosage forms, approved for use in veterinary practice of domestic animals in the Republic of Macedonia.

In veterinary medicine, as well as in human medicine, drugs must be of good quality, safe and effective. For a reliable quality control of the active compound's content it is essential to apply selective, accurate and precise analytical procedures. Potentiated sulfonamide combinations, like sulfamethoxazole/trimethoprim, are analyzed by a variety of chromatographic and conventional methods.

From an analytical point of view, methods for drugs analysis in commercial preparations are considerably less complex than methods for analysis of drugs and their metabolites in biological samples as blood, plasma, hair or urine. However, the unequivocal determination of a drug in veterinary formulations is as important as determination in complex matrices, because the veterinary product quality is directly related to safe levels of residues in food of animal origin.

Several UV/VIS spectrophotometric methods have been widely developed to quantify active drugs ingredients. As most active components possess chromophore groups, they can be determined directly in the ultraviolet region without the need for a derivatization reaction.

Multicomponent derivative spectroscopic method at 280 and 294 nm was employed for determination of the two substances in mixture [2]. A method for the simultaneous determination of sulfamethoxazole (SMX) and trimethoprim (TMP), based on a direct determination of SMX after diazotization and coupling with 2-naphthol by visible spectrophotometry and an indirect determination of TMP in the UV region by difference has been published [3]. By the suggested procedure, it was possible to analyze SMX and TMP in pharmaceutical preparations without separating from each other or from the excipients. Two methods, namely first derivative and classical last squares methods were selected and applied for comparative purposes to analyze UV-spectra of the methanol solutions of the SMX and TMP in synthetic binary mixtures and in number of antibacterial pharmaceutical preparations produced by Egyptian companies [4].

The formulation of Sulfamethoxazole and Trimethoprim in a combination mixture is very good pharmacologically since it enhances the efficacy of the individual drugs. However in these combination difficulties in analysis on ordinary UV spectrophotometry are introduced because the two components give overlapping spectral bands on zero-order. The method is rapid, simple and can be applied successfully to assay a mixture of the two drugs in pharmaceutical preparations [5].

A micellar electrokinetic capillary chromatography was performed for the determination of sulfamethoxazole and trimethoprim. Recoveries were optimal and acceptable after extraction with ethanol/deionized water for both investigated compounds from laboratory mixtures of standards. The method was applied to determine sulfamethoxazole and trimethoprim in tablets, powder and solution for infusion [6].

A rapid, reliable and sensitive UV-spectrophotometric method has been developed for the determination of the Sulphamethoxazole. The developed method was employed in *in-vitro* protein binding studies, the drug shows 50-60 % binding and it was good agreement with reported pharmacokinetic data and drug release kinetics [7].

A rapid, simple and sensitive spectrophotometric method for the determination of some sulfa drugs is described. The method is based on the formation of orange yellow colored azo product by the diazotization of sulfonamides: dapsone (DAP), sulfathiazole (SFT), sulfadiazine (SFD), sulfacetamide (SFA), sulfamethoxazole (SFMx), sulfamerazine (SFMr), sulfaguanidine (SFG) and sulfadimidine (SFDd) followed by a coupling reaction with 3-aminophenol in aqueous medium. The method is successfully employed for the determination of sulfonamides in various pharmaceutical preparations and common excipients used as additives in pharmaceuticals do not interfere in the proposed method [8].

The common availability of the instrumentation, the simplicity of procedures, economy, speed, precision and accuracy of the technique still make spectrophotometric methods attractive. So, in this present investigation, an attempt has been made to develop accurate, precise, environmental friendly and economically UV spectrophotometry method for the estimation of sulfamethoxazole in different dosage form.

MATERIALS AND METHODS

Standard stock solutions

Sulfamethoxazole (SMX) was purchased from Sigma-Aldrich. Standard stock solutions (1 mg mL^{-1}) were prepared by weighing 50 mg of SMX and by dissolving in 50 mL 96 % ethanol (Riedel-de Haën). Applying suitable dilutions, spiking solution for veterinary formulation samples with concentration of 100 µg mL^{-1} and calibration standards for the UV method in the concentration range of 1−30 µg mL^{-1} (1, 2, 5, 8, 10, 15, 20 and 30 µg mL^{-1}) were prepared using 10 % ethanol and acetate buffer (pH 4.8).

Commercial veterinary formulation

A commercial veterinary product Hemosul-P (oral powder) and Hemosul-S (inj. solution) from producer "Veterinarski zavod Subotica" was assayed. It's declared content was as follows: 100 mg sulfamethoxazole and 20 mg trimethoprim in 1 g powder of Hemosul-P; 200 mg sulfamethoxazole and 40 mg trimethoprim in 1 mL solution of Hemosul-S.

Sample solutions preparation

For the commercial sample analysis, an adequate mass portion (equal to concentration of 100 µg mL^{-1} SMX) of Hemosul-P (or volume of Hemosul-S) was transferred into 100 mL flask, 2 ml buffer solution (pH=4.8) and 10 % ethanol were added and the sample was sonificated in ultrasonic bath for 30 minutes. The flask was filled to the mark with 10 % ethanol. In the following step, an aliquot of the filtrate was transferred into 10 mL flask, then 2 ml buffer solution (pH=4.8) were added and the volume was filled up with 10 % ethanol, mechanically shaken and filtrated through white Whatman filter paper.

Spectrophotometric measurements and Data analysis

The UV spectra of the standard and sample solutions, and appropriate blanks were recorded on a Varian Carry 50 UV/Visible Spectrophotometer, at room temperature in 1 cm quartz cell, in the wavelength range from 190 to 350 nm, with resolution 0.5 nm and scan rate of 300 nm/min. Varian Carry software was used for the calculated derivative spectra and Microsoft Office Excel for determination of statistical parameters.

RESULTS AND DISCUSSION

The spectra of the standard solutions of SMX (Figure 1) are characterized by two absorption band, one at lower wavelengths, with a maximum of absorbance that exhibits bathochromic shift by increasing concentration of SMX (from 206 nm to 212 nm). Therefore this absorption band is not suitable for quantitative determination. On the other hand, the spectral analysis clearly indicates that the absorbance band at the higher wavelength (268 nm) is more suitable for quantitation.

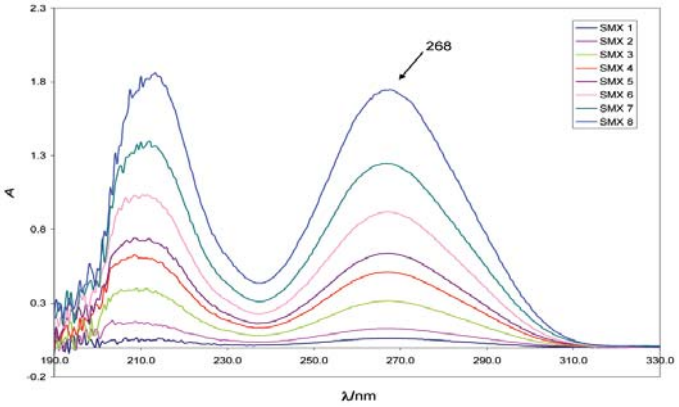


Figure 1. *UV spectra of standard solutions of sulfamethoxazole (1−30 μg mL^{-1})*

With aim to eliminate the possible interferences of the excipients, and improvement of the method precision, the first-order derivative spectra are obtained (Figure 2).

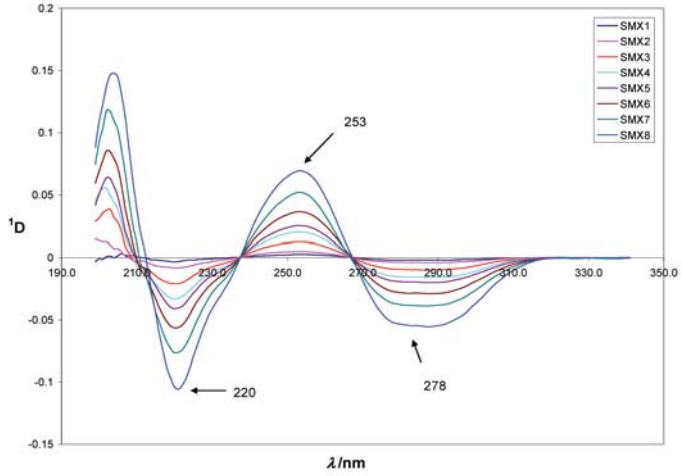


Figure 2. *First-order derivative spectra of standard solution of SMX (1–30 μg mL^{-1})*

The spectral analysis from first-order derivative spectra indicate that the obtained derivative signals at 220 nm, 253 nm and 288 nm are sufficiently well-defined for quantitative determination of the SMX. Under the optimum conditions described above, the linear correlation was obtained between the concentrations of SMX in the tested range and their corresponding absorbances at 268 nm, as well as and for the derivative signals of three selected wavelengths (Table 2). The equations of the calibration curves, correlation coefficients (R), number of data (n) and standard errors of estimate ($SE_{y,x}$) are listed in Table 1.

Table. 1. *Equation of calibration curves for zero-order and first-order spectroscopy at different wavelengths*

Signal*	y=ax+b #	$SE_{y,x}$	R	n	γ(SMX)/ μg mL^{-1}
A_{268}	y = 0.0586x + 0.0281	0.0295	0.9997	8	1 - 30
$^1D_{220}$	y = 0.0035x + 0.0033	0.0031	0.9985	8	1 - 30
$^1D_{253}$	y = 0.0024x + 0.0010	0.0020	0.9990	8	1 - 30
$^1D_{278}$	y = 0.0017x + 0.0007	0.0007	0.9999	8	1 - 30

** A – absorbance from UV spectra, ^1D – derivative signals from first derivative spectra*

 # a – slope, b – intercept, y – A or ^1D, x – mass concentration = γ(SMX)

From the data in the Table it may be clearly seen that the correlation coefficient for the calibration equation calculated from the $^1D_{278}$ signals is the highest. The standard error of the estimate is the lowest, indicating that the accuracy of prediction is highest.

Accuracy testing

To test the method suitability in terms of accuracy and precision, recovery experiments were conducted by adding known quantities of standard solution of SMX (5, 10 and 15 μgmL^{-1}) to the different sample formulations of SMX, and then the mixtures were analyzed by the proposed method. Figure 3 presents the spectra of solutions of Hemosul-P spiked with a standard solution of SMX. The results from the recovery experiment are shown in Table 2. The obtained satisfactory values for the recoveries (95−105 %), except for those obtained from derivative signal $^1D_{220}$, indicate that the method is accurate.

The same procedure was performed for Hemosul-S and the corresponding results are given in Table 3. In this case, the values for recovery are quite variable, probably due to insufficient homogeneity of Hemosul-S as a matrix. It may be concluded that the obtained values from the signals 1D220 and 1D253 are unacceptable.

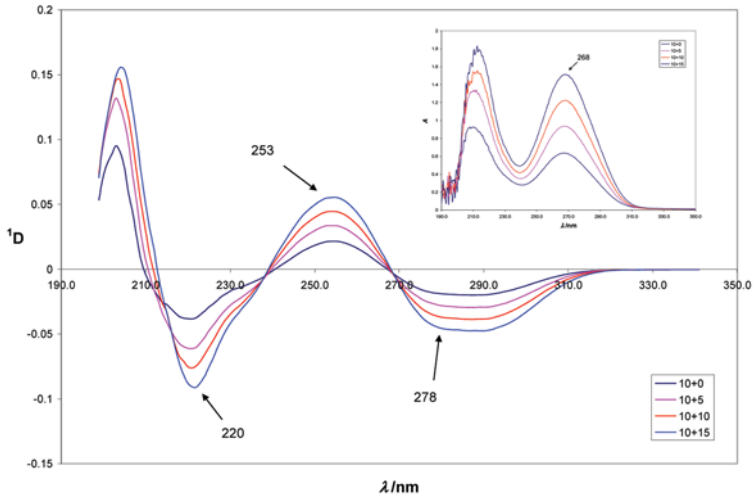


Figure 3. *First-order (and zero-order in small picture) derivative spectra of spiking solution (sample of Hemosul-P containing 10 μg mL^{-1} SMX + 0, 5, 10 and 15 μg mL^{-1} of standard solution SMX)*

Method Repeatability

The precision for the proposed methods were investigated by intra-day determination of six replicates [9] at working concentrations of 15 μg mL^{-1} SMX. The intra-day precisions are expressed as relative standard deviation and the data presented in Table 2 for Hemosul-P and Table 3 for Hemosul-S, respectively.

The obtained values for RSDs are compared with the maximal theoretical values (Horwitz criterion) calculated according to the declared content of SMX in both veterinary drugs.

Table 2. *Analytical Validation Parameters for Hemosul-P*

	A_{268}	$^1D_{220}$	$^1D_{253}$	$^1D_{278}$
Accuracy				
Recovery/%	100.82-101.45	93.53-110.60	98.80-103.59	96.14-96.23
Repeatability				
m(SMX) / mg	107.27	108.69	89.18	107.02
n	6	6	6	6
SD / mg	2.09	4.48	2.15	1.78
RSD / %	1.94	4.12	2.41	1.66
Horwitz criterion RSD ≤ 4.00 %				

The results for repeatability are quite expected, from the perspective of the previous test accuracy. For Hemosul-P, the results for the content of sulfamethoxazole obtained from the derivative signal $^1D_{220}$ are unsatisfactory according to the Horwitz criteria (4.00 % in case of Hamosul-P). The best results with lowest RSD were obtained by absorbance at 268 nm and the derivative signal at 278 nm.

In Hemosul-C, problems with insufficient homogeneity of the samples came to full expression. As a result, any RSD value, except that the $^1D_{278}$, failed Horvitz criterion, which in this case is lower (3.40 %) because of higher content of SMX in Hemosul-S, compared with the same in Hemosul-P.

Table 3. *Analytical Validation Parameters for Hemosul-S*

	A_{268}	$^1D_{220}$	$^1D_{253}$	$^1D_{278}$
Accuracy				
Recovery/%	96.62-103.48	66.77-103.59	84.83-88.08	87.51-93.77
Repeatability				
m(SMX) / mg	171.82	247.67	176.04	200.27
n	6	6	6	6
SD / mg	7.30	28.40	7.81	4.88
RSD / %	4.25	11.47	4.44	2.44
Horwitz criterion RSD ≤ 3.40 %				

Generally, it can be concluded that the best results are obtained from derivative signal $^1D_{278}$. This was expected and logical and is strongly related to previous conclusions for the calibration curves on different wavelengths (Table 1). Accordingly, it is confirmed that the possible effects of matrix or excipients are eliminated by application of first-order derivative spectroscopy *vs*. the classical (zero-order) spectroscopy.

CONCLUSION

The proposed UV method is suitable for quantitative analysis of commercial veterinary formulation Hemosul-P and Hemosul-S. Statistical analysis showed the method is accurate and precise. There was no interference from excipients in the tablets. Since there is no need for sample preparation or sophisticated apparatus, the proposed methods may be used successfully applied for quantitative determination of sulfamethoxazole, for performing the official quality control. The results obtained for the analyzed compound are in a very good agreement with the declared contents.

REFERENCES

1. Craig, G. R. (1972). The place for potentiated trimethoprim in the terapy of diseases of the skin in dogs and cats. *J. Small Animal Pract.* 13: 65−70.
2. Otman, S., (1990). Multicomponent derivative spectroscopic analysis of sulfamethoxazole and thrimethoprim, *International Journal of Pharmaceutic* 63(2): 173−176.
3. Samsa, F., Amani L. (2006). Determination of Sulfamethoxazole and Trimethoprim in Pharmaceuticals by Visible and UV Spectrophotometry, *Iranian Journal of Pharmaceutical Research* 1:31−36.
4. Hassounaa, M.E.M., (1997). Simultaneous Spectrophotometric Determination of Sulfamethoxazole and Trimethoprim in Drugs. *Analytical Letters* 30(13): 2341−2352.
5. Balyejjusa, S., Adome, R.O., Musoke, D. (2002). Spectrophotometric determination of Sulphamethoxazole and Trimethoprim (co-trimioxazole) in binary mixtures and in tablets, *African Health Sciences* 2(2): 56−62.
6. Injac, R., Kac, J., Rajic, K.K., Strukelj, B. (2008). Optimal Conditions for Extraction and Simultaneous Determination of Sulfamethoxazole and Trimethoprim in Pharmaceuticals by Micellar Electrokinetic Capillary Chromatography. *Journal of Food and Drug Analysis* 16(1): 18−25.
7. Himavani, K.V., Sudheer, M., Naresh, G., Padma, A., Ramalingam, P. (2011). Development of New Validated Spectrophotometric method for the estimation of Sulphamethoxazole & its application to protein binding studies, *International Journal of Pharma and Bio Sciences* 2(1): 565−570.
8. Nagaraja, P., Yathirajan, H.S., Raju, C.R., Vasantha, R.A., Nagendra, P., Kumar, M.S. H. (2003). 3-Aminophenol as a novel coupling agent for the spectrophotometric determination of sulfonamide derivatives, *Il Farmaco* 58(12): 1295–1300.
9. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology. Q2(R1). (2005). International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use.
10. Horwitz, W. (1982). Evaluation of analytical methods used for regulation of foods and drugs. Anal. Chem. 54(1): 67A−76A.

КОНТРОЛА НА КВАЛИТЕТ НА ПОТЕНЦИРАНИ СУЛФОНАМИДНИ КОМЕРЦИЈАЛНИ ВЕТЕРИНАРНИ ФОРМУЛАЦИИ СО УВ-СПЕКТРОФОТОМЕТРИЈА

Михајловиќ Јелена [1], Димитриеска-Стојковиќ Елизабета[2], Велев Ромел[2], Стојковиќ Горан[1]

[1]Институт за Хемија, Природно-математички факултет, Универзитет "Св. Кирил и Методиј", Скопје, Република Македонија
[2]Институт за храна, Факултет за ветеринарна медицина – Скопје, Универзитет "Св. Кирил и Методиј", Скопје, Република Македонија

*Автор за коресподенција: goranst@pmf.ukim.mk

АПСТРАКТ

Комбинацијата на сулфометоксазол и триметоприм во фиксен однос во различни комерцијални препарати, многу често се користи во хемотерапевската пракса во ветеринарната медицина. Како во хумаката, така и во ветеринарната медицина овие формулации на активни компоненти најчесто се нарекуваат "потенцирани сулфонамиди" кои мораат да бидат со добар квалитет, безбедни и ефикасни.

За сигурна контрола на активната компонента, од суштинско значење е применување на селективни, точни и прецизни аналитички постапки. Најчесто користени методи, кои вклучуваат специфична подготовка на примерокот, се течна хроматографија и спектрофотометрија. Во ова истражување развиен е едноставен УВ-ВИС спектрофотометриски метод за определување на содржината на сулфометоксазол во присуство на триметоприм.

Овој метод има одлична линеарност во концентрацискиот опсег од 1-30μg⁄mL. Коефициентот на корелација се движи од 0,9997 до 0,9999, одејќи од класична кон деривативна УВ спектроскопија. Со статистичката анализа утврдено е дека овој метод има задоволителна точност и прецизност во согласност со критериум на Horwitz, каде RSD<4,00% за комерцијални ветеринарни медицински производи Hemosul-P (прашок за орална примена) и RSD<3,40% за Hemosul-S (инјекција). Со примена на предложената постапка, можно е да се изврши селективна анализа на сулфометоксазол во фармацевските препарати, без отстранување на екципиенси. Бидејќи не постои потреба за подготовка на пробата и за софистицирани апарати, опишаниот метод претставува прилично евтина постапка за квантитативно определување на сулфометоксазолот во овие ветеринарни медицински производи. Добиенитие резултати се во согласност со декларираната содржина.

Предложениот УВ метод е погоден за користење на контрола на квалитетот на овие ветеринарни медицински производи во смисла на обезбедување на соодветна и ефикасна администрација на лекарства. На крајот, ова резултира со присуство на безбедно ниво на резидуи од лековите во храната од животинско потекло.

Клучни зборови: потенцирани сулфонамиди, сулфометоксазол⁄триметоприм, УВ спектрофотометрија, ветеринарни медицински производи

UDC: 636.09:[615.33.074:543.422.3

COMPARISON OF SPECTROPHOTOMETRIC AND COMPLEXOMETRIC – SPECTROPHOTOMETRIC ASSAY FOR DETERMINATION OF OXYTETRACYCLINE IN VETERINARY DRUGS

Naumoska Marina[1,3], Dimitrieska-Stojković Elizabeta [2], Stojković Goran[1]

[1]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Skopje, Republic of Macedonia*
[2]*Institute for Food, Faculty for Veterinary Medicine – Skopje, University "Ss. Cyril and Methodius", Republic of Macedonia*
[3]*Quality Control Department, REPLEK FARM, Ltd, Skopje, Republic of Macedonia*

*Corresponding author: mnaumoska@gmail.com

ABSTRACT

Two parallel methods for assay determination of oxytetracycline (OTC) in a final pharmaceutical product were optimized and compared. Both methods include solid-liquid extraction; the first one represents a simple extraction procedure; and the second employed Zirconium (IV) ion to obtain yellow-colored complex with OTC (1:4), whose composition was previously determined with application of the spectrophotometric method of Job. Water was chosen as a solvent in both of the cases, because of its availability; and because it is more ecological and cheaper solvent. The assay was determined UV-spectroscopically, with a VARIAN Carry Win 50 UV/Visible spectrophotometer, in a 1-cm cell at wavelength range 190−500 nm, with resolution 0.5 nm and scan rate of 300 nm/min.

The linear calibration function was established in the concentration range 1 to 40 μg/mL of OTC in respect of both cases (without and with Zr(IV) application). Of the analyzed data the following results for parameters were obtained linearity (both cases $R^2 > 0.999$), accuracy (without Zr - Recovery = 92.0−94.0 %; with Zr – Recovery = 97.0−100.0 %), sensitivity (LOD = 0.27 μg/mL; LOQ = 0.89 μg/mL without; and LOD = 0.58 μg/mL and LOQ = 1.96 μg/mL Zr(IV) addition) and precision (RSD ≤ 2.0 %) in the respective linear concentration ranges.

Both of the presented methods offer the simplicity needed for testing a large numbers of samples, but adding Zirconium is crucial in the assay determination procedure in order to exclude the possible interferences of the excipients or other active ingredients (in case of combined dosage forms) in the tested specimens, which improves the sensitivity, accuracy and the precision of the method.

Key words: UV-VIS spectroscopy, Oxytetracycline -Zirconium complex, method optimization and validation

INTRODUCTION

Since their isolation from different *Streptomyces* species in the late 1940s and early 1950s, the tetracyclines are widely and commonly used broad spectrum antibiotics in humans, to treat different diseases (acne and skin infections; systemic infections of the respiratory, urinary and gastrointestinal tract etc.), and also have a common and important application in veterinary medicine.

Tetracyclines, generally act as bacteriostatic antibiotics, by inhibiting the protein synthesis by reverse binding the 30S ribosomal subunits of susceptible organisms, and preventing access of aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex. Tetracyclines also are believed to reversibly bind to 50S ribosomes and additionally alter cytoplasmic membrane permeability in susceptible organisms. In high concentrations, tetracyclines can also inhibit protein synthesis by mammalian cells [1].

Due to its wide antibacterial spectrum, Oxytetracycline (OTC) is a common antibiotic used to treat different food-producing animals such as cattle, pigs, sheep and polutrty, as well as in dogs and cats and fish. Usually it is administrated orally with feed dosage rate of 25−700 mg/kg [1, 2].

Quality assurance and control of OTC in the veterinary products is important in order to prevent overdosage/toxicity or lack of the therapeutical effect; and usually performed with validated and standardized analytical methods. The official valid United States Pharmacopoeia monographs for OTC (and OTC hydrochloride) and British Pharmacopoeia, current edition, state High Performance Liquid Chromatography with UV-detection, as an analytical procedure for quantification of our analyte of interest. Other HPLC techniques include Photodiode array, MS or electrochemical detection. Also throughout the literature there are other methods reported for OTC determination, such as the ones based on microbiological assay which is a procedure with limitations (pH-dependence, low sensitivity, low stability and time consumption). Metal-chelate affinity chromatography, TLC with UV- or FL-detection, UV/FL-spectrophotometry (with or without derivations) are also reported and employed; as well as electrophoresis [2, 3, 4].

Spectrophotometric determination of tetracyclines with uranyl acetate, among which is OTC as well, was reported as well as by using sodium molibdate as analytical reagent [5, 6].

The aim of our study was to develop a simple, accurate, fast, sensitive and economical method for determination of OTC in veterinary drugs that can be readily

used in every day practice in the analytical and quality control laboratories. That is why two parallel methods of OTC determination in a final pharmaceutical (veterinary) product were optimized, then validated, compared and evaluated.

MATERIALS AND METHODS

Apparatus and spectrophotometric conditions

For content determination of OTC, two spectrophotometric methods were developed, which include "VARIAN Carry Win 50" UV-VIS-spectrophotometer, 1-cm quartz cell at wavelength range 190 - 500 nm, with resolution 0.5 nm and scan rate of 300 nm/min.

Chemicals and Reagents

The Oxytetracycline hydrochloride standard (98.1 %) was supplied from Sigma Aldrich. The water that was used as a solvent was de-ionized water supplied form the Faculty of Veterinary Medicine – Skopje, Institute for Food. Zirconium and Methanol gradient grade for liquid chromatography LiChrosolv® Reag. Ph. Eur, are supplied from Merck.

Commercial veterinary formulation

Two combined veterinary drugs (soluble oral powders) were tested: Neosulfox P where OTC represents 4 % of the powder (1 g powder contains 100 mg of Sulfadimidine, 60 mg Neomycin sulphate, 40 mg Oxytetracycline Hydrochloride) and Geomycin, where OTC represents 5 % of the powder (1 g powder contains 50 mg Oxytetracycline in form of hydrochloride and 35 mg Chlorhexidine digluconate).

Preparation of Standard Stock Solutions of OTC:

• OTC Standard Stock Solution:

20 mg of Oxytetracycline hydrochloride RS precise weight is transferred into 20 ml volumetric flask, then 12 mL of Methanol is added, mechanically shaken to achieve complete dissolution (sonicated if necessary) and filled up to the mark with the same solvent; concentration of about 1 mg/mL Oxytetracycline RS.

Then by applying suitable dilutions with water, deionized, calibration standard solutions for the range of the method were prepared, i.e. from 1 to 40 µg /mL.

• Zirconium – OTC (1:4) Standard Stock Solution:

The basic stock solution is prepared identically as previously mentioned with the difference when making the dilutions into suitable concentration range, i.e. from 1 to 40 µg/mL, 100 µL of the Zirconium(IV) solution is added to produce yellow colored complex. The solvent is water, de-ionized.

Test solution(s) preparation

Of the previously fine powdered granulates of the Neosulfox P and Geomycin, a quantity of the homogenous powder equivalent to 5 mg OTC hydrochloride and OTC respectively is weighed and transferred quantitatively into 50 mL volumetric flask. 30 mL water, deionized is added and the sample is sonificated about 20 minutes and mechanically shaken 15 minutes and then filled up to the mark with the same solvent.

The test solution then is filtered, discarding the first 10 ml of the filtrate.

The filtrate is then used for preparation of suitable dilutions for further testing.

Procedure

Both methods include solid-liquid extraction; the first one represents a simple extraction procedure; and the second emloyes Zirconium(IV) ion to obtain yellow-coloured complex with OTC (1:4), whose composition was previously determined with application of the spectrophotometric method of Job.

When performing the analysis with Zirconium, all the test solutions preparation steps are the same as previously, with a difference in the final dilutions, when Zr(IV) solution is added and then filled to the mark with water.

Data analysis

For determination of the statistical parameters, the Microsoft Office Excel is used.

RESULTS AND DISCUSSION

All of the analytical validation parameters for this proposed method were determined according to the ICH guidelines. A parallel summary for both veterinary drugs of the obtained validation parameters are presented in Table 1a and 1b. The comparison between the spectral curves of OTC standard solutions without and with Zirconium(IV) ion is presented in addition (Figure 1) [5, 8, 9].

1. LINEARITY& RANGE

The Linearity of the both spectroscopic methods was determined at ten concentration levels ranging from 1-40 µg Oxytetracycline hydrochloride RS/mL (1, 2, 3, 5, 10, 12, 15, 20, 30 and 40 µg/mL) and calibration curve was constructed for both cases by plotting the respective concentrationsvs. detector's response i.e. absorption maxima. Linear correlation in the above mentioned concentration range was confirmed with the coefficient of correlation of R^2 =1.000 in case of simple extraction without, and R^2 = 0.9998 in the case with Zirconium(IV) addition.

2. ACCURACY/ Analytical recovery

The Accuracy of the method with the study of analytical recovery was performed and confirmed at three concentration levels (5 -15 µg /mL) for both cases, by spiking the various pre-analyzed sample formulations of OTC with known quantities of OTC standard solution, and then analyzing the mixtures by the proposed method. The recovery results were found to be in the range of 92.0 - 94.0 % without and 97.0-100.0 % with Zirconium(IV) addition.

3. SENSITIVITY

Both the Limit of Detection (LOD) determination at lowest concentration giving response and Limit of Quantification (LOQ) determination were estimated from the standard deviation of the response and the slope, based on the data of the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.27 µg/mL and 0.89 µg/mL without; and 0.58 µg/mL and LOQ = 1.96 µg/mL, respectively, with Zirconium(IV) addition. The LOD and LOQ showed that both of the methods are sensitive for OTC determination.

4. PRECISION/ Repeatability

The method repeatability was performed using 9 de-

terminations covering the specified range of the procedure i.e. three concentrations with three replicates each. The obtained RSD is lower than 2.0 %, i.e. Geomycin 1.69 % without and 1.06 % with Zirconium(IV) addition and for Neosulfox P, 1.22 % and 1.49 % respectively. The calculated RSD value for Neosulfox P according to Horwitz [8] is 4.80 % and for Geomycin 4.60 %.

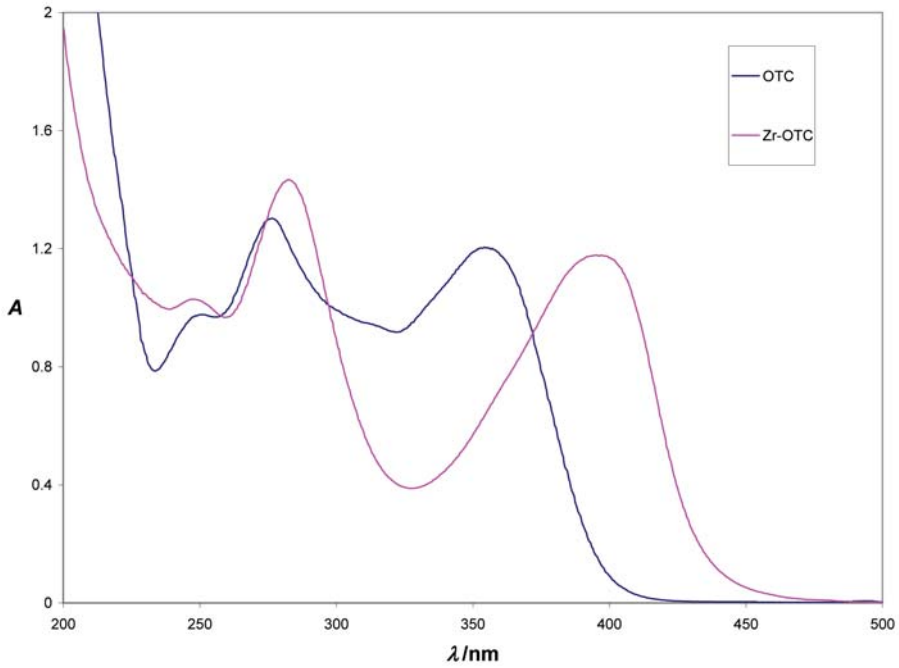


Figure 1. *Comparison between the UV spectrum of OTC standard solutions without (λmax = 275 and 350 nm) and with Zirconium(IV) ion is presented (λmax. = 280 and 395 nm)*

It can be noted that the spectral curves of OTC-Zr-complex are more defined than the ones of OTC solely. For results evaluation in both cases the data obtained from λmax. = 350 nm for OTC and 395 nm for OTC-Zr were used.

Table 1a. *Validation summary for NEOSULFOX P, without and with Zirconium(IV) introduction into the standard/test solution.*

Analytical technique		UV-Spectrophotometry	
Type of apparature for validation		UV – VIS Spectrophotometer VARIAN, Carry Win 50	
Validation parameters	**Acceptance criteria**	**Results**	
		Without Zirconium(IV)	With Zirconium(IV)
RANGE:	min. acceptable 80 – 120 %	1 to 40 µg/mL of OTC Oxytetracycline hydrochloride RS / mL	1 to 40 µg/mL of OTC Oxytetracycline hydrochloride RS / mL
LINEARITY: Correlation coefficient R^2:	≥ 0.9900	R^2 =1.0000	R^2 =0.9998
SENSITIVITY: LOD		0.27 µg/mL	0.58 µg/mL
LOQ		0.89 µg/mL	1.96 µg/mL
ACCURACY: Recovery:	95 – 105 %	Recovery = 93.6 %	Recovery = 97.02 %
PRECISION: (method repeatability)	RSD≤ 4.80 %	s = 0.1117 RSD = 1.22 %	s = 0.1517 RSD = 1.49 %

Table 1b: *Validation summary for GEOMYCIN, without and with Zirconium(IV) introduction into the standard/test solution.*

Analytical technique		UV-Spectrophotometry	
Type of apparature for validation		UV – VIS Spectrophotometer VARIAN, Carry Win 50	
Validation parameters	**Acceptance criteria**	**Results**	
		Without Zirconium(IV)	With Zirconium(IV)
RANGE:	min. acceptable 80 – 120 %	1 to 40 µg/mL of OTC Oxytetracycline hydrochloride RS	1 to 40 µg/mL of OTC Oxytetracycline hydrochloride RS
LINEARITY: Correlation coefficient R^2:	≥ 0.9900	R^2 =1.0000	R^2 =0.9998
SENSITIVITY: LOD		0.27 µg/mL	0.58 µg/mL
LOQ		0.89 µg/mL	1.96 µg/mL
ACCURACY: Recovery:	95 – 105 %	Recovery = 92.18 %	Recovery = 97.21 %
PRECISION: (method repeatability)	RSD≤ 4.60 %	s = 0.1240 RSD = 1.69 %	s = 0.0970 RSD = 1.06 %

CONCLUSION

Comparing the results obtained with statistical evaluation from both proposed methods for spectrophotometric content determination of Oxytetracycline in the combined veterinary drugs, it can be concluded that the method where Zirconium(IV) ion is added, is more selective, sensitive, accurate and reproducible, than the one that only includes simple extraction procedure with water, because when the OTC-Zr complex is made the absorption maxima excludes the possible additive effects and interferences of the other active compounds, as well as the excipients within the formulation.

REFERENCES

1. Plumb, Donald C. (1999). *Veterinary drug handbook*, 3rd Ed. Iowa State University Press, USA.
2. Fernandez-González, R., García-Falcón, M.S., Simal-Gándara, J. (2002). Quantitative analysis for oxytetracycline in medicated premixes and feeds by second-derivative synchronous spectrofluorimetry. *AnalyticaChimica Acta* 455: 143−148.
3. British Pharmacopoeia (Veterinary) (2012). Vol. VI. Her Majesty's Stationery Office, London 2011, p. 179.
4. United States Pharmacopoeia, 32 NF27, p. 3178−3182.
5. Saha, U., Sen, A.K., Das, T.K. (1990). Spectrophotometric determination of tetracyclines in pharmaceutical preparations, with uranyl acetate. *Talanta* 37(12): 1193–1196.
6. Jelkić-Stankova, M., Veselinović, D., Malešev, D., Radović, Z. Spectrophotometric determination of oxytetracycline in pharmaceutical preparations using sodium molibdate as analytical reagent. (1989). *Journal of Pharmaceutical and Biomedical Analysis* 7(12): 1565−1570.
7. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology. Q2 (R1). (2005). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use.
8. Horwitz, W. Evaluation of analytical methods used for regulation of foods and drugs. *Anal. Chem.* (1982). 54(1): 67A−76A.
9. Validation of Analytical Methods and Procedures http://www.labcompliance.com/tutorial/methods/default.aspx

СПОРЕДБА НА СПЕКТРОФОТОМЕТРИСКИ И КОМПЛЕКСОМЕТРИСКО-СПЕКТРОФОТОМЕТРИСКА АНАЛИЗА ЗА ОПРЕДЕЛУВАЊЕ НА ОКСИТЕТРАЦИКЛИН ВО ВЕТЕРИНАРНИ ЛЕКОВИ

Наумоска Марина[1, 3]*, Димитриеска-Стојковиќ Елизабета[2], Стојковиќ Горан[1]

[1]*Институт за хемија, Природно-математички факултет, Универзитет "Св. Кирил и Методиј ", Скопје, Република Македонија*
[2]*Институт за храна, Факултет за ветеринарна медицина - Скопје, Универзитет "Св. Кирил и Методиј ", Скопје, Република Македонија*
[3]*Оддел за контрола на квалитетот РЕПЛЕК ФАРМ, ДООЕЛ, Скопје, Република Македонија*

*Автор за коресподенција: mnaumoska@gmail.com

АПСТРАКТ

Оптимизирани се и споредени два паралелни метода за определување на окситетрациклин (OTC) во финален фармацевтски производ. И двата метода вклучуваат цврсто-течна екстракција; првиот претставува едноставна екстракциона постапка а вториот метод вклучува цирконium (IV) јон за добивање на жолто обоен комплекс со OTC (во однос 1:4), чиј состав беше претходно утврден со примена на спектрофотометрискиот Job-метод. И во двата случаи, како растворувач беше користена водата заради нејзината достапност, ниската цена и занемарливиот ефект врз екологијата. Анализата беше направена со УВ-спектроскопија, со VARIAN Carry Win 50 UV/Visible спектрофотометар, во 1-cm кварцна ќелија во област на бранови должини од 190-500 nm, со резолуција 0,5 nm и брзина на скенирање 300 nm/min.

Со двата метода (без и со Zr(IV) јон) конструирана е калибрациона права во концентрациски опсег од 1-40 μg/mL OTC. Од анализираните податоци беа добиени следниве резултати за параметрите на линеарност (и во двата случаја R^2> 0,999), точност (без Zr – аналитички принос = 92,0-94,0%; со Zr(IV) - аналитички принос = 97,0-100,0%), осетливост (LOD = 0,27 μg/mL ; LOQ = 0,89 μg/mL , без Zr(IV) и LOD = 0,58 μg/mL и LOQ = 1,96 μg/mL со Zr(IV)) и прецизност (RSD ≤ 2,0%) во соодветниот линеарен концентрациски опсег.

Двата претставени метода ја нудат потребната едноставност за тестирање на голем број на примероци. Додавањето на цирконium-јон е од клучно значење во постапката за анализа на OTC, со цел да се исклучат можните пречки од ексципиентите или другите активни состојки (во случај на комбинирани дозирани форми) во тестираните примероци, со што се подобрува осетливоста, точноста и прецизноста на методот.

Клучни зборови: УВ-ВИС спектроскопија, окситетрациклин-цирконium комплекс, оптимизација и валидација

UDC: 636.09:[615.33.074:543.422.3-76

APPLICATION OF ZERO- AND FIRST-ORDER DERIVATIVE SPECTROSCOPY IN THE QUALITY CONTROL OF VETERINARY DRUGS

Trajkovska Violeta [1], Dimitrieska-Stojković Elizabeta [2], Stojković Goran[1]

[1]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius", Skopje, Republic of Macedonia*
[2]*Institute for Food, Faculty for Veterinary Medicine – Skopje, University "Ss. Cyril and Methodius", Republic of Macedonia*

Corresponding author: goranst@pmf.ukim.mk

ABSTRACT
Sulfonamides are antimicrobial substances that have wide application in veterinary medicine. Sensitive to sulfonamides are only the microorganisms that synthesize their own folic acid. Quality control of veterinary medical product is particularly important for assuring proper and effective drug administration resulting in presence of safe levels of residues in food of animal origin, knowing that sulfonamides are among the most frequently administered drugs at food producing animals.
Sulfadimidin, Trimetosul and Neosulfox are some of registered veterinary medicinal products (VMPs) in Republic of Macedonia that contain sulfonamides. Active substances are single or in combination with trimethoprim or other antimicrobial active substances, and frequently sophisticated and expensive analytical techniques are the first choices for quality control of those VMPs. In this study a simple, rapid accurate, economically acceptable and environmental friendly method, based on zero- and first-order UV spetrophotometry, for the determination of sulfonamides on veterinarian drugs has been developed. The methods have good linearity in the concentration range 1–20 μg/ml for sulfadimidine and 0.8–40 μg/ml for sulfafurazole. The calibration curves demonstrated correlation coefficients of 0.9997 and 0.9947, respectively.
Statistical analysis confirmed that the method has satisfactory accuracy and precision according to the Horwitz criterion, and the maximal RSDs of 2,34 % for Sulfadimidin (tablet), and 4.00 % for Neosulfox (oral powder) and Trimetosul (oral powder) have not been exceeded. The obtained results for active substances content are in a good agreement with the declared values.
Key words: veterinary drugs, sulfadimidine, sulfafurazole, UV derivative, spectrophotometry

INTRODUCTION

Sulfonamides are a group of organic compounds that have played an important role as chemotherapeutics of bacterial infections in veterinary medicine. The authorized MVPs such as Trimetosul, Sulfadimidin and Neosulfox are often used in human and veterinary medical applications. Sulfadimidin act as bacteriostatic, and the base is built from p-amino benzoic acid in the folic acid molecule. Trimetosul is combined powder of trimethoprim with sulfafurazole, whose antibacterial action is based on blocking the synthesis of folic acid. Neosulfox is bacteriostatic chemotheraputic drug with highly antibacterial activity. His action is based on blocking the enzyme reductase which participates in the synthesis of proteins.

In many cases tablets contain a mixture of sulfonamides (sulfafurazole, sulfadimidine, sulfamethoxazole and sulfanilamide) with trimethoprim or other antibacterial active substances (tetracyclines, neomycin). These combinations are commonly termed as "potentiated sulfonamide antimicrobial agents". Because by overdosing of these substances side effects may be caused, it is important to perform control and constant checking of their content of the active components.

Physicochemical characteristics of these compounds are determined and their pharmacokinetic behavior and distribution to the animal tissues, or animal products such as milk or eggs. In a model study with 11 sulfonamides differing in pK_a value and lipid solubility, their distribution *in vivo* between yolk and white was determined [1].

A simple and sensitive spectrophotometric procedure for the determination of sulfacetamide sodium (I), sulfadiazine (II), sulfadimidine (III) and sulfathiazole (IV) is based on the reaction of the drug with acetylacetone-formaldehyde reagent to give a yellow product has been utilized [2]. A chlorocoulometric method for determination of sulfadimethoxine and sulfafurazole in pharmaceutical preparation is proposed and can be applied for determination of small quantities of sulfafurazole and sulfadimethoxine [3].

Binary mixtures of sulfathiazole and oxytetracycline can be resolved by using derivative spectrophotometry. The mixture was determined in honey without previous separation when the fourth-order derivative spectrum was utilized for eliminating the background absorbance [4].

In another investigation a rapid, simple and sensitive spectrophotometric method for the determination of some sulfa drugs is described. The method is based on the formation of orange yellow colored azo product by the diazotization of sulfonamides [5].

Multicomponent derivative spectroscopic method at 280 and 294 nm was employed for determination of the sulfonamide in mixture [6]. A method for the simultaneous determination of sulfamethoxazole (SMX) and tri-

methoprim (TMP), based on a direct determination of SMX after diazotization and coupling with 2-naphthol by visible spectrophotometry and an indirect determination of TMP in the UV region by difference has been published [7]. Derivative spectrophotometric method is reported for simultaneous determination of sulfadimidine and trimethoprim without the need the prior separation [8].

The common availability of the instrumentation, the simplicity of the procedures, low expences, speed, precision and accuracy of the technique still make UV spectrophotometric methods attractive for quality control of VMPs. The subject of this paper work is development, validation and application of zero- and first-order UV spectrophotometric methods for determination of active components sulfadimidine and sulfafurazole in three medicinal products: Sulfadimidin, Trimetosul and Neosulfox.

MATERIALS AND METHODS

Standard stock solutions

Sulfadimidine (SDM) was purchased from Fluka and sulfafurazole (SFZ) from Farmbase. Standard stock solutions (1 mg mL^{-1}) were prepared by weighing 50 mg of standards and by dissolving in 50 mL 96 % ethanol (Riedel-de Haën). Applying suitable dilutions, spiking solution, in 96 % ethanol, for veterinary formulation samples with concentration of 100 μg mL^{-1} was prepared. Calibration standards for the UV method in the concentration range of 1−20 μg mL^{-1} for SDM and 0.8−40 for SFZ, respectively, are prepared using double deionized water.

Commercial veterinary formulations

Sulfadimidin tablet, Veterina d.o.o, Hrvatska − 2.5 g sulfadimidine in 1 tablet (3.7 g);

Neosulfox powder, FM Pharm, Srbija − 100 mg sulfadimidine, 60 mg neomycin sulphate and 40 mg oxytetracycline hydrochloride in 1 g powder;

Trimetosul powder, Veterina d.o.o, Hrvatska − 100 mg sulfafurazole and 20 mg trimethoprim in 1 g powder.

Sample solutions preparation

For the sample analysis, appropriate mass of Neosulfox and Trimetosul (equal to concentration of 100 μg mL^{-1} SDM and SFZ, respectively) was transferred into 50 mL flask, double deionized water was added and sonificated in the ultrasonic bath 30 for minutes in the case of Neosulfox and 1 hour for Trimetosul. Volume was completed with double deionized water. The content of the flask was mechanically shaken and filtrated through disk filter RC 0.45 μ for Neosulfox. In case of Trimetosul procedure without filtering were used.

For the Sulfadimidin sample analysis, an adequate mass portion was transferred into 100 mL flask, then added double deionized water and sonificated in the ultrasonic bath for 30 minutes. Volume was completed with double deionized water. In the following step, an aliquot of this solution was transferred into 25 mL flask (for desired concentration of 100 μg mL^{-1}) and the volume was filled up with double deionized water, mechanically shaken and filtrated through disc filter (RC 0.45 μ).

Spectrophotometric measurements and Data analysis

The UV spectra of the standard and sample solutions, and appropriate blanks were recorded on a Varian Carry 50 UV/Visible Spectrophotometer, at room temperature in 1 cm quartz cell, in the wavelength range from 190 to 350 nm, with resolution 0.5 nm and scan rate of 300 nm/min. Varian Carry software was used for the calculated derivative spectra and Microsoft Office Excel for determination of statistical parameters.

RESULTS AND DISCUSSION

1. Sulfadimidine (SDM)

The spectra of the standard solutions of SDM (Figure 1) are characterized by two overlapping absorption band, with maximum at 240 nm and 263 nm. Absorption band at lower wavelength (240 nm) is not suitable for quantitative determination. On the other hand, the spectral analysis clearly indicates that the absorbance band at the higher wavelength (263 nm) is more suitable for calibration curve construction (Table 1).

Because of the evident absorption bands overlapping, in order to eliminate possible interferences, and improve the method precision, the first-order derivative spectra were obtained (Figure 1).

The spectral analysis from first-order derivative spectra of SDM indicates that the obtained derivative signals at 233, 245, 256 and 276 nm are sufficiently well-defined for quantitative determination of the SDM. The linear correlation was obtained between the concentrations of SDM in the tested range and their corresponding absorbance's at 263 nm, as well as and for the derivative signals of four selected wavelengths. The equations of the calibration curves, correlation coefficients (R), number of data (n) and standard errors of estimate ($SE_{y,x}$) are listed in Table 1.

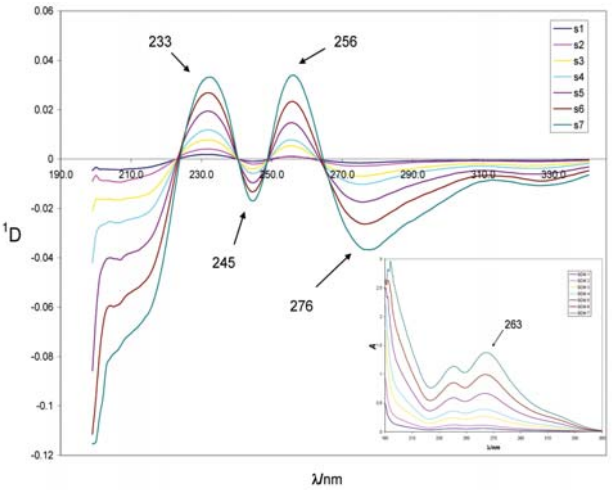


Figure 1. *[1]D and UV spectra (in small picture) of sulfadimidin of standard solution of SDM in concentration range 1–20 mg mL^{-1}*

Table. 1. *Equation of calibration curves for SDM using zero-order and first-order spectroscopy at different wavelengths*

Signal*	y=ax+b #	$SE_{y,x}$	R	n	γ(SDM)/ µg mL^{-1}
A_{263}	y = 0.0689x - 0.0171	0.0132	0.9997	7	1-20
$^1D_{233}$	y = 0.0017x + 0.0011	0.0010	0.9969	7	1-20
$^1D_{245}$	y = 0.0008x + 0.0006	0.0004	0.9980	7	1-20
$^1D_{256}$	y = 0.0017x - 0.0020	0.0008	0.9974	7	1-20
$^1D_{276}$	y = 0.0018x - 0.0007	0.0004	0.9996	7	1-20

* A – absorbance from UV spectra, ^{1}D – derivative signals from first derivative spectra

a – slope, b – intercept, y – A or ^{1}D, x – mass concentration = γ(SDM)

The data presented in Table 1 show that the highest coefficient of correlation was determined for the calibration line constructed from absorbances at 263 nm, and the derivative signal at 276 nm. The standard error of the estimate is the lowest for the derivative spectra data, indicating that the accuracy of prediction is highest from $^1D_{276}$ signal, compared with A_{263}.

Accuracy testing

To test the method suitability in terms of accuracy and precision, recovery experiments were conducted by adding known quantities of standard solution of SDM (5, 10 and 15 µgmL^{-1}) to the different sample formulations of SDM, and then the mixtures were analyzed by the proposed method. Figure 2 presents the spectra of sample of Sulfadimidin tablet spiked with a standard solution of SDM. The results from the recovery experiment are shown in Table 2. The obtained satisfactory values for the recoveries, except for the ones obtained from derivative signals $^1D_{233}$ and $^1D_{245}$, have proven that the method is accurate within the acceptable interval of recoveries (95−105%).

The same procedure was performed for Neosulfox and the corresponding results are given in Table 3. In this case, values for recovery are more variable, due to the complexity of the matrix. Namely, it contains oxytetracycline, which exhibits absorption maximum in the same wavelength range, resulting in a presence of certain interferences.

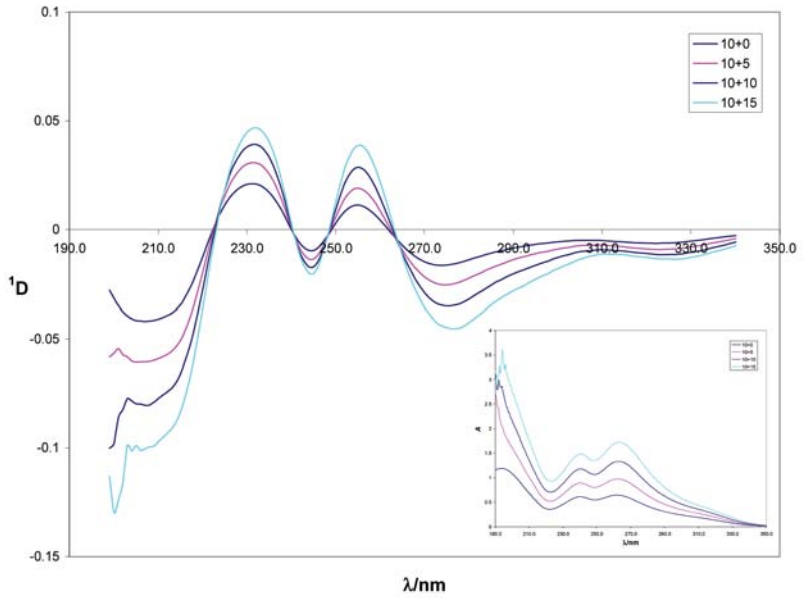


Figure 3. *First-order (and zero-order in small picture) derivative spectra of spiking solution (sample of Sulfadimidin tablet containing 10 µg mL^{-1} SDM + 0, 5, 10 and 15 µg mL^{-1} of standard solution SDM)*

Method Repeatability

The precision for the proposed methods were investigated by intra-day determination of six replicates [9] at working concentrations of 15 µg mL^{-1} of SDM. The intra-day precisions are expressed as relative standard deviation and the data presented in Table 2 for Sulfadimidin and Table 3 for Neosulfox, respectively.

The obtained values for RSDs are compared with the maximal theoretical values (Horwitz criterion) calculated according to the declared content of SDM in both veterinary drugs.

Table 2. *Analytical Validation Parameters for SDM in Sulfadimidin*

	A_{263}	$^1D_{233}$	$^1D_{245}$	$^1D_{256}$	$^1D_{276}$
Accuracy					
Recovery/%	100.03-104.42	99.15-109.11	89.93-92.57	97.19-102.72	103.43-108.75
Repeatability					
m(SDM) / g founded	2.59	3.24	2.77	1.90	2.49
n	6	6	6	6	6
SD / g	0.04	0.13	0.07	0.06	0.05
RSD / %	1.66	4.03	2.46	3.09	1.96
Horwitz criterion RSD ≤ 2.34 % Declared content = 2.5 g SDM in tablet (3.7 g)					

Table 3. *Analytical Validation Parameters for SDM in Neosulfox*

	A_{263}	$^1D_{233}$	$^1D_{245}$	$^1D_{256}$	$^1D_{276}$
Accuracy					
Recovery/%	101.54-102.05	104.73-109.96	92.91-109.91	95.55-104.90	103.41-112.71
Repeatability					
m(SDM) / mg founded	94.17	99.97	77.44	69.54	78.74
n	6	6	6	6	6
SD / mg	2.93	3.30	1.71	6.24	2.26
RSD / %	3.11	3.30	2.21	8.97	2.87
Horwitz criterion RSD ≤ 4.00 % Declared content = 100 mg SDM in 1 g powder					

However, if we want to choose an appropriate signal for both drugs, it undoubtedly would be the absorbance of 263 nm, i.e. classical UV spectrophotometry before derivatives would be a method of choice.

2. **Sulfafurazole (SFZ)**

On Figure 3 the first derivative spectra and the selected derivative signals of sulfafurazole standard solution at 216 nm and 289 nm for minimum and 240 nm for maximum, respectively are presented.

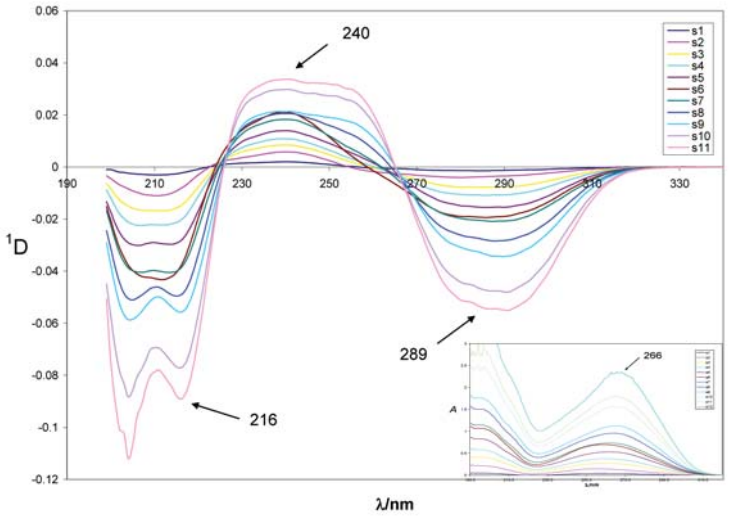


Figure 4. *[1]D and UV spectra (in small picture) of standard solution of SFZ in concentration range 0.8–40 µg mL[-1]*

The spectral analysis from zero- and first-order derivative spectra of SFZ indicates that the absorbance at 265 nm and obtained derivative signals at 216 nm and 289 nm are sufficiently well-defined for quantitative determination of the SFZ. The equations of the calibration curves, correlation coefficients (R), number of data (n) and standard errors of estimate ($SE_{y,x}$) are listed in Table 4.

Table. 4. *Equation of calibration curves for SFZ using zero-order and first-order spectroscopy at different wavelengths*

Signal*	*y*=a*x*+b #	$SE_{y,x}$	R	n	*γ*(SFZ)/ µg mL^{-1}
A_{265}	y = 0.0846x - 0.1438	0.0637	0.9947	13	0.8 - 40
$^1D_{216}$	y = 0.0038x - 0.0023	0.0024	0.9981	13	0.8 - 40
$^1D_{289}$	y = 0.0025x + 0.0002	0.0075	0.9732	13	0.8 - 40

A – absorbance from UV spectra, ^{1}D – derivative signals from first derivative spectra

a – slope, b – intercept, *y* – *A* or ^{1}D, x – mass concentration = *γ*(SFZ)

Accuracy testing

To test the method suitability in terms of accuracy and precision, recovery experiments were conducted by adding known quantities of standard solution of SFZ (3, 5 and 10 µg mL^{-1}) to the sample of Trimetosul, and then the mixtures were analyzed by the proposed method. The results from the recovery experiment are shown in Table 5. Given the acceptable interval of recoveries (95−105%), the obtained values indicate that the classical UV method is more accurate than derivative method.

Table 5. *Analytical Validation Parameters for SFZ in Trimetosul*

	A_{265}	$^1D_{216}$	$^1D_{289}$
Accuracy			
Recovery/%	100.68-103.86	91.12-112.93	101.43-108.28
Repeatability			
m(SFZ) / mg founded	92.52	110.42	92.98
n	9	9	9
SD / mg	1.54	2.94	1.54
RSD / %	1.66	2.66	1.65
Horwitz criterion RSD ≤ 4.00 % Declared content = 100 mg SFZ in 1 g powder			

Method Repeatability

The precision for the proposed methods were investigated by intra-day determination of nine replicates [9] at working concentrations of 10 μg mL^{-1} of SFZ. The intra-day precisions for Trimetosul are expressed as relative standard deviation and the data presented in Table 5. RSD values satisfy the criterion of Horwitz (RSD ≤ 4.00 %) calculated according to the declared value for SFZ in Trimetosul.

CONCLUSIONS

The proposed method is simple, fast and non-expensive for determination of sulfadimidine and sulfafurazole in commercial veterinary formulations Sulfadimidin, Neosulfox and Trimetosul. Generally, statistical analysis confirmed that the method is accurate and precise. The results obtained for the analyzed active substances are in a agreement with the declared contents. Since there is no need for sample preparation, the proposed methods may be successfully applied for performing the quality control of formulations mentioned above.

REFERENCES

1. Kan, C.A., Petz, M. (2000). Residues of veterinary drugs in eggs and their distribution between yolk and white. *J Agric Food Chem.* 48(12): 6397–403.
2. Amin, A.S., M.M., Zareh, (1996). Acetylacetone-formaldehyde reagent for the spectrophotometric determination of some sulfa drugs in pure and dosage forms, *Microchimica Acta*, 124(3-4): 227–233.
3. Nikolic, K., Medenica, M., (1990). Coulometric determination od sulfadimethoxine and sulfafurazole, *Acta Pol Pharm*,; 47(1-2): 11–3.
4. Salinas, F. Espinosa, J.J.Berzas, M. A. N. (1991). Simultaneous determination of sulfathiazole and oxytetracycline in honey by derivative spectrophotometry, 06071 Badajoz, Spain, *Microchemical Journal*, 43(3): 244–252.
5. Nagaraja, P., Yathirajan, H.S., Raju, C.R., Vasantha, R.A., Nagendra, P., Kumar, M.S. H. (2003) 3-Aminophenol as a novel coupling agent for the spectrophotometric determination of sulfonamide derivatives, *Il Farmaco,* 58 (12): 1295–1300.
6. Otman, S., (1990). Multicomponent derivative spectroscopic analysis of sulfamethoxazole and thrimethoprim, *International Journal of Pharmaceutic* 63(2): 173−176.
7. Samsa, F., Amani L. (2006). Determination of Sulfamethoxazole and Trimethoprim in Pharmaceuticals by Visible and UV Spectrophotometry, *Iranian Journal of Pharmaceutical Research* 1:31−36.
8. Zimmer, L., Czarnecki, W. (2010). Derivative spectrophotometric method for simultaneous determination of sulfadimidine and trimethoprim. *Annales Universitatis Mariae Curie-Sklodowska* XXIII (1, 3) Sectio DDD.
9. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology. Q2(R1). (2005). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use.
10. Horwitz, W. (1982). Evaluation of analytical methods used for regulation of foods and drugs. *Anal. Chem*. 54(1): 67A−76A.

ПРИМЕНА НА ДЕРИВАТИВНА СПЕКТРОСКОПИЈА ОД НУЛТИ И ОД ПРВ РЕД ВО КОНТРОЛА НА КВАЛИТЕТ НА ВЕТЕРИНАРНИТЕ ЛЕКОВИ

Трајковска Виолета[1], Димитриеска-Стојковиќ Елизабета[2], Стојковиќ Горан[1]

[1]Институт за Хемија, Природно-математички факултет, Универзитет "Св. Кирил и Методиј", Скопје, Република Македонија
[2]Институт за Храна, Факултет за ветеринарна медицина – Скопје, Универзитет "Св. Кирил и Методиј", Скопје, Република Македонија

Автор за коресподенција: goranst@pmf.ukim.mk

АПСТРАКТ

Сулфонамидите се антимикробни супстанции кои имаат широка примена во ветеринарната медицина. Чувствителни на сулфонамидите се само оние микроорганизми кои ја синтетизираат фолната киселина. Контролата на квалитетот на еден ветеринарен медицински производ е особено важна за обезбедување соодветна и ефикасна употреба на лекот што резултира со присуство на безбедно ниво на резидуи во храната од животинско потекло, имајќи го во предвид фактот дека сулфонамидите се помеѓу најчесто употребуваните лекови за животни од кои се произведува храна.

Sulfadimidin, Trimetosul и Neosulfox се некои од регистрираните ветеринарни медицински производи (ВМП) во Република Македонија кои содржат сулфонамиди. Активните супстанции се поединечни или во комбинација со триметоприм или други антимикробни активни супстанции, па според тоа софистицирани и скапи аналитички техники често се прв избор за контрола на квалитетот на овие ВМП.

Во оваа студија развиена е едноставна, брза, точна, економски и еколошки прифатлива метода за определување на сулфонамиди во ветеринарните лекови, базирана на дериватива УВ спектрофотометрија од нулти и од прв ред. Методите имаат добра линеарност во концентрациски опсег од 1–20 μg/mL за сулфадимидин и 0,8–40 μg/mL за сулфафуразол. Калибрационите криви покажуваат коефициент на корелација 0,9997 и 0,9947, соодветно.

Статистичката анализа потврдува дека методот има задоволителна точност и прецизност во согласност со критериумот на Horwitz, при што максималните RSD вредности од 2,34% за Sulfadimidin (таблета) и 4,00% за Neosulfox (прашак за орална примена) и Trimetosul (прашак за орална примена) не се надминати. Добиените резултати за содржината на активните супстанции се во добра согласност со декларираните вредности.

Клучни зборови: ветеринарни лекови, сулфадимидин, сулфафуразол, УВ дериватива спектрофотометрија.

UDC: 615.281:615.322

ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS ON SOME FOOD BORNE PATHOGENIC AND SAPROPHITIC BACTERIA

Ratkova Marija[1], Sekulovski Pavle[1], Jankuloski Dean[1], Angelovski Ljupco[1], Mojsova Sandra[1], Prodanov Mirko[1]

[1] Food Institute, Faculty of veterinary medicine – Skopje, University „Ss Cyril and Methodius„ – Skopje, R. Macedonia

ABSTRACT

The antimicrobial activity of some plants and their extracts are known for centuries, and they are still examined for detection of new effects and new applications in various industries.

Seven strains of bacteria (*S. enteritidis, S agona, S. pullorum, S. panama, E. coli, L. monocytogenes, St. aureus*) were exposed to the effects of ten different pure plant extracts : gallic acid, rutin, chrysin, chlorogenic acid, quercetin, catechin, epicathechin, naringin, naringenin and morin. The survey was conducted using the Muller Hinton agar and measuring of the inhibition zone of every extract in two concentrations using the disc diffusion method altered with wells.

There was no effect on the tested strain of *E.coli*, and also there was a very insensible effect on the strains of *Salmonella spp*. Only 2 substances (catechin and naringenin) with concentration of 10 µg. shown some notable inhibitory effect on *L. monocytogenes*, and there was an evident inhibitory effect from 5 extracts on *St. aureus*, and 3 of them (quercetin, gallic acid and naringenin) showed inhibition of 24, 26 and 26 mm. respectively for the extract concentration of 10 µg.

The examined plant extracts have some inhibitory effect, especially to the Gram positive bacteria, and they can be eventually used for applications as antimicrobial substances and preservatives of food in bigger concentrations.

Key words : extracts, bacteria, disc diffusion method, antimicrobial effect

INTRODUCTION

Because of the increasing antibiotic resistance of the bacteria, researches are done to find a new efficient source for their replacement, and consequently plant extracts are in the focus of the scientific interest for examining of their antiseptical and antimicrobial effects and the option of such their application (1).

Antimicrobial agents from plants and spices are used in the food to control spoilage, extend food`s shelf life and inhibit or prevent the growth of the microorganisms (2). There are more than 1340 known plants with defined antimicrobial compounds, and over 30.000 substances isolated from phenol-group containing plant-oil compounds that are used in the food industry (2). Some benefits of their antimicrobial usage are reducing the need of antibiotics, controlling the microbial contamination in the food, decreasing of the development of the antibiotic resistance of the microorganisms and strengthening of the animal and human immune cells.

There is a considerable potential for utilization of the plant antimicrobials in food, especially the application on fresh fruits and vegetables, for their improvement of the quality and nutritional value of the food, in addition to their strong antifungal and antibacterial effects. Natural antimicrobials are also used incorporated into the packaging materials, first of all to protect the food surface rather than the food itself.

Plant extracts with antimicrobial action are usually found in various types of fruits, herbs and vegetables. A group of flavones and organic acids, that have antibacterial properties are interesting for further and continuous examing of their effects on food borne bacteria, in order to wide the range of options for their use in the food industry. Gallic acid is a type of phenolic acid, an organic acid, found in gallnuts, sumac, witch hazel, tea leaves, oka bark, and other plants. Gallic acid acts an antioxidant and shows cytotoxicity against cancer cells, without harming healthy cells. Gallic acid is used as a remote astringent, for treating albuminuria and diabetes, psoriasis and external haemorrhoids (8). Rutin is a flavonoid and contains approximately a half concentration of quercetin, and it is found in apricots, buckwheat, cherries, prunes, rose hips, the whitish rind of citrus fruits, and the core of green peppers. Naringin is a flavanone glycoside. It is a major flavonoid in grapefruit and gives the grapefruit juice its bitter taste. It is metabolized to the flavanone naringenin in human organism. Naringenin occurs naturally in citrus fruits. Chrysin is a naturally occurring flavone chemically extracted from the blue passion flower (*Passiflora caerulea*) and honeycomb in small amounts. Chlorogenic acid (CGA) is one of the major phenolic compounds identified in peach, prunes and coffee. Known as antioxidant, also slows the release of glucose into the bloodstream after a meal, and has laxative effect in prunes. It is a food additive used in coffee, chewing gum, and mints. Quercetin is a plant-derived flavonoid found in fruits, vegetables, leaves and grains. It also may be used as an ingredient in supplements, beverages or foods. Catechin is a simple flavonol, a type of natural phenol and antioxidant. It is found in peach, vinegar, and as natural phenols in argan oil. Morin is a yellow color substance that can be isolated from *Maclura pomifera* (Osage orange), *Maclura tinctoria* (old fustic) and from leaves of *Psidium guajava* (common guava).

Having all these information in mind, the aim of this study was to evaluate and compare the antimicrobial effects of 10 plant extracts to seven common food borne bacteria, both pathogenic and non-pathogenic.

MATERIALS AND METHODS

For the purposes of this study, disc diffusion method (recommended from FDA) was used with variation of making wells in the agar plates instead of using discs (2,3). The bacterial strains which were used were reference materials : *S. agona, S. enteritidis* NCBB 100284*, S panama, E coli* NCCB 100297 *, L. monocytogenes* NCCB 100286 and *St. aureus* NCCB 100294 (Food consumer and Product safety Authority, VWA, the Netherlands), only *S. pullorum* was isolated and stored at -20 ° C in our laboratory for food and feed microbiology at the Faculty of veterinary medicine - Skopje . All of the strains were in concentrations of 10^5 cfu/ml.

The plant extracts were in pure substance. For the cold extraction, there were used 5 gr. of the substance and mixed with 45 ml. 75% ethanol, after that were evaporised with nitrogen on 40º C. Then for every extract, 10 ml. of destilated water was added and they were filtered. At the end a concentrations of 5 and 10 µg were gained and used in the extracts study.

For the well diffusion method, Muller Hinton agar (Oxoid) was used, the bacterial suspensions of 10^5 cfu/ml were suspended in the agar which was molten to 48º C, and then was poured into plates, 15-20 ml. in each. After 20 minutes the medium was solidified and the wells were made with sterile cylinder with diameter of 6 mm.. With sterile pipette in the wells were transferred 100 µl of the exctracts with the concentration of 5 µg and then on other plate of 10 µg, and were incubated on 37ºC for 24 h (4). After the incubation, the zone of inhibition was measured together with the diameter of the wells. For the quality control, one well was filled with sterile water, and also one with solution of gentamicin with concentration of 10 µg (Oxoid).

RESULTS

The results of the examination showed that the Gram positive bacteria have more susceptibility towards the extracts than the Gram negative. *E .coli* was the only strain that showed resistance for the two concentrations of all examined extracts. From the rest of the strains, *S. panama* showed the lowest susceptibility for the extracts, only to quercetin. The results of the well diffusion test and inhibition zones of the ten examined extracts can be seen in Table 1.

Table 1. *Inhibition effect of the examined extracts*

		PLANT EXTRACT (diameter of inhibition zone in mm.)									
Bacterial strain	Concentr. of the extract	Gallic acid	Rutin	Chrysin	Chlorogenic acid	Quercetin	Catechin	Epicathechin	Naringin	Naringenin	Morin
S. enteritidis	5 µg	0	0	0	0	0	0	0	0	8	0
	10 µg	0	0	0	0	0	0	0	0	14	0
S. agona	5 µg	0	0	0	0	0	0	0	0	7	0
	10 µg	0	0	0	0	0	8	0	0	10	0
S. pullorum	5 µg	0	0	0	0	8	0	0	0	7	0
	10 µg	0	0	10	0	10	0	0	0	10	0
S. panama	5 µg	0	0	0	0	0	0	0	0	0	0
	10 µg	0	0	0	0	8	0	0	0	0	0
E. coli	5 µg	0	0	0	0	0	0	0	0	0	0
	10 µg	0	0	0	0	0	0	0	0	0	0
St. aureus	5 µg	12	10	8	8	12	10	8	7	16	0
	10 µg	26	14	16	10	24	18	10	8	26	0
L. monocytogenes	5 µg	0	0	7	0	8	8	0	0	10	0
	10 µg	0	8	12	0	12	18	0	8	20	0

The only extract that gave no inhibition to any of the bacteria was morin. *L. monocytogenes* showed susceptibility towards rutin, chrysin, naringin, quercetin and more evident towards catechin and naringenin.

All the exctracts except morin, showed antimicrobial activity towards *St. aureus*, and the most notable was for gallic acid, quercetin, catechin and naringenin.

Naringenin was the extract that gave the most remarkable antimicrobial effect in both concentrations, towards 5 of the examined bacteria, and had best effect on *L. monocytogenes* and *St aureus*.

CONCLUSION

Some of the tested extracts were chosen as substances which already have use in the pharmacology and in the food industry as food additives. They can be found in various fruits, vegetables and herbs used as tees. They belong in the group of flavones and organic acids with some kind of antimicrobial action against the microorganisms.

After the analyzing of the results of the study for the antimicrobial effects of the 10 extracts, it can be concluded that they have shown a type of antimicrobial activity (dependending on the concentrations and the individial properties of the bacterial strains) especially on the Gram positive bacteria, but there is also a slight activity towards the *Salmonella* strains, which is showing a option for antimicrobial effect with bigger concentrations of the extract. It is known that plant substances affect microbial cells by various antimicrobial mechanisms, including attacking the phospholipid bilayer of the cell membrane, disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids (2,7). Generally, Gram-negative bacteria are less sensitive to the antimicrobials because of the lipopolysaccharide outer membrane of this group, which restricts diffusion of hydrophobic compounds. However, this does not mean that Gram-positive bacteria are always more susceptible. Gram-negative bacteria are usually more resistant to the plant-origin antimicrobials and even show no effect, compared to Gram-positive bacteria (2,7).

The best results had quercentin with inhibitory effect on 4 and naringenin with inhibitory effect on 5 bacterial strains. Naringenin had the most visible antimicrobial effect, especially towards *L. monocytogenes, St aureus,* and it was the only extract that had inhibitory action on three Gram negative bacteria : *S. enteritidis, S agona* and *S. pullorum*. So they can be considered for their antimicrobial activity, and used in bigger concentrations for more effective inhibition on the food borne bacteria.

It can be concluded that the herb extracts may provide alternatives and be used as supplements to conventional antimicrobial additives in foods, food preserving substances and disinfectants (3,5). But if this is the case there must be a control in the use because of the possible changes of the flavor due to their application.

Therefore it is in the biggest importance to understand how these extracts affect on the taste of food, quality of food and especially on the undesired microbial growth and spoilage of the food products, so it can lead us to new technologies for their applications in maintaining of the food safety and quality (6).

REFERENCES

1. Alipour M., Khanmohammadi O. : Antibacterial activity of plant extracts against oral and skin pathogens, African Journal of Microbiology Research vol. 5 (19), 2011.
2. Tajkarimi M.M., Ibrahim S.A. and Cliver D.O. : Antimicrobial herb and spice compounds in food, Food Control 21, 2010.
3. Dorman H.J.D. and Deans S.G.: Antimicrobial agents from plants:antibacterial activity of plant volatile oils, Journal of Applied Microbiology vol. 88 pp 308-316, 2000.
4. Hsieh P.-C., Mau J.-L., Huang S.-H. : Antimicrobial effects of various combinations of plant extracts, Food Microbiology vol. 18, 2001.
5. Eldayyar M., Draughon F.A., Golden D.A. and Mount J.R. : Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms, Journal of Food Protection 64 (7), 2001
6. Min B. R., Pinchak W.E., Merkel R., Walker S., Tomita G. and Anderson R.C.: Comparative antimicrobial activity to tannin extracts from perennial plants on mastitis pathogens, Scientific research and Essay, vol. 3 (2) pp. 66-73, 2008.
7. Wei L. S. et al. : Antimicrobial properties of tropical plants against 12 pathogenic bacteria isolated from aquatic organisms, African Journal of Microbiology Research vol. 7 (13), 2008.
8. LD Reynolds and NG Wilson, “Scribes and Scholars” 3rd Ed. Oxford: 1991. pp193–4

АНТИМИКРОБНА АКТИВНОСТ НА РАСТИТЕЛНИ ЕКСТРАКТИ ВРЗ ОДРЕДЕНИ ПАТОГЕНИ И САПРОФИТСКИ БАКТЕРИИ КОИ ШТО ПОТЕКНУВААТ ОД ХРАНА

Раткова Марија[1], Секуловски Павле[1], Јанкулоски Деан[1], Ангеловски Љупчо[1], Мојсова Сандра[1], Проданов Мирко[1]

[1]*Институт за храна, Факултет за ветеринарна медицина – Скопје, Универзитет „Св. Кирил и Методиј„ – Скопје, Р. Македонија*

АПСТРАКТ

Антимиробната активност на некои растенија и нивните екстракти се добро познати со векови, и тие се уште се испитуваат со цел да се откријат нови ефекти и нови примени во различни индустрии.

Седум соеви на бактерии (*S. enteritidis, S agona, S. pullorum, S. panama, E. coli, L. monocytogenes, St. aureus*) беа изложени на дејството на десет чисти растителни екстракти: галична киселина, рутин, хрисин, хлорогена киселина, кверцентин, катехин, епикатехин, нарингин, нарингенин и морин. Испитувањето се изведуваше со употреба на Muller Hinton agar и мерење на зоната на инхибиција за секој екстракт во двете концентрации со употреба на диск дифузониот метод изменет со употреба на бунарчиња.

Немаше никаков ефект врз испитуваниот сој на *E.coli*, и истотака имаше незабележителен ефект на соевите *Salmonella spp*. Единствено 2 супстанции (катехин и нарингенин) со концентрации од 10 µg. покажаа забележителен инхибиторен ефект врз *L. monocytogenes*, и имаше евидентен инхибиторен ефект од 5 екстракти врз *St. aureus*, и 3 од нив (кверцетин, галична киселина и нарингенин) покажаа инхибиција од 24, 26 and 26 mm. соодветно за концентрација на екстрактите од 10 µg.

Испитуваните растителни екстракти покажаа некаков инхибиторен ефект, особено врз Грам позитивните бактерии и тие евентуално би можеле да се применат и аплицираат како антимикробни супстанции или како конзерванси во храната употребени во поголеми концентрации

Клучни зборови : екстракти, бактерии, диск дифузионен метод, антимикробен ефект

UDC: 636.087.1:340.13

LEGISLATION AND HEALTH ASPECT OF NUTRITIONAL FEED SUPPLEMENTS

Crceva Nikolovska Radmila[1], Sekulovski Pavle[1], Prodanov Risto[1], Hajrulai Musliu Zehra[1], Angelovski Ljupco[1], Arsova Gordana[1], Nikolovski Aleksandar[2]

[1]*Food Institute, Faculty of veterinary medicine Skopje*
[2]*Food and veterinary agency, Skopje*

rnikolovska@fvm.ukim.edu.mk

ABSTRACT

Feed manufacturing today can be hardly imagined without the use of nutritional feed supplements. Due to technological reasons, feed supplements are intentionally added during production, processing, preparing, packaging, transport or storage, where they become a part of the final product. Regulation of feed additives usage in the European legislation is a "living" process which is not left freely to feed producers, but there are differences in the application of feed supplements on the national level in the countries outside of EU.

As a reaction on food and feed safety crisis at the beginning of the new millennium European Union adopted sets of new rules establishing the new safety system of the food chain „from farm to fork„ to increase the level of protection of both human and animal health. The key Regulation in this area No. 178/2002 has established the principle of risk analysis which consists of three components (risk assessment, risk management and risk communication). Those differences necessitate a change in professional approach, raising awareness of feed manufacturers aimed at greater competitiveness and effect-tiveness, as well as faster discovery of all possible risks of uncontrolled feed supplementation. Increasing level of responsibility of feed manufacturers in countries outside the EU is generated from the demands of EU for harmonization of national legislation with European legislation. Changes in regulations related to the use of feed supplements are in the process of being prepared within the regulatory structures of the European Union. They are based on the results of monitoring and review of documentation during the renewal of registration of feed supplements in the process of which it can be expected that the licenses to use some, until now essential supplements to be revoked. By monitoring the impact of feed on the safety and quality of food of animal origin, certain parameters are obtained which are used as the basis for defining the standards of the international programme for food safety (Codex Allimentarius).

The aim is to be involved in risk assessment of feed supplements as well as in the consideration of all possible problems that may arise, depending on the process in during which the supplements are used in the production process. This will decrease the adverse effect of this production process on the environment and overall food safety.

Key words: feed, nutritional supplements, legislation

INTRODUCTION

Livestock production occupies a very important position in agriculture. Satisfactory results in terms of farming animals and financial gain in livestock as well as public and animal health, animal welfare and environment, depend largely the use of appropriate quality and safe animal feed (1). From the market perspective, more attention is being paid to processes with a goal of livestock development with Euro-pean Union. Therefore, there is a need for the review, that is a "rebalancing", of Common Agricultural Policy that benefits livestock, towards the improvement of production technologies for balanced and precise animal nutrition which promotes intensive livestock production. The Code of Good manufacturing practice in addition to implementation of HACCP principles when used in the production of specific feed supplements and components of animal origin, must provide higher level of sustainable livestock production and total agricultural productivity. Therefore, there is an increasing change in the opinions of individual producers and farmers even when they are not involved in commercial production. Analogous to the definition of food from EU regulations, the feed is defined as any substance or product that will be consumed by animals. Therefore, feed supplements are also considered as feed.

HEALTH ASPECT

Feed supplements are substances, microorganisms or preparations, together with finished products of animal feed and premixes, which are intentionally added to animal feed or drinking water for animals with the aim of conducting one or multiple functions (2). They are also used for more efficient tehnology production process in preparation of the mixture, the improvement of the characteristics of products of animal origin, as well as increasing the efficiency of environmental protection. What may be the greatest problem during technically incorrect use of feed supplements, is the fact that in practice all categories of feed additives are called the same - nutritional supplements. Nutritional supplements in feed, whose specific effects on the animal organism are dosedependent, are the chemicals necessary for life and growth of organisms. Many in-gredients in food naturally contain in themselves certain nutritional values (they are rich in specific vitamins, minerals…). With regard to supplements used in feed, it is important to exclude

„natural ingredients“ and mention that the nutritional supplements are added afterwards in order to achieve a specific effect.

To understand feed supplements / additives, their nature, use and role in animal husbandry promotion, we have to understand, first, the concept of nutritionally balanced animal feed, its safety and high quality / efficiency in optimizing the genetic potential of farm animals and maximizing their production. Considerable research into animal nutrition has helped us in identifying factors that perfectly balance the feed to be fed to animals for enabling them to perform at the highest level of output. Animal feed supplements, which are also called as animal feed additives or premixes enhance the quality of animal feed by balancing and enriching all required nutrients.

Animal Feed Supplements are a group of various organic and inorganic substances, and also chemicals and antibiotics, which when added, have been found to enhance the production potential and boost up the production by animals. Today, the feed supplements are playing such a vital role in the animal feed industry, that no balanced animal feed can be manufactured without proper supplementation of animal feeds, if the performance of the nimal has to be enhanced. Feed supplements, feed premixes and feed additives, are all part and parcel of balanced animal feed with varying nomenclature. All these are complementary to each other, forming an inseparable and indispensable part of the complete feed that is well balanced in all required nutrients. The objective of all these commodities is also the same, viz. to enhance the productivity of producing animals.

Presently, the following feed supplements are used exclusively in the manufacture of animal feeds:

1. Feed grade vitamin premixes
2. Mineral mixes
3. Feed grade amino acids / mixes
4. Antibiotic feed supplements
5. Pro-Biotics
6. Enzyme preparations specific to animal feeding
7. Anti-oxidants
8. Mould inhibitors
9. Taste enhancers
10. Feed Flavours
11. Coccidiostats
12. Feed preservatives

In line with Annex III of EC Regulation 1831/2003, the trade in nutritional supplements reguires declaration of teh level of active ingredients and its expiration date, as well as the storage time limit of a given level of active ingredients up until date of manufacturing (3). The combination of the above substances also can be classified under feed supplements. These feed supplements can be mixed in feed & can also be offered through water. Their physical state could be in powder, pellet or liquid form.

Whereas it is theoretically adequate to feed animals, merely on fodder and other feed ingredients for sustenance, the intention of the animal husbandry, and therefore, of animal feed industry, is not merely to keep animals alive, but to make them highly productive. Thus, the argument that feed supplements / additives are not essential in animal ration, does not stand to reason. This view had been vindicated even by law courts. Likewise, animal feed supplements by themselves cannot keep the animal alive or sufficient to enhance production; they will have to be in combination with whole feed. This is how they are complementary to each other, and are a part and parcel of the end product -- complete animal feed. In other words, all of them are supplementary feeds.

The register of records of EU includes around 2800 supplements that are used in animal feed, from which 2711 undergo re-evaluation (data from year 2008) which is conducted in line with the guidelines of the EU Commission (7).

The assessment of Annex IV of regulation No 767/2010 on permitted tolerances for nutrients and feed additives performed by EU national reference laboratories, including the National Feed Laboratory, revealed that analytical tolerances, particularly for feed additives, with higher content, were higher that the tolerated ones. In such cases meeting the reguirements of regulation No 767/2010 could have been difficult and inconsistencies found within official control could possibly refer even to high guality feeds. The problem was solved by publishing the regulation of the Council (EC) No 939/2010 of 20 October 2010 modifying Annex IV to regulation 767/2009.

LEGISLATION, EU-R.MACEDONIA

Stabilisation and Association Agreement concluded between the European Community and its Member States on the one hand and the Republic of Macedonia on the other hand, obliged Macedonia to harmonize national legislation with applicable and appropriate EU legislation (EU Acquis). Furthermore, the Council Decision 2006/57/EC on the principles, priorities and conditions contained in the Accession Partnership with the Republic of Macedonia, determine short-term priorities, including harmonization of legislation with EU veterinary legislation. Law on Veterinary Health (“Official Gazette of the Republic of Macedonia” no. 21/1998), which regulates the use and trade of food for animals, is already changed a new Law on Veterinary Health (“Official Gazette of the Republic of Macedonia” No. 113 / 2007) which, although basically contains the principles of EU legislation is necessary to precise definition of the specific areas and segments. In Macedonia there are regulations governing the said area, but it is passing a law that will apply in all stages of production, processing and distribution of food for animals other than primary production intended for personal household needs.

Namely the adoption of this law tends to align the basic principles and responsibilities relating to: the use of additives in feedingstuffs comply with the procedures in Regulation of the European Parliament and Council (EC) br.1831/2003 of September 22, 2003, hygiene of food for animals comply with the procedures in Regulation of the European Parliament and Council Regulation (EC) no. 183/2005 of 12 January 2005 and compliance with the procedures of Regulation of the European Parliament and Council (EC) 882/2004 of April 29, 2004 for performing official controls to ensure verification of compliance with legislation on animal food and food for people and the rules for animal health and animal welfare (1,4,9).

The basis of the European legislation in this area is Regulation 1831/2003 on additives for use in feed replaced the former Directive 70/254, inclusion of amino acids and equipment for conservation of silage, introduction of compulsory registration for coccidiostatic, as feed supplements, provision for withdrawal of agents that promote growth, that is antibiotic as of 1 January 2006, introduction of five categories of feed supplements, as the main legal text for feed additives. This

Regulation divides all additives into categories and then into functional groups. There are four categories – technological, sensory, nutritional and zootechnical. Each of these is divided into functional groups. At the same time, there are ongoing changes made by appropriate legislative structures of the European union related to the use of feed supplements. They are based on the results of monitoring, as well as documentation requested for the renewal of registration of feed supplements, and could lead to potential abolition of licenses for the use of, till then, some important additives.

In the national legislation, the area of feed supplements is accurately, comprehensively defined and covered by legal provisions in laws and regulations. Legislation on drugs defines concepts of veterinary drug and premixes.

CONCLUSION

The development of feed technology and regulating the use of nutritional supplements in feed will result in reducing the harmful effects of production on the environment and food safety in general. In the end, incresing attention is being paid to the strategy for sustainable development through the resolution of the greenhouse gas emissions and contribution to the miligation of climate changes.

By accepting the rules, guidelines and recommendations of European law, the field of nutritional feed supplements will be more perisely defined. The goal is to engage in risk assessment of feed supplements and consideration of all the possible problems that arise, depending on the way in which the supplements are used in the production process.

REFERENCES

1. Regulation (EC)183/2005 laying down the legal reguirements for the hygiene of feed.
2. ANNEX III Regulation (EC) 1831/2003 Specific Labelling Reguirements for Certain Feed Additives and for Premixtures.
3. Aneks II Regulation 84/587/EEC-a the use of antibiotics, coccidiostatic and veterinary medical substances.
4. Regulation (EC) 429/2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorization of feed additives.
5. Annex II, Regulation (EC) No 429/2008, General reguirements to be satisfied by the dossier provided for in article 3 General aspects; 3.2.2.7. Determination of No Observed Adverse Effect Levels (NOAEL).
6. Regulation (EC) No 1272/2008 on classification, labelling and packaging of dangerous substances and mixtures.
7. Закон за безбедност на храната за животни (Службен весник бр. 145/10, 53/11).
8. Правилник за квалитет на добиточна храна (Службен весник бр. 15/89).
9. Regulation (EC) 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.
10. Regulation (EC) 767/2009 on the placing on the market and use of feed.

ЛЕГИСЛАТИВА И ЗДРАВСТВЕНИ АСПЕКТИ НА НУТРИТИВНИТЕ АДИТИВИ ВО ХРАНА

Чрчева Николовска Радмила[1], Секуловски Павле[1], Проданов Ристо[1], Хајрулаи Муслиу Зехра[1], Ангеловски Љупчо[1], Арсова Гордана[1], Николовски Александар[2]

[1]Институт за храна, Факултет за ветеринарна медицина, Универзитет "Св. Кирил и Методиј", Скопје, Македонија
[2]Агенција за храна и ветеринарство

e-mail: rnikolovska@fvm.ukim.edu.mk

АПСТРАКТ

Производството на добиточна храна во денешно време тешко може да се замисли без употреба на додатоци за храна. Поради технолошки потреби, додатоците намерно се додаваат за време на производството, преработката, припремата, пакувањето, транспортот и складирањето каде тие стануваат интегрален дел од финалниот производ. Регулирањето на употребата на додатоците за храна во Европската легислатива е "жив" процес кој не е поставен за слободно толкување од страна на производителите, но постојат разлики во апликацијата на додатоците за храна на национално ниво во земјите што не се членки на ЕУ.

Како реакција на кризите со безбедноста на храната и добиточната храна на почетокот на новиот милениум Европската унија усвои нови правила офорувајќи нов систем за безбедност на ланецот на храна "од фарма до трпеза" како би се зголемило нивото на заштита на здравјето и на луѓето и на животните. Клучната регулатива во оваа област Регулативата бр. 178/2002 го воспостави принципот на анализа на ризик кој се состои од три компоненти (процена, менаџирање и комуникација на ризиците). Пројавените разлики со стариот систем бараат промени во професионалниот пристап, зголемена будност на производителите на добиточна храна со цел поголема конкурентност и ефективност, како и побрзо откривање на можните ризици на неконтролирани додатоци во храната. Зголеменото ниво на одговорност на производителите од земјите вон ЕУ е генерирано од барањата на ЕУ за хармонизација на националните легислативи со Европската легислатива. Промените во регулативите поврзани со употребата на додатоците во добиточната храна се во процес на припрема во телата на ЕУ. Тие се базираат на резултатите од моиторингот и прегледот на документацијата за време на обновата на регистрацијата на додатоците за храна во процес во кој може да се очекува да лиценците на некои досега дозволени додатоци бидат повлечени. Со мониторинг на влијанието на добиточната храна врз производите од анимално потекло се добиваат одредени параметри кои се користат како основа за дефинирање на стандардите на интернационалните програми за безбедност на храна (Codex Allimentarius).

Крајна цел е учество во проценатa на ризикот кај додатоците во добиточната храна како и во разгледувањето на сите можни проблеми кои може да се појават, зависно од процесот при кој додатоците се додаваат во храната. Ова ќе го намали негативниот ефект кој производствениот процес го има врз околината и целоосно врз безбедноста на храната.

Клучни зборови: добиточна храна, хранливи додатоци, легислатива

UDC: 637.1.055(497.7)

CONTROL OF PROCES HYGIENE IN FERMENTED DAIRY PRODUCTS IN THE R. MACEDONIA

Prodanov Mirko[1], Ratkova Marija[1], Angelovksi Ljupčo[1], Mojsova Sandra[1], Jankuloski Dean[1], Sekulovski Pavle[1]

[1]*Food Institute, Faculty of Veterinary Medicine, University "Ss. Cyril and Methodius,,, Skopje, Republic of Macedonia*

e-mail: m.prodanov@fvm.ukim.edu.mk

ABSTRACT
Microbiological safety of milk and dairy products is a major issue in public health. In order to place dairy products on the market, producers must meet the requirements for process hygiene from the Book of rules for specific requirements for food safety in terms of microbiological criteria (Off .Gaz. of R.M. No.78/2008). The goal of this study was to determine the process hygiene during production of fermented dairy products from various producers in Republic Macedonia. A total of 241 samples of fermented dairy products, 138 yogurt and 103 sour milk were examined. The detection and enumeration of the Enterobacteriaceae was done by the method ISO 21528-2, and detection and enumeration of coagulase positive staphylococci was done following the ISO method 6888-1:1999.More than one third (36,92%) of the samples did not meet the requirements of the book of rules. This is an indicator that the hygiene of the production process needs to be improved.
Key words: yogurt, sour milk, Enterobacteriaceae, coagulase positive staphylococci, process hygiene

INTRODUCTION

Yogurt and sour milk are the most widely consumed fermented milk products today, but there production process differs mainly in a production capacity, from large scale industrial dairy factories to small scale producers and eve sometimes in homes, for local consumption [1]. Those products are good sources of minerals and vitamins and they contain only small amounts of lipids. In addition, fermented dairy products have their role in regulation the absorption of dietary nitrogen components in the body and in supply with many lactic acid bacteria, which may offer other health benefits as well [2]. Yogurt is one of the best-known types of food that contain probiotics. Yogurt is defined by the *Codex Alimentarius* in 1992 as a coagulated milk product that results from the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [3].

Microbiological safety of milk and dairy products is a major issue in public health. Microbiological contamination may occur by direct excretion of micro-organisms from the udder, fecal contamination during milking or inadequate hygienic measures during further processing steps.

Milk is an excellent substrate for growth of all microorganisms present, including pathogens and spoilage organisms. Levels of microorganisms provide information on the hygiene level during milking and subsequent steps. Coliforms and others Gram–negative bacteria, indicator of bad hygiene practice in the production process can be found in the milk. Various human pathogens and zoonozes organisms like *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *Mycobacterium tuberculosis* can also contaminate milk and present hazard to human health [4].

In order to place dairy products on the market they must meet the criteria from the Book of rules for specific requirements for food safety in terms of microbiological criteria (Official gazette of Republic of Macedonia No.78/2008). This is very important for yogurt and sour milk because they are very popular dairy product among consumers who have problems with digestion [5].

The control of the process hygiene of this diary products is especially important because in a previous study it was determined that the process of pasteurization of the milk in 58,09% of the cases was not sufficient to reduce the microbial load to required levels and the pasteurized products did not meet the requirements of the Book of rules (Off. Gaz. of R.M. No.78/2008). During the fermentation of the yogurt the temperature needed for the growth of the lactic-acid bacteria, also supports the growth of the most pathogenic bacteria [6].

The goal of this study was to determine the process hygiene during the production of fermented dairy milks from various producers in Republic Macedonia.

MATERIALS AND METHODS

For the purpose of this study a total of 241 samples fermented dairy products were examined, during the year of 2011. From the total number, 138 of the samples examined were yogurt and 103 of the samples were sour milk.

The samples were collected and brought to the Faculty of Veterinary Medicine – Skopje, by the producers from around the country as regular periodical control for process hygiene. The samples were stored in a refrigerator at temperature of 4°C and were analyzed the same day they were brought in the laboratory.

In accordance with the Book of rules, the samples were analyzed for 2 parameters, detection and enumeration of Enterobacteriaceae and detection and enumera-

tion of coagulase positive staphylococci [5].

Detection and enumeration of the Enterobacteriacae was done according to the method ISO 21528-2. One milliliter of the sample was aseptically transferred to empty sterile Petri dish. Approximately 15 ml of Violet red bile-glucose (VRB-G) agar was poured over and mixed by swirling. The plates were then incubated for 24 hours at 37°C. Following incubation plates were checked for the presence of characteristic colonies witch are pink to red or purple, with or without precipitation haloes, oxidase negative and glucose positive. The characteristic colonies were enumerated [7].

Detection and enumeration of coagulase positive staphylococci was done according to the method ISO 6888-1. Half a milliliter of the sample was aseptically transferred to a Petri dish with Baird-Parker (BP) agar. The sample was spreaded on the surface of the agar using a sterile hockey stick. The plates were then incubated 48 hours at 37°C. After the incubation the plates were examined for typical colonies and were enumerated. Typical colonies are black or gray, shining, and convex and are surrounded with a opalescent ring. The confirmation of the typical colonies was done with rabbit plasma coagulase test.[8].

RESULTS

Of the 241 analyzed samples 36,92% (89) were positive for at least one of the tested hygiene parameters and did not meet the requirements of the Book of rules. One hundred fifty two (63,07%) samples met the requirements (Table 1).

Eighty five samples (35,27%) were found to be positive for Enterobacteriaceae, from which 47 were yogurt samples and 38 sour milk samples. Only two samples (0,83%) (one yogurt and one sour milk) were found to be positive for presence of coagulase positive staphylococci. Additional two samples (0,83%) were found to be positive for both tested parameters: high number of Enterobacteriaceae and presence of coagulase positive staphylococci.

Table 1. *Results from process hygiene testing of fermented dairy products*

	Total Tested	Positive for Enterobacteriaceae	Positive for CPS	Positive for Ent + CPS	Total positive
Yogurt	138	47 (19.5%)	1 (0.41%)	0	48 (19.92%)
Sour Milk	103	38 (15.77%)	1 (0.41%)	2 (0.83%)	41 (17.01%)
Total samples	241	85 (35.27%)	2 (0.83%)	2 (0.83%)	89 (36.9%)

CONCLUSION

1. More than 1/3 (36,92%) of the samples did not meet the requirement of the book of rules. This can be interpreted that fermented dairy products in Republic of Macedonia are relatively safe for consumption for the general public, but the process hygiene practices must to be improved.
2. According to the book of rules the samples that did not meet the requirements were not withdrawn from the market, but producers are to be advised for improvement and monitoring of the process hygiene.
3. The high percentage of samples contaminated with Enterobacteriaceae is in correlation with the results of the bad hygiene of the milk that is used for yogurt production [6].

REFERENCES

1. Elmer H. Marth, James L. Steele (2001): Applied dairy microbiology. Fermented Milks and Cream, Vikram V. Mistry, 9:301-325, Marcel Dekker
2. Corinne Guerin-Danan, Claire Chabanet, Christophe Pedone, Françoise Popot, Pierre Vaissade, Christine Bouley, Odette Szylit, and Claude Andrieux. Milk fermented with yogurt cultures and Lactobacillus casei compared with yogurt and gelled milk: influence on intestinal microflora in healthy infants. Am J Clin Nutr January 1998 vol. 67 no. 1 111-117
3. Bourlioux P, Pochart P. Nutritional and health properties of yogurt. World Rev Nutr Diet 1988;56:217–58
4. Elmer H. Marth, James L. Steele (2001): Applied dairy microbiology. Raw Milk and Fluid Milk Products, 2:59-76, Marcel Dekker.
5. Book of rules on the microbiological criteria for food regarding the specific requerments for food safety (Official journal of R.M. 78/2008)
6. Prodanov M., Sekulovski P., Jankuloski D., Angelovski Lj., Kostova S., Ratkova M. : Control of the hygiene proces in the production of the pasterized milk in R. Macedonia; II Scientific Conference „Quality and safety of food„-2010, Bitola, R.Macedonia
7. ISO 21528-2:2004, Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae -- Part 2: Colony-count method
8. ISO 6888-1:1999, Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) -- Part 1: Technique using Baird-Parker agar medium

КОНТРОЛА НА ПРОЦЕС НА ХИГИЕНА КАЈ ФЕРМЕНТИРАНИ МЛЕЧНИ ПРОИЗВОДИ ВО Р. МАКЕДОНИЈА

Проданов Мирко[1], Раткова Марија[1], Ангеловкси Љупчо[1], Мојсова Сандра[1], Јанкулоски Деан[1], Секуловски Павле[1]

[1]*Институт за Храна, Факултет за Ветеринарна Медицина, Универзитет „Св. Кирил и Методиј“, Скопје, Република Македонија*

e-mail: m.prodanov@fvm.ukim.edu.mk

АПСТРАКТ

Микробиолошката безбедност на млекото и млечните производи е од голема важност за јавното здравство. За да може млечниот производ да се пласира на пазарот, производителот мора да ги исполни условите за хигиена на процес на производство од Правилникот за посебните барања за безбедност на храна во однос на микробиолошките критериуми (Сл. Весник на Р.М. бр. 78/2008). Целта за овај труд е да се одреди хигиената на процесот во производството на ферментираните млечни производи од разни производители од Република Македонија. Беа испитани вкупно 241 примероци од фермантирани млечни производи, од кои 138 јогурт и 103 кисело млеко. Детекцијата и броењето на Enterobacteriaceaебеше изведена по методот ISO 21528-2, а детекцијата и броењето на коагулаза позитивните стафилококи по методот ISO 6888-1:1999.Повеќе од една третина (36,92%) од примероците не ги исполнуваа условите пропишани со Правилникот. Ова е индикатор дека хигиената во производствениот процес треба да се подобри.

Клучни зборови:јогурт, кисело млеко, Enterobacteriaceae, коагулаза позитивни стафилококи, хигиена на процес

UDC: 636.52/.58.09:615.272.015

PHARMACOKINETICS OF THE FERROUS SULPHATE IN BROILER CHICKENS

Arnaudova-Matey Anna[1], Mehmedov Tanju[1]

[1]*Department of Non-infectious Diseases and Pharmacology, Faculty of Veterinary Medicine, University of Forestry, Sofia, Bulgaria*

ABSTRACT

The pharmacokinetics of the iron contained in the ferrous sulphate ($Fe_2SO_4.7H_2O$) in broiler chickens was studied. Sixteen birds from the same population, fed and raised in the same conditions, linear Anglo-American hybrid ROSS-IKOV – the birds were divided into 2 groups of equal in number of both genders, at the age of 35 days, all chickens in good clinical health, were involved in the test. 1 % aqueous solution of ferrous sulphate ($Fe_2SO_4.7H_2O$) was used. It was injected in eight broiler chickens intravenously in the vein of the right wing. The ferrous sulphate in the same concentration was also administered intraingluvial to the other group of eight birds. The solution was administered directly into the crop of the birds through elastic silicone tube. Serum samples were obtained immediately before the treatment of the birds, as well as after 0.17, 0.33, 0.50, 1, 2, 4, 6, 8,10, 12 and 24 h. The quantity of the iron in blood serum was determined through bioanalyzer, using the spectrophotometric and direct ferrene method. Some basic pharmacokinetic parameters were determined using the compartmental method and the non-compartmental analysis by using specialized pharmacokinetic software. In the intravenous injection they were as follows: $T_{1/2\beta}$ = 11.19 h and 5.26 h; $AUC_{0\rightarrow\infty}$ = 693.13 mmol.h/L и 561.57 mmol.h/L; $Vd_{(area)}$) = 0.107 mg/mmol/L и 0.130 mg/mmol/L. In the intraingluvial treatment the pharmacokinetic parameters determined by the two methods were as follows: $T_{1/2\beta}$ = 12.13 h and 10.66 h: $AUC_{0\rightarrow\infty}$ = 523.38 mmol.h/L and 451.68 mmol.h/L; C_{max} = 27.65 mmol/L и 28.59 mmol/L; T_{max} = 0.54 h и 1.56 h. In the intraingluvial administration of 1% solution of ferrous sulphate the relative bioavailability (F) determined by both pharmacokinetic methods showed good values, which were respectively 40.70 % and 40.36 %.

INTRODUCTION

From pharmacopeiacal point of view (EP7, R.3653, etc.) the ferrous sulphate ($Fe_2SO_4.7H_2O$) consists of prismatic transparent crystals with a bright aquamarine colour or pale green crystalline powder (green stone) with a molecular weight of 278.02, which is easily soluble in water (1:2.2). It evaporates in the air and contains 20 % of iron. Before the synthesizing and introduction of the ferrodextran complexes in the practice (Imferon 50 is the first commercial product introduced in England in 1954) the ferrous sulphate was one of the most commonly used iron compounds in the pathogenic (iron replacement) prevention and treatment of the anaemia in the growing animals, in particular in the pigs. Later on, its application in this area was limited. The ferrous sulphate, however, was included as a permanent component in the mineral feed additives of the mammals and birds, developed by the U.S.A. (National Research Council) and other countries in the 50s and 60s of the last century (5). In the recent years the inorganic iron salts intended for the feed have been replaced by organic chelate complexes of the iron with a single amino acid (US Patent # 4,067,994 regarding Feric Methionine Complex) or by an acid complex (US Patent # 5,698,724 regarding Iron Amino acid complex). It is assumed that the chelates have pharmacological and technological advantage over the inorganic salts. The commercial product Bioplex Fe (Alltech) is well-known. Unfortunately, there is insufficient scientific basis for comparison of the organic iron complexes and the inorganic salts (mainly the ferrous sulphate). The new products should be characterized by their bioavailability and bioactivity in the corresponding target animals. The iron-containing products, in particular the ferrodextran complexes were tested in the pigs. In our country they were tested in rabbits and pigs and detailed pharmacokinetic studies, which led to the development of rapidly absorbed products (1, 2) were conducted. Furthermore, good clinical results the in pigs were obtained in the studies of the iron proteinate synthesized in Bulgaria (4) and of the iron methionate (6).

The optimization of the mineral nutrition in the broiler chickens has a significant value. Depending on the product the iron participates in the broiler chicken feed from 25 to 40 ppm (5, 7) The scientific literature contains no detailed pharmacokinetic studies of the iron products in the chickens. The objective of this study was to carry out pharmacokinetic modelling of the ferrous sulphate in tests with broiler chickens.

MATERIALS AND METHODS

Sixteen broiler chickens from the same population, fed and raised in the same conditions, linear Anglo-American hybrid ROSS-IKOV - all of them equal in number of both genders weighing

1, 280 – 1, 420 kg were involved in the pharmacological study conducted. We bred the birds for 35 days in individual metal cages at a room temperature within the

[*] **Recipe for broiler chickens:**
Content of the ingredients for 1000 kg:
corn + hostazym 20 %; wheat + hostazym 42.03 %; (soybean meal 44 %) – 23 %; (sunflower meal 34 %) – 6.5 %, sunflower oil 1.2 %; oil emulsion 1.2%; (endox 0.0125 %) – 0.0125; (hostazym 0.05 %) 0.05 %, (11 BK - 4010 6 %) – 6 %.
crude protein – 210.00 g/kg; crude fat – 35.00 g/kg; crude fibre – 0.00 g/kg; ME (metabolizable energy) of the broilers – 2750.00 Kcal / kg

range 20-22 ˚C and air humidity 50 %. The broiler chickens were not previously treated with other drug products. They were fed with pelleted fodder for growing birds[1*]. Their water was provided *ad libitum*.

The substance of ferrous sulphate ($Fe_2SO_4.7H_2O$) was used for the preparation of working solutions with a concentration of 1% immediately before the treatment. Eight birds were injected with the sterile solution in the vein (*v. brachialis*) of the right wing. We administered the solution of ferrous sulphate in the same concentration to the other gourp of eight birds directly into the crop by using elastic silicone tube and syringe. The blood samples were received through venipuncture of *v. brachialis* on the left wing in Eppendorf tubes. We have used the solution of ferrous sulphate after 16 hour starvation diet. The blood samples were received immediately before the treatment of the birds and after that at 0.17, 0.33, 0.50, 1, 2, 4, 6, 8, 10, 12 и 24 h. The samples were storred 2 h at a room temperature, centrifuged them at 1500 rpm for 15 min and then kept them in a refrigerator at a temperature -20° C until the analysis.

The quantity of the iron in the blood serum was determined through bioanalyzer (spectrophotometer) (BS-200, Mindray Co LTD, China), using direct ferrene method at wavelength l = 600 nm.

The pharmacokinetic parameters were determined through the compartmental method and the non-compartanmental analysis (7), which necessitated the use of specialized pharmacokinetic software - pharmacokinetic programme TopFit, v.2.0. The pharmacokinetic parameters were determined in accordance with the Akaike's criterion (8).

RESULTS AND DISCUSSION

Table 1 shows the serum concentrations of the iron after single intravenous and intraingluvial application of the inorganic salt of the iron subject to our study. We should confirm that the serum concentrations of the iron in both methods of treatment were determined by the 12th hour after the application of ferrous sulphate to the chickens.

The serum concentration curves of Fe^{2+} *in the intravenous* injection of 1 % solution of ferrous sulphate corresponded to the two-compartmental pharmacokinetic model and those obtained at the intraingluvial administration - corresponded to the compartmental open pharmacokinetic model.

In the *intravenous injection* of ferrous sulphate the values of the speed and time of distribution of the iron ($T_{1/2a}$) showed that the inorganic iron product applied was distributed very fast in the body of the broiler chickens (Table 2). The pharmacokinetic parameters corresponding to the level of distribution of the iron - the volume of distribution ($Vd_{(area)}$), the steady state volume of distribution (V_{ss}), the volume of distribution in the central compartment (Vc) and the volume of distribution in the tissue compartment (V_t) indicated the poor distribution of the iron into the bird body (Table 2).

The rate constants featuring the passage of the iron from the central to the peripheral compartment (K_{12}) and from the peripheral to the central compartment (K_{21}) and their ratio (K_{12}/K_{21}) were evidence of the faster passage of the iron ions from the blood and perfused organs to the tissues and organs included in the periferal compartment, and *vice versa*, of their significantly slow return back to the central compartment (Table 2).

An important pharmacokinetic parameter for each drug, which does not depend on the methods used, is its elimination half-life, also known as biological half-life ($T_{1/2b}$). The biological half-life ($T_{1/2b}$) of the iron after intravenous bolus injection of ferrous sulphate determined by the compartmental method was significantly longer than that determined through the non-compartanmental analysis (Table 2). There was a similar trend in the mean residence time (MRT) (Table 2).

In the *intraingluvial administration* of iron sulphate the iron was absorbed rapidly and continuously by the digestive tract of the broiler chickens. This was proved by the values of the absorption half-life time ($T_{1/2abs}$) and those of the mean absorption time (MAT) (Table 3).

The elimination half-life ($T_{1/2b}$) of the iron out of the chicken body determined by both pharmacokinetic methods showed a longer stay of the iron in the body of the treated birds than that in the intravenous injection of the drug product tested (Table 2 and Table 3). There was a similar trend in the values of the mean residence time (MRT) (Table 2 and Table 3).

The maximum serum concentrations (C_{max}) of the absorbed iron (Table 3) were reached relatively fast - in approximately 1.5 h after the intraingluvial treatment of the broiler chickens with 1 % solution of ferrous sulphate.

The bioavailability (F) of the iron determined by the two pharmacokinetic methods showed good bioavailability in this non-intravenous mode of administration in the broiler chickens (Table 3).

The information in the literature showed that no studies on the pharmacokinetics of the iron in birds, including broiler chickens, were conducted. The information about the pharmacokinetics of the iron in pigs after intravenous injection of organic iron product - iron methionate is of great interest. The maximum serum concentrations (C_{max}) of the iron in chicken broilers, which we determined using both pharmacokinetic methods, were significantly lower than those determined by Petrichev (5) in pigs treated with iron methionate C_{max} = 104.50 mmol / L, which could be explained, on the one hand, by the better absorption ability of the organic iron product compared to the inorganic one that we used and, on the other hand, by the 5-fold higher dose of the drug product used in the pigs. Moreover, we should take into account the differences in the digestive system in these two different animal species.

Similar differences may be found in the values of the time required to reach the maximum serum concentrations (T_{max}) in broiler chickens compared to those in the pigs (5) - T_{max} = 2.67 h.

The values of the biological half-life ($T_{1/2b}$) and the mean resident time (MRT) in the broiler chickens (Table 3) were higher than those determined by Petrichev (5) in the pigs $T_{1/2b}$ = 9.41 h and MRT = 13.56 h.

REFERENCES

1. Antonov, S.I. (1971). Study of the iron metabolism in relation to the anaemia in the mammal pigs. PhD Thesis, Faculty of Veterinary Medicine, Sofia, Bulgaria.
2. Dilov, P. (1976). Pharmacological properties and biological standardization of the ferrodextran preparations. PhD Thesis, Faculty of Veterinary Medicine, Sofia, Bulgaria.
3. Gibaldi, M., D. Perrier, 2007. Pharmacokinetics, Second edition, Revised and Expanded, Informa-Healthcare USA Inc., New York, London.
4. Ivanov, A.L. (2001). Manifestations of the alimentary hy-

potrophic syndrome in newborn pigs and pportunities for prevention and treatment. Doctoral dissertation, Central Research Institute of Veterinary Medicine, Sofia, Bulgaria.
5. Lyons T.P. (2006). It is time to use the new technologies. Tenth Scientific Workshop (Alltech) in Bulgaria, 6-18.
6. Petrichev, M.H., 2006. Study of the toxicity, bioavailability and antianaemic effect of the methionine iron complex fodder additive in laboratory animals and pigs. PhD Thesis, Faculty of Veterinary Medicine, University of Forestry, Sofia.
7. Surdzhiyska S. (1996) Standards for bird feeding, ed. Trakia University, Stara Zagora.
8. Yamaoka, K., T. Nakagawa, T. Uno, 1978. Application of Akaike's Information Criterion (AIC) in the evaluation of linear pharmakcokinetic equations. *J Pharmacokinet Biopharm*, 6, 166-175.

Table 1. *Serum concentrations of 1% ferrous sulphate solutions after single intravenous and intraingluvial treatment of broiler chickens*

Time (h)	Intravenous injection		Intraingluvial administration	
	Mean	SEM	Mean	SEM
0.17	143.713	19.612	23.400	1.309
0.33	110.838	13.653	25.175	1.378
0.50	91.738	13.359	27.075	1.392
1	77.038	12.900	26.113	1.923
2	61.613	12.270	25.800	2.224
4	43.738	8.277	25.450	2.270
6	31.600	4.943	22.100	1.164
8	19.825	1.986	17.650	1.015
10	15.875	1.088	15.475	0.793
12	12.875	0.827	13.113	0.985

Table 2*. Pharmacokinetic parameters of the ferrous sulphate after single i.v. injection of broiler chickens with 1% solution*

Parameter	Units	Ferrous sulphate	
		Mean	SEM
Compartmental method			
K_{12}	h^{-1}	6.2944	1.890
K_{21}	h^{-1}	1.1003	0.355
K_{12}/K_{21}	-	13.892	7.517
$T_{1/2a}$	h	0.162	0.085
$T_{1/2b}$	h	11.190	3.957
V_c	mg/mmol x l	0.0230	7.214
V_t	mg/mmol x l	0.075	0.020
V_{ss}	mg/mmol x l	0.182	0.083
$Vd_{(area)}$	mg/mmol x l	0.107	0.021
MRT	h	14.911	5.243
$AUC_{0®¥}$	mmol x h/l	693.125	100.759
Non-compartmental analysis			
$T_{1/2b}$	h	5.255	0.926
MRT	h	7.116	1.275
$AUC_{0®¥}$	mmol x h/l	561.566	56.296
Vz	mg x l/ mmol	0.130	0.056
V_{ss}	mg x l/ mmol	0.167	0.014
r^2	-	0.963	0.008

Table 3. *Pharmacokinetic parameters of the ferrous sulphate after single intraingluvial treatment of broiler chickens with 1% solution*

Parameter	Units	Ferrous sulphate	
		Mean	SEM
Compartmental method			
$T_{1/2b}$	h	13.058	1.457
$T_{1/2abs.}$	h	0.073	0.007
MRT	h	18.938	2.104
$AUC_{0®¥}$	mmol x h/l	523.375	42.975
T_{max}	h	0.543	0.039
C_{max}	mmol/l	27.650	1.627
MAT	h	9.376	2.803
F	%	40.697	4.010
Non-compartmental analysis			
$T_{1/2b}$	h	10.661	0.820
MRT	h	14.838	1.308
$AUC_{0®¥}$	mmol x h/l	451.684	28.626
C_{max}	mmol/l	28.588	1.812
T_{max}	h	1.563	0.678
MAT	h	6.496	1.581
F	%	40.361	5.252
r^2	-	0.925	0.021

ФАРМАКОКИНЕТИКА НА ЖЕЛЕЗО СУЛФАТ ВО БРОЈЛЕРСКИ ПИЛИЊА

Арнаудова-Матеј Ана [1], Мехмедов Тању[1]

[1]*Катедра за Неинфективни Болести и Фармакологија, Факултет за Ветеринарна Медицина, Универзитет за Шумарство, Софија, Бугарија*

АПСТРАКТ

Во трудов беше проучувана фармакокинетиката на железото од железниот сулфат ($Fe_2SO_4.7H_2O$) во бројлерски пилиња. Во тестот беа вклучени шестнаесет птици од иста популација, хранети и одгледувани под исти услови, линеарен Англо-Американски хибрид ROSS-IKOV - птиците беа поделени во две групи со исти број од двата пола, на возраст од 35 дена, сите пилиња беа клинички со добро здравје. Се користеше 1% воден раствор од железниот сулфат. Растворот беше инјектиран интравенозно под десното крило во осум бројлерски пилиња. Во другата група од осум бројлерски пилиња, раствор на железо сулфат со иста концентрација беше администриран интраинглувиално. Растворот беше внесен директно во бапката на птиците преку еластична силиконска цевка. Примероци од серумот беа земени веднаш по третманот на птиците, како и по 0.17, 0.33, 0.50, 1, 2, 4, 6, 8,10, 12 и 24 часа. Количеството на железо во крвниот серум беше одредена со биоанализатор, со помош на спектрофотометрија и преку директен Ферене метод. Некои фармакокинетски параметри беа одредени со помош на компарметнтален метод и некомпармeнтална анализа со помош на специјализиран фармакокинетски софтвер. Кај интравенозната инјекција ги добивме следните резултати: $T_{1/2\beta}$ = 11.19 h и 5.26 h; $AUC_{0®¥}$ = 693.13 mmol.h/L и 561.57 mmol.h/L; $Vd_{(area)}$) = 0.107 mg/mmol/L и 0.130 mg/mmol/L. Кај интраинглувијалниот третман фармакокинетските параметри одредени од двата метода беа следните: $T_{1/2\beta}$ = 12.13 h и 10.66 h: $AUC_{0®¥}$ = 523.38 mmol.h/L и 451.68 mmol.h/L; C_{max} = 27.65 mmol/L и 28.59 mmol/L; T_{max} = 0.54 h и 1.56 h. Кај интраинглувиалната администрација на 1% раствор на железо сулфат релативната биодостапност (F) одредена по двата фармакокинетски методи покажаа добри вредности, кои беа 40,70% и 40,36% соодветно.

UDC: 637.5`65.06:[636.09:615.33.074

GATIFLOXACIN RESIDUES IN CHICKEN MEAT, SKIN AND GIBLETS

Kyuchukova Ralica[1]

[1]*Department. of Food Hygiene, Technology and Control, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria*

ABSTRACT

Gatifloxacin is a newer quinolone used in food-producing animals. The aim of the study was to investigate the residues of this antibiotic in chicken tissues (meat, skin and giblets). Chicken (n=30) were treated with gatifloxacin orally at a dose of 10 mg/kg BW for 5 days. Birds were divided into 5 groups and humanely killed in the following dynamics: on day 0 (the day after last administration of gatifloxacin), 2-nd, 4-th, 6-th and 8-th day. It was found that the highest concentration of the antibiotic was in the skin on day 0 (716μg/kg) and in liver on 2-nd day (690μg/kg). The residues in skin and liver gradually decreased and on 8-th day were 24,6μg/kg and 57μg/kg respectively. In breast muscle, heart and gizzard concentrations decreased quickly and were less than 100μg/kg on the 2-nd day.

Key words: gatifloxacin, chicken, residues

INTRODUCTION

The frequent use of antibiotics for livestock treatment and prevention leads to residues in meat and organs of slaughtered poultry (Donoghue, 2003). Fluoroquinolones are antimicrobial agents widely used, and most of them are approved for use in food-producing animals (Martinez et al., 2006; Okerman et al., 2007; Reyes-Herrera & Donoghue, 2008). Studies of Reyes-Herrera et al. (2005) showed that these antibiotics have different residues in different tissues.

Gatifloxacin is a fourth generation fluoroquinolone and it is used relatively recently (Patel et al. 2011). It is a broad-spectrum drug effective against gram-negative, gram-positive bacteria, mycoplasmas and those that are resistant against other agents (Saravolatz and Leggett, 2003). The European Union has established maximum residue limits (MRLs) for 7 quinolones – danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, oxolinic acid and sarafloxacin (Anonymous 2004).

There were no studies about gatifloxacin residue in tissues and organs of birds after repeated treatment with therapeutic doses; therefore we decided to do research in this direction.

MATERIALS AND METHODS

The study was conducted on 30 two months of age male chickens with approximately the same body weight (BW). Chickens were treated orally with gatifloxacin at a dose of 10 mg/kg BW in the drinking water during 5 days. Six birds were humanely killed on days 0 (the day after last administration of gatifloxacin), 2-nd, 4-th, 6-th and 8-th after the last treatment. Breast muscle, liver, gizzard, heart and skin (with fats) were separated from each carcass. Samples were weighed and homogenized with Maximum Recovery Diluent (MRD, HIMEDIA, India) in an amount equal to the mass of the sample, then were centrifuged for 15 min at 2500/min-1 (for liver samples 20 min). The supernatant was collected and dropped (100 μl) on a medium with test microorganism E. coli ATCC 25922. It was seeded on plain agar (HIMEDIA, India), which was sterilized and cooled to 50°C with concentration of cells 0.5 of McFarland standard. 90 mm sterile plates filled with 14 ml infected agar with E.coli were used as described Okerman et al. (2007). The diameters of the rings of inhibition zones were measured after incubation at 37°C for 24 h. The data for residues of gatifloxacin were processed statistically using the t-test by GraphPad program.

RESULTS

The results of the studies on gatifloxacin residues in meat, skin and giblets are presented on table1.

It is shown that the residues decrease in all the tissues, but with different rates. Gatiflixacin level in breast muscles from chicken slaughtered on day 0 was 211.83μg/kg, whereas in chicken from day 2 the levels were significantly lower with 36.33μg/kg (83% difference vs. day zero level). In the chicken, killed in the next days residues continued decreasing, but with slower rate, reaching 14.5μg/kg on the last day (day 8). The final residue levels in breast muscles were 6.8% from the initial level (on day 0).

Liver samples analysis showed lower gatifloxacin levels on day 0 than level on the next days. In chicken slaughtered on day 2 we found the highest levels – 690 μg/kg, which is two times higher than the level on day 0 (351,4 μg/kg). Probably there was a process of accumulation of the antibiotic in the liver after final treatment of the animals, in combination with slower elimination, and after that we determine relatively high levels up to day 6 (about 45% of highest level).

Table 1. *Gatifloxacin residues in meat and organs from chicken (µg/kg)*

day	Meat X ± SE	Liver X ± SE	Gizzard X ± SE	Heart X ± SE	Skin X ± SE
0	211.83 ± 23.66	351.4 ± 96.34	158.83 ± 26.51	169.83 ± 7.12	716 ± 332.95
2	36.33 ±4.65	690 ± 178.74	36.67 ± 5.03	14 ± 1.34	158 ± 38.64
4	27.33 ± 0.67	661 ± 109.43	16.17 ± 0.83	26.17 ± 3.34	170.5 ± 31.11
6	24 ± 1.03	313 ± 111.52	23.5 ± 1.78	19.17 ± 1.99	131.17 ± 23.59
8	14.5 ± 1.03	57 ± 18.88	13 ± 1.41	9.17 ± 0.98	24.6 ± 7.09

Data for antibiotic residues of gizzard showed 158.83µg/kg initial concentration and lightly decreasing on day 4 to 16.17µg/kg and increasing up to 23.5µg/kg on day 6. We found similar results for residue levels in the heart, as the concentration of the antibiotic on day 8 was 5% from the initial level, and there were fluctuations on day 4.

The highest initial levels of gatifloxacin we found in skin with fat (716µg/kg). In these samples we note decreasing sharply on day 2 (22%) and very slow from day 2 to day 6.

These results confirm the data of Reyes-Herrera & Donoghue(2008), who point out that different tissues in chickens can have different residue levels of antibiotics. Anadón et al. (2008) stated higher concentrations fluoriquinolones in liver and kidneys in comparison of meat, but lower in skin and fat. Data of Anadón et al. (1995) for orally treated broilers with enrofloxacin showed residues only in liver 12 days after administration.

CONCLUSION

In conclusion it has to underline that the data show the importance of using the right withdrawal periods, because the residues in different tissues differ significantly. In muscles, gizzard and heart on the second day there were minimal levels of gatifloxacin, but in skin and especially liver we found high levels almost one week after stopping treatment.

REFERENCES

1. Donoghue, DJ., 2003. Antibiotic residues in poultry tissues and eggs: human health concerns? Poultry Sci. 82(4), 618-21.
2. Martinez, M., McDermott, P., Walker, R., 2006. Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. *The Veterinary Journal 172*, 10–28.
3. Okerman, L., Noppe, H., Cornet, V., Zutter, L., 2007. Microbiological detection of 10 quinolone antibiotic residues and its application to artificially contaminated poultry samples. *Food Additives and Contaminants*, 252-257.
4. Reyes-Herrera, I., Donoghue, D., 2008. Antibiotic residues distribute uniformly in broiler chicken breast muscle tissue. *Journal of Food Protection*, 71 (1), 223-225.
5. Reyes-Herrera, I., Schneider, M.J., Cole, K., Farnell, M.B., Blore, P.J., Donoghue D.J., 2005. Concentrations of antibiotic residues vary between different edible muscle tissues in poultry. *Journal of Food Protection,* 68(10), 2217-2219.
6. Patel, S., Devada, S., Patel, H., Patel, N., Bhavsar, S., Thaker, A., 2011. Influence of co-administration of piperine on pharmacokinetic profile of gatifloxacin in layer birds. *Global Veterinaria* 7 (5), 427-432.
7. Saravolatz, LD., Leggett, J., 2003. Gatifloxacin, gemifloxacin, and moxifloxacin: the role of 3 newer fluoroquinolones. *Clin Infect Dis* 37 (9), 1210-1215.
8. Anonymous, 2004. Commission Regulation (EU) No 37/2010, Available: http://emea.eu.int
9. Anadón, M.A., Martínez, M., Martínez, C., De La Cruz, C., Díaz M.J., Martínez-Larrañaga, M.R., 2008. Oral bioavailability, tissue distribution and depletion of flumequine in the food producing animal, chicken for fattening. Food and Chemical Toxicology 46 (2), 662-670.
10. Anadon, A., Martinez-Larranaga, M.R., Diaz, M.J., Bringas, P., Martinez, M.A., Fernandez-Cruz, M.L., Fernandez, M.C., Fernandez, R.,1995. Pharmacokinetics and residues of enrofloxacin in chickens. American Journal of Veterinary Research 56 (4), 501-506.

ОСТАТОЦИ ОД ГАТИФЛОКСАЦИН ВО ПИЛЕШКО МЕСО, КОЖА И ВНАТРЕШНИ ОРГАНИ

Кјучукова Ралица[1]

[1]Одделение за хигиена на храна, технологија и контрола, Факултет за ветеринарна медицина, Универзитет на Тракиа, Стара Загора, Бугарија

АПСТРАКТ

Гатифлоксацин е хинолон од понова генерација кој се користи кај животните кои се одгледуваат за производство на храна. Цел на оваа студија беше испитување на остатоците од овој антибиотик во пилешки ткива (месо, кожа и внатрешни органи). Пилињата (n=30) беа третирани орално со гатифлоксацин со доза од 10 mg/kg телесна маса во тек на 5 дена. Птиците беа поделени во пет групи и жртвувани со следната динамика: нулти ден (денот после последното аплицирање на гатифлоксацин), втор, четврти, шести и осми ден. Највисока концентрација на антибиотикот е најдена во кожата на нултиот ден (716 µg/kg) и во црниот дероб на вториот ден (690 µg/kg). Резидуите во кожата и црниот дроб постепено опаѓаа и на осмиот ден беа 24,6 µg/kg и 57 µg/kg, соодветно. Во градниот мускул, срцето и желудникот концентрациите опаѓаа побрзо и на вториот ден беа помалку од 100 µg/kg.

Клучни зборови: гатифлоксацин, пилиња, резидуи.

ANIMAL WELFARE
&
GENETICS

Plenary Lecture

UDC: 637.512/.513

DEVELOPMENTS IN FARM ANIMAL WELFARE AS AFFECTED BY HANDLING, TRANSPORT AND SLAUGHTER

Anil Haluk[1]

[1]*School of City and Regional Planning, Cardiff University, Cardiff, UK*

ABSTRACT

Following primary production farm animals receive interventions that include handling, transport and slaughter processes during which animal welfare can be adversely affected with consequences for carcass and meat quality if optimum conditions are not met. In order to protect animal welfare, legislation is in place in most European countries. Council decisions such as Regulations 1099_2009_ (EC 2009) and (EC) No 1/2005 (EC 2005) provide guidance for national governments to prepare and enforce regulations. Various improvements to animal welfare have been made to improve conditions during transport to meat plants. Detailed requirements relating to loading, travel and unloading and resting, water and feed of animals as well as vehicle design, requirements for space, temperature control, training of personnel have recently been introduced. Humane slaughter has also been an important issue dealing with conventional and religious slaughter. Understanding animal behaviour of different species could help design abattoirs so that stress is minimised during passage of animals. Slaughter operations are also expected to induce insensibility in order to bleed out animals without pain and distress by applying stunning methods (Anil and Lambooij, 2009). Poor welfare can manifest itself in carcasses as acute problems such as fractures, bruising, petechial haemorrhages and chronic effects of stress and tiredness resulting in downgrading of products.In recent years the controversial religious slaughter has received much attention Dialrel, a European Commission funded project collected information on legislation, rules, practices, markets, consumer demands and highlighted problem areas of animal welfare (Dialrel).

Key words: Animal welfare, preslaughter handling, slaughter

INTRODUCTION

Farm animals are subjected to a series of procedures that affect them physically and mentally at the end of the production period. These events include handling, transport and slaughter by which animal welfare can be compromised with adverse effects on carcass and meat quality if optimum methods are not applied. Legislation prepared in most European countries aim to protect animal welfare, Council decisions such as Regulations 1099_2009_ (EC 2009) and (EC) No 1/2005 (EC 2005) provide guidance for national governments to prepare and enforce regulations.

Preslaughter handling, stunning and slaughter methods

In regard to transport, Council Regulation (EC) No 1/2005 amending directives came into force on 7 January 2011 with the aim of protecting animals during transport and related operations. Implications of this legislation include the following requirements:

- Advance journey planning
- Regular check points
- Vehicle design to protect animals at loading/unloading
- Space and height requirements for different species and stocking densities
- Certificates of approval for vehicles and containers
- Compulsory training of personnel
- Certificates of competence required
- Provision of rest, food and water

However, certain exceptions such as journeys to and from veterinary practices apply and farmers can use own vehicles to move animals within 50km radius.

No transport or restrictions apply in cases of:

- Very young animals e.g. calves less than10 days only allowed up to 100km
- Animals less than 14 days allowed 8 hours maximum
- In last third of gestation within 10% date of birth
- Females less than 1 week
- If journey is 65 km, then approval required

Specific problems highlighted during surveys in certain parts, in particular Eastern parts of Europe include:

Absent or unsuitable loading ramps, open and unsafe lorries, insufficient ventilation and temperature control; sheep being held by legs, legs tied together, dragged by legs, horn, fleece (wool pull damage) and excessive coercion. There are recent reports of animal welfare compromises during movement of meat animals between countries. These concern animals with incorrect identification, young, injured or pregnant animals at border posts causing undue delays and death.

On arrival at an abattoir or meat plant, handling and humane slaughter, both conventional and religious, are also important issues affecting welfare and quality. Understanding animal behaviour could help design abattoirs for different species to reduce stress during passage and stunning and slaughter.

Animals have to be transferred from the lairage pens either directly or through a race into an area where stunning and slaughter is carried out.

In order to facilitate stunning and also to protect the operatives some kind of restraint is necessary. Restraint should allow correct application of stunning equipment and protect animal welfare as well as providing protection from potential injury for the operatives especially from large animals. This could be achieved in a number of ways:

- Manual restraint in an open pen

This is usually done by manually handling the

free standing animal in an open area or a pen. Animal can enter the pen either directly from holding areas or through raceways. Electrical or captive bolt stunning in sheep and religious slaughter can be carried out this way. However, safety and welfare problems can be common features especially when handling cattle.

- Restraint in a squeeze / crush pen.

This principle involves holding the animal by pressure from the sides. Usually one side moves, not commonly used.

- Cattle stunning pens

Different designs of cattle restraint pens can be used. The objective is to confine the animal in a pen so that stunning and slaughter can be carried out effectively and safely. Animals usually enter the pen after going through a race. Pens must have gates to close after entry. Race should have smooth curved sides if long, have sufficient light. Use of prods should be minimal.

For captive bolt stunning, facilities to present the head for correct stunning at the front would be useful. Some cattle pens are specially constructed for captive bolt, electrical stunning and/or religious slaughter. Upright and Facomia pen designs have additional features for extra restraint such as belly lift, back push and chin-lift. Facomia pen tilts the animal around 45 degrees. Rotary pens that turn the animal 180 degrees are more stressful and banned in the UK.

The new impending European COUNCIL REGULATION ((EC) No 1099/2009) requires a study of cattle restraint systems and a report to be submitted by the end of 2012. Its aim is to establish whether certain optimum types of restraint apparatus employed for cattle as some existing ones may have inherent undue stress factors. Although this development has implications for both conventional as well as religious slaughter, the latter could be more affected. In particular restraint periods before and after a neck can be long in some systems. For example, some rotary pens take unduly long to rotate and present cattle for slaughter.

- V – type restrainers

These use the principle of suspending the animals in a funnel shape apparatus often having a conveyor system commonly used for pigs and sheep. It seems to work better for sheep than pigs. Sheep can be electrically stunned, either head-only or head-to-back at the end of the conveyor either manually or automatically.

- Monorail restrainers

This system holds the animal in a straddle position over a rail. Combined with a conveyor system, animals re moved to the point of stunning with possibly less stress than with V- restraint. This system is successfully used in pigs.

Slaughter operations must be able to induce rapid insensibility and bleed out without pain and distress (Anil and Lambooij, 2009). All stunning methods have disadvantages relating to quality, public health as well as possible misstuns. There is a need for research to develop alternative, ideally non invasive, stunning methods. A non-invasive method that does not result in tissue damage before death could also be acceptable by Jewish and Muslim communities. Magnetic stunning is based on passing a large current through a copper coil by which an intense magnetic field is generated. The coil is positioned close to the head so that the brain lies within this magnetic field. Transcranial magnetic stimulation (TMS) has been used in humans for years. The technique also reliably initiates seizures in humans as an alternative to ECT for the treatment of depression (Lisanby, 2002). Bristol research has provided evidence for insensibility during the TMS application (Anil et al, 2000). Using similar technology, studies aimed at producing seizure activity and prolonged insensibility without a painful induction have been conducted using new equipment and special coils in sheep, pigs and broilers (Lambooij, Anil et al, 2011). If fully developed, magnetic stimulation, a potential technique for stunning animals, could be used in future.

Poor welfare can manifest itself in carcasses as acute problems such as fractures, bruising, petechial haemorrhages (blood splash) and chronic effects of stress and tiredness resulting in downgrading of products. These could be visual effects such as bruising and haemorrhages, pelt burn in sheep, bone fractures, colour changes caused by PSE and DFD as well as those manifested in eating quality such as toughness. Stunning methods can also have adverse effects on carcass and meat quality and cause downgrading. One of the causes of reduced meat quality is related to pH decline. If pH level of 5.5 to 5.7 is reached within 48 minutes it is regarded as PSE. Conversely, glycogen depletion during chronic stress results in less lactic acid production. In this case the meat will be very dry and dark in colour. This condition is known as Dark Firm Dry (DFD) meat.

In order to reduce petechial haemorrhages and bruising following can be considered:

Shorten stunning to sticking interval so that blood leakage through ruptured vessels is reduced

- Captive bolt stunning may be preferable to electrical stunning if blood splash is a problem as muscle spasms are less pronounced after captive bolt
- Electrical stunning currents are applied in a continuous and uninterrupted manner
- In lambs electrical stunning with cardiac arrest may reduce blood pressure and blood splash.

Although, stunning methods have effects on animal welfare, in some instances, public health measures taken and concerns, especially as a result of the BSE threat, have inevitable welfare consequences too. Potential public health concerns from TSE infected animals have been considered and reviewed (Anil and Austin, 2001). CNS embolism of 4 and 2 per cent in jugular blood of cattle stunned with penetrating and non-penetrating captive bolts, respectively, has been reported (Coore et al, 2004; 2005).

Religious slaughter

In recent years the controversial religious slaughter has received much attention. Dialrel, a European Commission funded project collected information on legislation, rules, practices, markets, consumer demands and highlighted problem areas of animal welfare (Dialrel).

Most religious slaughter in Europe and the Western countries, where allowed by law, is carried out either by mostly the Muslim/Halal and to a lesser extent by the Jewish (Shechita) methods. As a result of the above, an EC funded project, DIALREL, has attempted to consult stakeholders, collect information and stimulated a debate on religious slaughter (http://www.dialrel.eu)

Although legislation in most European countries requires preslaughter stunning, there can be exemptions for animals slaughtered by religious methods if individual countries so decide. Several countries in Europe (EU

and others) do not allow slaughter without stunning (e.g. Sweden, Denmark, Norway and Switzerland).

Debate and concerns about religious slaughter focus on three questions:

i) Is there undue stress during handling prior to religious slaughter (Dunn 1990; Grandin, 1994; Grandin and Regenstein, 1994);
ii) Is the neck incision painful during the cut and/or immediately afterwards (Gibson et al, 2009a,b);
iii) Is sensibility and consciousness lost quickly enough following exsanguination ["sticking"] (Kalweit et al, 1989; Grandin and Regenstein, 1994; Anil et al, 1995a,b; Rosen, 2004)

In regard to stress of handling, as no specific religious requirements exist, the first question also applies to all other methods of slaughter. Some traditional practices however are still reported such as tying legs of sheep probably before religious slaughter that would be of concern. Others in cattle include the use of a casting pen (no longer permitted in the UK) and hoisting cattle by one of the hind legs prior to slaughter. It is argued that above practices are unduly stressful if practised.

The second and third questions are related. C. Johnson and his team in Zealand have recently developed a new technique to study pain in slaughter animals. Their series of publications report examination of EEG patterns in calves following neck cutting (Gibson et al, 2009a,b). Their reported comparative analysis concludes that ventral neck cutting results in responses to noxious stimuli, in particular when blood vessels are severed.

Halal slaughter and Shechita

In practice Muslim method of slaughter, now commonly referred to as Halal method, is shown to vary in the way it is applied. The variations are possibly due to differences in the interpretation of the Koran and the Hadith (the sayings of the prophet Mohammed), different traditions as well as lack of sufficiently trained slaughtermen, interested individuals and certifiers. This situation is in contrast with the approach of Shechita organisations that have strict and more consistent rules and applications.

The act of slaughter (Al-Dhabh) is allowed in the name of God; therefore pronouncing the name of Allah is the usual practice. This is to remind the slaughterer that he is taking the life of a living creature. Animals are restrained but there are no specific religious regulations as to how this should be done other than traditional methods employed. Following restraint, slaughter is carried out by severing the neck to achieve instant and copious exsanguination using a sharp knife. The usual type of incision is transverse severance of the vessels in the retrograde fashion following an initial stab incision in the neck.

Muslims believe that they are required to ensure rapid and maximum blood loss and that this is crucially important during and after Halal slaughter, because consumption of blood is forbidden. Effective exsanguination however, has been a source of concern in that in some cases occlusions can impede bleed out rate and delay loss of consciousness (Anil *et al*, 1995a,b). Another claim was that stunning methods could impede blood loss during Halal slaughter. Comparative studies in sheep and cattle have shown, however, that there is no significant difference between stunned and non-stunned sheep (Anil *et al,* 2004) and cattle (Anil *et al*, 2006).

Dialrel report aimed to summarise rules relating to Halal slaughter based on religious written sources and recommendations as well as consultations with a variety of interested parties and scholars from different countries and background. Although religious rules regarding Halal slaughter are still controversial, consultations carried out by Dialrel project have tried to shed some light on this important issue. The main findings of the consultations in Egypt as well as contrasting views are listed below:.

- Alive animal is required before death by exsanguination
- Flow of blood before death is essential
- Tasmiyyah (prayer) is required during slaughter
- Eating of any meat in necessity and from people of the books is acceptable
- More flexibility in rules than thought
- Kible (facing Mecca) is not necessary, but optional
- Recommendation for latest techniques confirmed
- Stunning acceptable if above conditions are met
- Misunderstandings of techniques and effects still exist that require addressing globally

The main difference between the conventional and Halal slaughter is the bleed out. In order to obtain Halal meat animal's death must be the result of exsanguination after a neck cut. Earlier stopping of the heart would render the carcass unacceptable.

The main controversy, undoubtedly, is still whether or not preslaughter stunning is acceptable. Although, the consultations and research in Egypt and Europe have revealed that reversible stunning would be permissible, subject to conditions, there are and will be objections. Some of those are based on legitimate concerns about stunning effects, however, the others stem from misunderstanding or lack of knowledge about stunning techniques. Unless these challenges are addressed satisfactorily, a two-tier application involving both stunning and slaughter and neck cutting without stunning, where allowed nationally, will be used in practice. To this end, Dialrel, following research and consultations has produced a set of recommendations for good practices under different scenarios.

The other religious slaughter is Shechita-Jewish method. Jews consume beef, veal, mutton, lamb and poultry, but not pork. These meats must be slaughtered and prepared in accordance with the rabbinical laws (Levinger, 1995).

The slaughter is carried out by a Shocet. A single, transverse cut is made across the neck using a very sharp, special knife (chalaf). The knife has to be examined for its sharpness between each cut

Once an animal is dead, an incision is made through the abdominal wall and a Jewish Inspector feels at arm's length into the thorax to check for pleural adhesions or any other signs of abnormality. If any abnormality is found, the entire carcass is rejected for Jewish consumption on the ground that the animal was not healthy at the time of slaughter.

In Europe religious slaughter has been practices for centuries, however, objections on welfare grounds started in the 1900s. Consumers in Europe now have more concern for food quality and safety as well as animal welfare. As regards legislation some EU members, such as Sweden, have banned slaughter without stunning in recent years. Nevertheless, Council Directive 93/119/EC (European Community, 1993) of the European Union al-

lows derogations so that Member States can authorise religious slaughter without pre-slaughter stunning in their own territory. However, it is also required that welfare of animals slaughtered by religious methods shall be protected and a mechanical form of restraint be used to prevent injury when the animal is killed.

Dialrel project has collected national legislation documents on religious slaughter from European Countries and prepared a report on this issue (Ferrari and Bottoni, 2010) showing existing gaps and differences. The new 1099/2009 (European Community, 2009) regulation is aimed at bringing in further important changes. Some examples are:

- Individual restraint of bovine and ovine animals if slaughtered without stunning and checks on recovery
- A report on systems restraining bovine animals by inversion to be submitted before 2013 (This method is banned in the UK)
- Ban on hoisting and clamping legs of animals (other than poultry) before slaughter
- Requirement for training slaughterman

Codes of practices and recommendations

Dialrel project concluded its activities with a set of recommendation for improved practices to be adopted during religious slaughter. This document was the result of detailed discussion between project partners, advisory board members, and representatives of the meat industry, religious organisations, groups and individuals. Although it does not impose legal requirements, it is hoped that the recommendations are observed up as much as possible with a view to protecting animal welfare as much as possible in practice. The following are extracts from the final document posted on the Dialrel website:

1. The slaughter person must be ready to perform the cut before the animal is restrained.
2. The neck cut must be performed without any delay.
3. Both carotid arteries and both jugular veins must be cut without touching the bones of the spine (vertebrae) with the knife.
4. Each animal should be neck cut by a single swift or continuous back and forward movement of the knife without interruption.
5. The knife used must be sufficiently long for each type of animal to minimize the need for multiple cuts. Ideally, the length of the knife blade should be at least twice that of the width of the animal's neck.
6. The knife must be sharp for each animal. The knife should be checked by the slaughtermen (Shochetim for Shechita) as frequently as required for nicks and bluntness and sharpened accordingly. Emphasis on training slaughter persons to improve their knife sharpness is recommended.
7. Neck breaking must not be performed together with the cut.

Several clinical signs have been suggested to recognize unconsciousness:

- Complete loss of posture.
- No attempts to regain or to retain upright body posture.
- No reactions (e.g. retraction) to mechanical impacts on the wound (e.g. contact of the wound to parts of the head-holder or pen).
- Absence of tracking by the eye of movements in the vicinity often accompanied by spontaneous closure of the eyelid.
- Absence of response to threatening movements (e.g. rushing the hand towards the eyes leading to closing of the eyes or moving the head backwards does not occur).

These are the clinical signs of brain death:

- Permanent absence of cardiac activity (e.g. pulse or heart-beat) when bleeding has ceased.
- Permanent absence of brain stem reflexes such as pupillary light reflex, corneal reflex, rhythmic breathing and gagging.

Post cut checks:

1. There must be no interference with the wound until the animal is unconscious, except for procedures involved with checking the adequacy of the cut.
2. The cut should be inspected carefully for complete sectioning of both carotid arteries and both jugular veins, and for the efficiency of bleeding through the strong flow and seeing the pulsating effect of the heart-beat on this flow..
3. The animal must be assessed to be unconscious by the slaughter persons (or the shochet) before it can be released from the restraint. It is suggested that the signs of unconsciousness are checked at least twice, for cattle between 30 and 40 seconds post-cut, and for sheep between 15 and 25 seconds post-cut. The following clinical signs should be used as a guide for monitoring:

- No attempts to regain or retain upright body posture.
- No reactions (e.g. retraction) to mechanical impacts on the wound (e.g. contact of the wound with parts of the head-holder or pen).
- Absence of tracking by the eye movements in the vicinity often accompanied by spontaneous closure of the eyelid.
- Absence of response to threatening movements (e.g. rushing of the hand towards the eyes leading to closing of the eyes or moving of the head backwards does not occur).

4. In the event of inefficient bleeding or prolonged consciousness being exhibited during repeated checks after neck cutting, animals should be stunned with a suitable method as soon as possible, even if this requires the religious authorities to declare the animal as non-kosher or haram.
5. As prolonged consciousness is an indicator of poor procedures, in the event of prolonged consciousness, the problem should immediately be investigated and necessary corrective action taken. Records of failure should also be documented for monitoring purposes.
6. Further dressing or scalding or electro-stimulation shall only be performed after brain death of the animal has been verified as indicated above.
7. When the cut is performed in a 180º inverted position in cattle, it may be preferable to turn the box to a position between 180º and 90º directly after the cut for better access to the head of the animal and a more relaxed position.

REFERENCES

1. Anil, Haluk and Lambooij, Bert 2009. Stunning and slaughter methods. In: Welfare of production animals: Assessment and management of risks, Volume 5, Food safety Assurance and veterinary Public Health, Eds: Smulders, FJM and Algers, Bo. Wageningen Publs."
2. Anil, M.H., Yesildere, T., Aksu, H., Matur, E., McKinstry, J.L., Erdogan, O., Hughes, S. and Mason, C., 2006. Comparison of Halal slaughter with captive bolt stunning and neck cutting in cattle: exsanguination and quality parameters. Animal Welfare 15, 325-330.
3. Anil, M.H., Yesildere, T., Aksu, H., Matur, E., McKinstry, J.L., Erdogan, O., Hughes, S.and Mason, C., 2004. Comparison of religious slaughter of sheep with methods that include preslaughter stunning and the lack of differences in exsanguination, packed cell volume and quality parameters. Animal Welfare 13 (4), 387-392.
4. Anil, M.H. and Austin, A., 2003. Bovine Spongiform Encephalopathy: A Review of some factors that influence meat safety. Agrippa.http://www.fao.org//DOCREP/ ARTICLE/AGRIPPA/590_en.htm#P11_1213
5. Anil, M.H., Butler, S.R., Johnson, C.B. and McKinstry, J.L., 2000. Suppression of somatosensory evoked potentials by Transcranial magnetic stimulation in the rabbit. Proc. Physiological Society, 526p.
6. Anil, M.H., McKinstry, J.L., Wotton, S.B. and Gregory, N.G. 1995a. Welfare of calves – 1.Investigation into some Aspects of Calf Slaughter. Meat Science, 41 (2), 101-112.
7. Anil, M.H., McKinstry, J.L., Gregory, N.G., Wotton, S.B. and Symonds, H., 1995b. Welfare of calves. 2. Increase in vertebral artery blood flow following exsanguination by neck sticking and evaluation of chest sticking as an alternative slaughter method. Meat Science 41, 113-123
8. Coore, R.R., Love, S., McKinstry, J.L., Weaver, H.R., Philips, A., Hillman, T., Hiles, M., Helps, C.R. and Anil, M.H., 2005. Brain tissue fragments in jugular-vein blood of cattle stunned by use of penetrating or non-penetrating captive bolt guns. Journal of Food Protection 68 (4), 882-884.
9. Coore, R.R., Love, S., McKinstry, J.L., Weaver, H.R., Phillips, A., Hillman, T., Hiles, M.J., Shand, A., Helps, C.R. and Anil, M.H., 2004. Research note: Dissemination of brain emboli following captive bolt stunning of sheep: Capacity for entry into the systemic arterial system. Journal of Food Protection 67, 1050-1052.
10. DIALREL. http://www.dialrel.eu
11. EC 2005. Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97
12. EC 2009. European Community, 2009. COUNCIL REGULATION (EC) No 1099/2009 http://ec.europa.eu/food/animal/welfare/slaughter/regulation_1099_2009_en.pdf
13. EC 1993. European Community, 1993. Directive 93/119/EC on the protection of animals at the time of slaughter or killing. European Community Official Journal 340, 21–34.
14. EFSA 2004. Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals http://www.efsa.europa.eu/de/scdocs/doc/45.pdf
15. DIALREL. Reports and factsheet. http://www.dialrel.eu/dialrel-results
16. Ferrari, S. And Bottoni, R. 2010. Legislation regarding religious slaughter in the EU member, candidate and associated countries. Dialrel Deliverable 1.4 http://www.dialrel.eu/images/report-legislation.pdf
17. Gibson, T.J., Johnson, C.B., Murrel, J.C., Hull, C.M., Mitchinson, S.L., Stafford, K.J.,Johnstone, A.C. and Mellor, D.J., 2009a. Electroencephalographic responses of halothane anaesthetized calves to slaughter by ventral-neck incision without prior stunning. New Zealand Veterinary Journal 57(2), 77-83.
18. Gibson, T.J., Johnson C.B., Murrell, J.C., Chambers, J.P., Stafford, K.J. and Mellor, D.J.,2009b. Components of electroencephalographic responses to slaughter in halothane-anaesthetized calves: Effects of cutting neck tissues compared with major blood vessels. New Zealand Veterinary Journal 57(2), 84-89.
19. Grandin, T. and Regenstein, J.M., 1994. Religious slaughter and animal welfare: a discussion for meat scientists. Meat Focus International, March 1994: 115-123. http://www.grandin.com/ritual/kosher.slaugh.html
20. Kalweit E., Ellendorf F., Daly, C. and Schmidt, D. 1989 1 Physiological reactions during slaughter of cattle and sheep with and without stunning. Deutsch. Tierärztl. Wochenschr. 96, 89-92.
21. Levinger, I.M., 1995. Shechita in the Light of the Year 2000. Maskil, L. David (Publ).
22. Lisanby, S.H., 2002. Update on magnetic seizure therapy: a novel form of convulsive therapy. J. Ect. 18(4), 182-188.
23. Masri. Al-Hafiz, B.A. 1989. Animals in Islam. The Athene Trust. ISBN 1-870603-01-X
24. Lambooij, E., Anil, H. S.R. Butler , H. Reimert, L. Workel and V. Hindle (2011).Transcranial Magnetic Stunning of Broilers; a preliminary trial to induce unconsciousness. Animal Welfare 20, 407-412
25. Rosen, S.D., 2004. Physiological insights into Shechita. The Veterinary Record 154, 759-765.
26. WASK 1995. The Welfare of Animals (Slaughter or Killing) Regulations 1995) UK

ПОДОБРУВАЊА НА БЛАГОСОСТОЈБАТА КАЈ ФАРМСКИТЕ ЖИВОТНИ ПРИ МАНУПУЛАЦИЈА,ТРАНСПОРТ И КОЛЕЊЕ

Анил Халук[1]

[1] Гратско Училиште и Регионално Планирање, Универзитет во Кардиф, Кардиф, У.К

АПСТРАКТ

Во процесот на примарно произвотсво на фармски животни, истите се подложни на интервенции кои вклучуваат манипулација, транспорт и колење, каде благосостојбата на животните може негативно да влијае врз квалитетот на целиот труп и месото, доколку оптималните услови не се запазени. Со цел да се заштити благосотојбата на животните, направена е легислатива во повеќето Европски земји. Одлуката на советот како што е Регулативата 1099_2009- (EC 2009) и (EC) No 1/2005 (EC 2005) која обезбедува насоки за националните влади за да се подготват и ги споведат прописите. Направени се разни подобрувања на благосостојбата на животните за подобрување на условите во текот на транспортот на месото до кланиците. Неодамна беа воведени детални услови кои се однесуваат на товарење, патување, истовар и одмор, вода и храна на животните, како и дизајн на возилото, барања за простор, за контрола на температурата, и обуки на персоналот. Хуманото колење исто така е важно прашање кое се занимава со конвенционални и религиозни колења. Разбирање на однесувањето на животните од различни видови може да помогне во дизајн на кланиците, со што би се минимизирал стресот за време на минување на животните Се очекува операциите на колење да се изведуваат со безболно и безстресно искрварување на животните, со користење на методи за зашеметување (Anil and Lambooij, 2009). Слабата благосостојба може да се манифестира во самите трупови како акутни процеси како што се фрактури, модрици, петехијални хеморагии и хронични ефекти на стрес и замор што резултира со смален квалитет на производи. Во последните години, особено големо внимание се посветува на религиозните аспекти во процесот на колење. Dialrel, проект кој е финансиран од Европската комисија, собра информации во однос на законодавството, правилата, практиките, пазарите, побарувачката и значајните проблематични точки во однос на благосостојбата кај животните (Dialrel).

Клучни зборови: благосостојба на животните**,** манипулација пред колење, колење

Plenary Lecture

UDC: 636.2.034.082:575.22

RELEVANCE OF GENETIC RESEARCH FOR MILK PRODUCTION AND UDDER HEALTH

Ogorevc Jernej[1], Prpar Sonja[1] , Kunej Tanja[1] and Dovc Peter[1]

[1]*Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Slovenia*
**Corresponding author:peter.dovc@bf.uni-lj.si*

ABSTRACT

Development of molecular genetics and in the last period rapid advancement of genomic thechnology enabled research towards molecular architecture of mammary gland related traits. A number of genetic markers, associated with functional mutations in candidate genes allows application of DNA polymorphisms in selection procedures leadingt o marker assisted selection. In addition to specific milk trait related genes, there is also an important field of research covering health traits and improvement of animal welfare and robustness. Combination of large amounts of data with bioinformatics tools opens a new approach in mammary glkand biology research, which is heavily dependent on large amounts of data and enables efficient combination of different types of data for more profound analysis of biological problems. For practical animal breeding and veterinary medicine these new technologies offer for the first time the possibility to select directly for favourable genetic vaiants, rather for phenotypic traits which might be heavily biased by environmental factors.

Key words: casein genes, mammary gland, milk QTL, mastitis, cell renewall

INTRODUCTION

Milk plays an important role in human nutrition and therefore milk production is one of the most important branches of animal production. Dairy industry is oriented in the production of the increasing number of different milk products and, consequently more and more interested in the improvement if technological properties of milk. For the production of cheese, for example, milk with higher content of proteins with favorable cheese making properties is preferred (Buchberger and Dovc, 2000). The early detection of polymorphisms in amino acid sequence of caseins allowed classical linkage studies revealing clustering of casein loci on BTA6. Application of recombinant DNA technology revealed cDNA sequences for major lactoproteins and further development of molecular genetics enabled study of the genomic organization of lactoprotein gene loci (Debeljak e tal., 2000). These studies revealed an insight into molecular architecture of casein gene expression (Rijnkels et al., 1997). Further development of genomic tools in combination with advanced statistical methods introduced the concept of QTL which allowed identification of genomic regions affecting complex milk production traits (Bovenhuis and Spelman, 2000).

The progress in cattle genome sequencing and public availability of whole genome maps led to the identification of important polymorphic sites with an impact on the lactoprotein gene function and signal transduction in the mammary gland. Recently, the development of a high resolution whole genome map of the cattle genome and comparative mapping approach utilizing human genome information, facilitated identification of genes responsible for several important quantitative traits, among them also quantitative trait loci for milk production. Considerable effort has been spent in order to produce genetic maps of different farm animal species, which have enabled localization of selected loci into syntenic groups (Gellin et al., 2000). Synteny maps provided valuable early information for practical animal breeding facilitating haplotype selection rather than simple selection for desired genotypes. For the practical animal breeding, the information about the genetic variation in some functionally important regions is of great importance, therefore numerous population studies analyzing genetic polymorphisms within crucial genomic regions were performed. Further, deposition of large stretches of genomic DNA sequences in public databases allowed searching for DNA sequences from different species and powerful bioinformatics tools made analysis of complex genomic data accessible for a wider scientific community. The introduction of micro array technology enabled analysis of complex tissue specific gene expression profiles in a single experiment. This approach can provide the information about differential gene expression in different developmental and physiological stages as well as reaction to different environmental stimuli. Further development of high density SNP chips allowed large association studies which gave the information about genome regions associated with important production aand health traits.

The concept of forward genetics, starting with phenotypic traits, which was the only possible concept in the past, was complemented with reverse genetics approaches offering identification of gene function based on sequence and mapping information. Historically, pedigree analyses and establishing of suitable mapping populations was an important goal of animal genetic research. In farm animals creation of special mapping populations is often too costly, therefore suitable statistical models (e.g. daughter design) were developed in order to extract genetic information from already available population structure. Genomic libraries containing ordered collection of large genomic fragments (mostly BAC clones) represented one of the most important resources

for genomic research in every species and BAC libraries with several fold coverage of the genome are now available for all farm animal species. The information from EST libraries has been also used for assignment of gene ontology, shedding a new light into the functional organization of the genome. Since polygenic traits are of crucial importance for animal breeding, statistical methods for identification of genomic regions with significant phenotypic impact on quantitative traits have been developed. A recent development in RNA sequencing and new generation of DNA sequencing strategies open a new horizon in individual genetic analysis of the complex architecture of milk production traits. Due to the permanantly growing costs for veterinary interventions in high producing herds, the interest for improvement of health status, especially improved mastitis resistance and improved robustness in milk herds is an important issue.

The impact of casein loci on milk traits

Introduction of molecular genetics, mainly polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis enabled identification of different lactoprotein gene variants at DNA level. The number of different variants, from which were some present in all breeds but other only in some local breeds, has rapidly increased. Table 1 summarises the known genetic variants of milk proteins.

Table 1. *Genetic variants of milk proteins in cattle**

Milk protein	Common variants	Rare variants in Bos taurus breeds	Variants found only in non-European breeds
as1-Casein	B, C	A, D, F, G	E, H, X, X', Y
as2-Casein	A	B, D	C
b-Casein	A1, A2, B	A3, A5, C, E, F, G A4, A', $A3_{Mongolie}$, B2_	BZ, D, H
k-Casein	A, B	C, E, F, G, H A1, B2, F, I	A_{1SS}, G, H, J, X, Y
b-Lactoglobulin	A, B	C, D, H, I, J, W	Dr, D_{Yak}, E, F, G, H, X, Y, Z
a-Lactalbumin	B	A	C

** Buchberger and Dovc, 2000*

The common variants of lactoproteins are present in all cattle breeds. However, many minor variants occur only in non-western breeds such as yaks, Indonesian breeds (Bali cattle/banteng), zebus or in *Bos indicus* x *Bos taurus* crosses. The frequency of genetic variants differs from breed to breed and the frequency distribution of the various milk protein variants in different breeds has been studied comprehensively. In the last decades numerous investigations have focused on the association between certain genetic variants of milk proteins and yield traits, milk composition and technological properties of milk. The scope of these studies was to explore the possibility to select for specific protein variants related to economically important traits. Several polymorphisms within the coding and regulatory regions of lactoprotein genes were associated with technological properties of milk and differences in milk composition. Probably the most well known effect of lactoprotein variants on technological properties of milk is positive effect of CSN3 allele B on micelle size, coagulation time, curd firmness and cheese yield (Buchberger and Dovc 2000). In addition to the quantitative effect of CSN3 allele B on milk composition, there is a positive effect on cheese making properties of milk which exceeds the change which would be expected from the changed milk composition. The higher expression of CSN3 allele B at protein and at RNA level could not be explained with allele specific polymorphisms in the CSN3 gene proximal promoter. As a possible molecular reason for higher expression of CSN3 allele B has been suggested a more stable mRNA, due to the absence of destabilizing element in the CSN3 B 3'-UTR region which might represent a target region for miRNA.

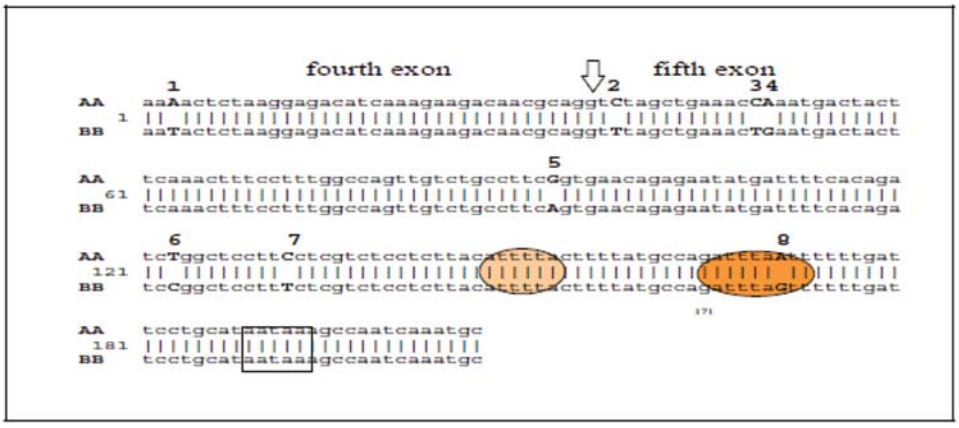


Figure 1. *Allelic differences between bovine CSN3 last two exons and 3'-UTR sequence having a potential impact on mRNA stability. Allele specific mutations are numbered and mutation number 8 abolishes destabilizing element in the CSN3 allele B.*

Beta lactoglobulin locus – a candidate locus

The level of expression of beta lactoglobulin (BLG) gene and casein genes demonstrates a competitive character. So have the animals with higher expression of BLG gene lower casein number. The molecular reason for differential allelic expression at the BLG locus is promoter mutation which represents a candidate mutation for BLG expression and casein number. The G to C transversion in the promoter region of the *BLG* gene at position -430 within the AP-2 binding site was proposed to be associated with the reduced binding efficiency of AP-2 transcription factor (Lum et al., 1997). The reduction of BLG expression as the consequence of the lower binding affinity of AP-2 to the mutated promoter site was demonstrated *in vitro*. However, Kuss et al (2003) were able to show that G to C transversion within the AP-2 binding site of the BLG promoter is associated with lower expression of BLG and ALA genes and higher casein number in the German HF and Simmental population.

DGAT and ABCG2

Large genomic sections represented by QTLs, interactions between environment and genotype, epistatic effects and genetic imprinting compromise reliability of QTL detection. These are the reasons why the precision of QTL mapping is significantly reduced compared with a detection of causal loci for the single locus traits. Therefore, for reliable proof for identification of a causal gene for QTL in commercial animal populations multiple pieces of evidence are necessary, of which no single one is convincing, but all together consistently point to the candidate gene. Although a number of QTLs affecting milk production traits was identified in cattle and other mammalian species, there are only a few reports demonstrating convincing molecular evidence for the presence of milk trait related QTLs in cattle at the nucleotide level. The first reported positional cloning of a QTL in an outbred mammal revealed the missense mutation in the bovine *DGAT1* gene with the major effect on milk yield and composition (Grisart et al., 2002). The gene identification was based on bioinformatics, comparative mapping and functional analysis. Bovine *DGAT1* gene was cloned by means of positional candidate cloning targeting the 3 cM chromosomal region on the telomeric end of BTA14. The presence of the milk trait QTL in this chromosomal region was detected earlier but Grisart et al. (2002) detected nonconservative K232A substitution in the *DGAT1* (acylCoA:diacylglycerol acylransferase 1) gene as a molecular cause for the BTA14 QTL effect. The bovine *DGAT1* gene spans 8.6 Kb and comprises 17 exons and has 89.5% DNA sequence identity with the human homologue. Recent gene knock out experiments showed that absence of functional *DGAT1* gene in mouse completely inhibits lactation. In cattle, four *DGAT1* haplotypes were identified, where three (sHQ-D, sHQ-NZ and sHQ-III) had positive and one (shq) negative effect of milk fat content. The positive Q alleles for milk fat, characterized by K at position 232 had negative effect on milk and protein yield (-158 kg and -2.82 kg, respectively) and positive effect on fat and protein % (+ 0.17% and + 0.04%, respectively). However, in spite of the known role of the *DGAT1* gene in fat metabolism and effect of its inactivation in knock out mice, the mechanism of action of the K323A mutation remains unclear. The second example for identified missense mutation as a cause of QTL effect in an outbred cattle population is the Y581S mutation in the bovine *ABCG2* gene at BTA6. The phenotypic effect of allele substitution on *ABCG2* locus based on genetic evaluation of 335 Israeli Holstein sires was -341 kg milk, +0.16% fat and +0.13% protein (Cohen-Zinder et al., 2005). The allele substitution effect, measured on 670 cows, daughters of two heterozygous sires was -226 kg milk, +0.09% fat and +0.08% protein. Within the 31,655 bp genomic region at BTA6 bovine *SPP1, PKD2* and *ABCG2* genes were sequenced and *SPP1* and *ABCG2* were further considered as candidates due to their differential expression during the lactation and dry period. Multiple evidence e.g. significant phenotypic effect, gene expression during lactation, concordance with the previously mapped QTL region at BTA6 and robustness in two outbred populations made tyrosine to serine mutation at position 581 in the bovine *ABCG2* gene a good candidate for QTL on BTA6. In addition, the physiological function of the *ABCG2* gene in mouse, where it is responsible for the active secretion of clinically and toxicologically important substances into the mouse milk, suggests the possible role of this transmembrane protein, which belongs to the ATP binding cassette transmembrane protein, in cholesterol transport into milk.

Mastits resistance

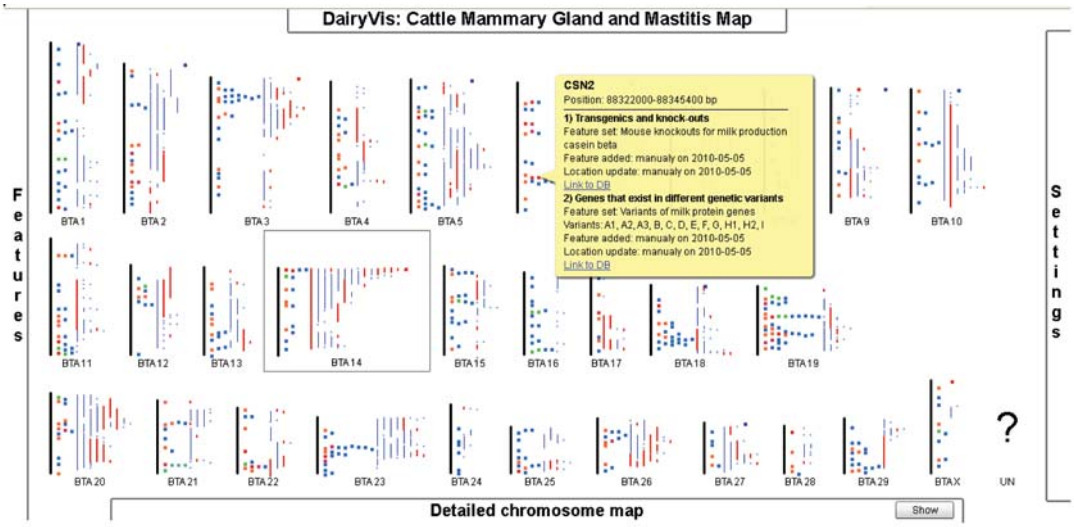
Mastitis is the most costly disease in dairy cattle. It has been estimated that economic loses caused by mastitis range from $100 to $200 per cow per lactation. That is why a fair amount of genetic research related to udder health has been performed. Milk production and manufacturing significantly supported genetic research related to milk production and udder health traits in the past. During last years many experiments have identified different QTL regions in cattle affecting functional traits such as mastitis. QTLs cover large chromosomal regions averagely spreading from 10 to 40 cM, involving hundreds of genes. The ultimate goal of the QTL analysis is the identification of causal gene itself, therefore a fine mapping of the mastitis associated QTLs could make marker assisted selection (MAS) possible and eventually facilitate identification of resistance genes and alleles. Beside QTLs, a large number of genetic polymorphisms within the causal gene regions or genetic markers associated with mastitis traits have been identified in cattle. The high throughput technologies such as microarray analysis offers the possibility to study changes in expression profiles of thousands of genes simultaneously as a response to infection with the pathogen. The release of the cattle genome sequence enabled discovery of new markers and creation of synteny maps including data from other species. miRNAs have been isolated from mammary gland tissue and proposed to play important role in regulatory pathways in mastitis resistance or susceptibility. Additionally, epigenetic chromatin modifications (remethylation) were also demonstrated to be involved in clinical mastitis.

To facilitate development of new genetic markers for mastitis resistance or susceptibility we used genome-wide comparative approach to review mastitis associated loci. Mastitis traits were studied using different approaches, including QTL approach, association studies and candidate gene approach. Information extracted from these methodologically focused studies is often fragmented and controversial. To integrate information from different sources we created gene map of mastitis

resistance or susceptibility candidate genes. Gene map approach reveals positional overlaps of loci found with different approaches and exposes regions with high density of candidate loci. Candidate loci associated with different approaches or with different studies using the same approach and/or in regions overlapping with QTLs represent especially strong candidate genes for association with mastitis resistance or susceptibility. Best mastitis resistance or susceptibility candidates were selected and *in silico* searched for genetic variability and miRNA target sites in their 3' UTR. We aimed to establish data of candidate loci applicable for use in further functional studies for mastitis resistance or susceptibility traits and understanding of mastitis regulatory pathways. A mastitis resistance or susceptibility candidate gene map was created incorporating different study approaches (QTLs, association studies, expression experiments, AFLP studies, miRNAs, and epigenetic factors) consisting of 233 loci. To date there are 60 cattle QTLs associated with mastitis traits (clinical mastitis and somatic cell score). Six genes showed association between sequence variation and mastitis resistance or susceptibility. 107 genes with expression patterns associated with mastitis resistance or susceptibility were reported in 12 publications in cattle and mouse. Additionally 27 AFLP markers associated with mastitis were found and the most promising marker named *CGIL4* was further characterized and mapped to BTA22 q24. To date 32 miRNA genes were reported to be expressed in bovine mammary gland, but their involvement in mastitis is not known yet. Epigenetic factors; DNA-remethylation around the *STAT5*-binding enhancer in the *CSN1S1* promoter was shown to be associated with shutdown of α_{S1}-casein synthesis during acute mastitis.

We developed also a web based tool for in silico analysis of mammary gland expressed genes, DairyVis, which enables holistic map based analysis of candidate regions. The application of DairyVis is shown in Fig. 3.

Figure 3. *An expample of candidate region (CSN locus) analysis using DairyVis tool.*



Renewal of the mammary gland

The caprine epithelial cells were transplanted under the renal capsule of NOD/SCID mice which were treated with hormones administered through a silicon pellet and recovered after 4–6 weeks. In the immunostained xenograft sections (Fig. 4) the spatial distribution of cells is similar to that seen in caprine mammary tissue. Cells are organized as a polarized bilayered epithelium enclosing a lumen. The mammary origin of these cells was confirmed by progesterone receptor expression and by presence of milk proteins in the lumen of the outgrowths. Both stainings also show that the regenerated structures underwent a functional differentiation and were able to perform as proper mammary alveoli (Prpar et al., in press). This experiment demonstrated the regenerative capacity of ruminant mammary gland epithelial cells *in vivo*.

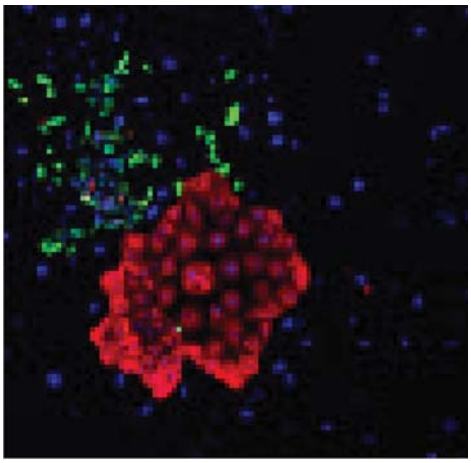


Figure 4. ***I****mmunostained xenograft sections and spatial distribution of cells similar to that seen in caprine mammary tissue.*

Conclusion and further prospectives

Development of genomic tools and public availability of cattle genome sequence represent a great challenge for further improvement of lactation related traits. Presented examples of QTLs which have been characterized at the nucleotide level demonstrate the power of comparative approach using bioinformatics and functional genomics. The realistic expectation is that more and more, well defined molecular markers will be discovered and applied for marker assisted selection. Better understanding of physiological impact of natural variation in lactoprotein genes and genes responsible for synthesis of different milk components, will allow selection for milk with optimized characteristics. In this context play very important role bioactive substances in milk, which have an impact beyond the nutritional value of milk. Different oligopeptides, CLA and glutathione are only some of the substances which could have an impact on major health threats in the modern society: blood pressure, obesity and cancer. Application of genomics, proteomics and metabolomics for identification of bioactive substances in milk will enable production of dairy products with health claims. Reduction of lactose content, desaturation of milk fat and modification of milk protein fraction as well as improvement of technological properties of milk are only a few challenges for the milk production in the future. However, the profound knowledge about genetic architecture of milk related traits opens a wide field of oportunities for marker assisted selection and tergeted introduction of favourable genetic variants usoing conventional breeding strategies.

REFERENCES

1. Buchberger, J., Dovc, P. (2000) Food technol. biotechnol. 38 : 91-98.
2. Debeljak, M., Susnik, S., Milosevic Berlic, T., Medrano, J.F., Dovc, P. (2000) Food technol.biotechnol. 38 : 83-89.
3. Rijnkels, M., Kooiman, P.M., De Boer, H.A., Pieper, F.R. (1997) Mamm Genome 8 : 148-52.
4. Bovenhuis, H., Spelman, R.J. (2000) J Dairy Sci. 83 : 173-80.
5. Gellin, J., Brown, S., Marshall Graves, J.A., Rothschild, M., Schook, L., Womack, J., Yerle, M. (2000) Mamm Genome 11 : 140-144.
6. Lum, L.S., Dovc, P., Medrano, J.F. (1997) J. Dairy Sci. 80 : 1389-1397.
7. Kuss, A.W., Gogol, J., Geldermann, H. (2003) J Dairy Sci. 86 : 2213-2218.
8. Grisart, B; Coppieters, W; Farnir, F; Karim, L; Ford, C; Berzi, P;Cambisano, N; Mni, M; Reid, S; Simon, P; Spelman, R; Georges, M; Snell, R. (2002) Genome Research 12: 222-231
9. Cohen-Zinder, M., Reichenstein, M., Everts-van der Wind, A., Heon-Lee, J., Shani, M., Lewin, H.A., Weller, J.I., Ron, M., and Seroussi, E. (2005) Genomics 84: 374–383.
10. Prpar, S., Martignani, E., Dovc, P., Baratta, M. Biology of reproduction (in press).

ЗНАЧЕЊЕ НА ГЕНЕТСКИТЕ ИСТРАЖУВАЊА ЗА ПРОИЗВОДСТВОТО НА МЛЕКО И ЗДРАВЈЕТО НА ВИМЕТО

Огоревц Јернеј[1], Прпар Соња[1] , Кунеј Тања[1], Довч Петер[1]

[1]*Катедра за сточарство, Биотехнички факултет, Универзитет во Љубљана, Словенија*

*Автор за кореспонденција: peter.dovc@bf.uni-lj.si

АПСТРАКТ

Развојот на молекуларната генетика а во последно време и брзиот напредок на геномската технологија овозможија да се истражува молекуларната основа на својствата како што се производството на млеко и здравјето на вимето. Постојат неколку генетски маркери поврзани со функционални мутации кои овозможуваат директна примена на ДНК полиморфизми во селекциски цели, метода која денес е позната и како селекција со помош на генетски маркери (MAS-Marker Assisted Selection). Покрај истражувањата на својствата кои се поврзани со хемискиот состав и количината на произведеното млеко, се повеќе се спроведуваат и истражувања на својствата поврзани со здравјето на вимето, благосостојбата и отпорноста на животните. Со комбинирање на се поголемиот број податоци со достапните биоинформатичките методи се овозможува еден нов начин на истражување на биологијата на млечната жлезда, за кој е потребно што е можно поголем број на податоци со цел да се добие што е можно појасна претстава за биолошките проблеми. За практична примена во одгледувачките програми и ветеринарната медицина овие нови технологии за прв пат нудат можност директно да се селектираат посакуваните генотипови, наместо досегашната пракса да се селектира на фенотипски својства кои во голема мера можат да бидат под влијание на условите на средината.

Клучни зборови: казеински гени, млечна жлезда, QTL за млеко, маститис, клеточна регенерација

UDC: 639.3:597.551.2]:637.513

INFLUENCE OF TRANSPORTATION AND SLAUGHTER METHOD ON STRESS REACTION OF COMMON CARP (*CYPRINUS CARPIO* L.) FOR HUMAN CONSUMPTION

Daskalova Alexandra[1], Pavlov Alexander[1]

[1]*Deptartment of Food Hygiene, Technology and Control, Faculty of Veterinary Medicine, Trakia University, Stara Zagora 6000, Bulgaria*

ABSTRACT

Fish welfare during transport, keeping of live fish and slaughter process is important to produce quality product for human consumption. The aim of this study was to evaluate the effect of transportation, keeping in tanks for 6 days and slaughter method (by percussive blow on the head immediately after removal from the water tank and by asphyxia for 3-3.5 hours before death). This study evaluates stress reaction and postmortem changes in muscle pH in common carp (*Cyprinus carpio* L.). Stress reaction of the fish was assessed by measuring blood glucose and blood cortisol levels. Blood samples were taken immediately after transport and after six days rest in tap water with constant aeration. For assessment of postmortem changes in muscle pH some fish were killed by percussive blow on the head and another by asphyxia. Postmortem pH levels were measured at 0, 3, 6, 12, 24, 48 and 72 h post-slaughter. Data showed significant increase in blood glucose level after transportation of fish and significant decrease ($P \leq 0.001$) after six days rest in tap water. Cortisol levels after transport and after rest in tanks did not show statistically significant differences ($P \geq 0.05$). Fish flesh after asphyxia had significantly lower initial pH level (6.93), compared to fish killed by percussive blow (7.29) ($P \leq 0.001$). Muscle pH levels of fish after asphyxia were similar from 0 to 48 h, stored at 4°C, and grew up significantly at 72 h of storage ($P \leq 0.05$). Musculature of fish killed by percussive blow showed slow decrease of pH and after 12 hours of storage at 4°C the result was statistically significant ($P \leq 0.05$), and the same low levels were presented to the end of the study (72 h).

The results indicate the possible influence of fish stress (after transport and asphyxia) on fish meat quality during storage at refrigerated temperatures.

Key words: fish stress, common carp, pH, fish meat, fish slaughter

INTRODUCTION

In recent years, there is a growing interest in animal welfare, including fish welfare during transport, stunning and slaughter. According to Council Regulation (EC) №1099/2009 on the protection of animals at the time of killing fish is physiologically different from terrestrial animals and research on the stunning of fish is far less developed than for other farmed species. Nevertheless, there are a lot of recommendations of different authorities concerning fish welfare during transport and slaughter (OIE, 2011; EFSA, 2004). Some authors consider that fish is not able to feel pain and emotional distress (Rose, 2002) but others assume that there is anatomical, physiological and behavioral evidence that fish can suffer (Chandroo et al., 2004). Remains the question how we could evaluate the level of pain and stress. Primary stress response in most of the fish species includes releasing of cortisol which leads to the increase in blood glucose level as a secondary response to stress factors (Barton, 2002). Slaughter method can cause stress reaction as well. A lot of different methods for stunning and killing fish are used in practice. According to the EFSA Scientific Panel on Animal Health and Welfare (2004) only percussive stunning, electrical stunning and spiking methods can be considered humane if correctly applied. Methods as asphyxia, asphyxia in ice, thermal shock, gas solutions, CO_2 narcosis, decapitation, exsanguination are not recommended because they cause avoidable suffering before death. Aversive reactions and increased muscle activity before death leads to muscle glycogen depletion which causes rapid reduction of postmortem muscle pH and earlier Rigor mortis onset. These changes shorten the pre-rigor period when the fish is processed and reduce the keeping quality of fish (Poli et al., 2005).

The aim of this study was to assess the influence of transportation, new life environment and slaughter method on stress response and changes in postmortem muscle pH in common carp.

MATERIALS AND METHODS

Fish and sampling

A batch of 24 carps (body weight 1,0 – 1,5 kg) was obtained from a commercial fish farm in Nikolaevo county, Bulgaria. Immediately after arrival blood samples (1 ml) were taken from caudal vessel of each fish. Blood samples were used for measuring cortisol and glucose levels. After that carps were placed in tanks with 800 l tap water and constant aeration. Fish were let rest for six days without feeding and then blood samples were taken again for measuring cortisol and glucose levels. For assessing the influence of slaughter method on postmortem muscle pH 6 carps were removed from the water and let die by asphyxia which took 3 to 3,5 hours (asphyxia group) and another 8 fish were killed by percussive blow on the head with a hammer (percussion group). Heads, tails and internal organs of each fish were removed and each carcass were divided into two halves along the spinal column and kept in a fridge at 4°C. Muscle pH was measured direct in the dorsal muscle at 0, 3, 6, 12, 24, 48 and 72 h postmortem.

Analytical methods

Blood for analyzing glucose levels was placed in tubes containing EDTA and centrifuged for 10 min at 3000 rpm^{-1}. Plasma was separated and blood glucose was measured by automatic biochemical analyzer BS-120 (Mindray, China). For measuring cortisol levels

blood samples were placed in tubes without anticoagulant and let coagulate at room temperature until serum could be separated. Cortisol level in the blood serum was estimated by Cortisol ELISA kit EIA 1887® (DRG Instruments GmbH, Germany). Sample dispense into Microtiter wells, as well as results calculation were carried out by automatic system SUNRISE® (Tecan, Austria). Muscle pH was measured at 0, 3, 6, 12, 24, 48 and 72 h using pH-meter Consort C532 (Belgium) by inserting a penetration electrode directly into the dorsal muscle.

RESULTS

Our results indicate that there are clear differences in the level of tested blood parameters in carps immediately after transportation and after 6 days resting. There was a 2-fold decrease ($P < 0.0001$) in blood glucose level after fish had rested for six days. Immediately after transport we estimated 10.773±2.235 mmol/L glucose, which decreased to 5.247±1.156 mmol/L after 6 days rest. Cortisol level dropped from 433.16 ± 147.07 ng/mL (after transport) to 363.33±253.98 ng/mL (after rest) but this difference was not statistically significant ($P \geq 0.05$). Muscle pH (Figure 1) of the fish died by asphyxia did not significantly change from 0 to 48 h, but at 72 h pH increased significantly ($P < 0,05$). Fish killed by percussion showed more regular postmortem muscle pH which decreased at 12 h ($P < 0,05$) and kept almost unchanged to the end of the study. Furthermore, asphyxia group showed significantly lower initial pH ($P < 0,001$) than percussion group - 6.93 and 7.29, respectively.

CONCLUSIONS

The results of blood glucose and cortisol measurements show that resting for six days is not enough for fish to overcome the stress caused by transport and accommodation to new life conditions. In our study carps kept extremely high levels of cortisol (both before and after rest) compared with the values reported by Barton (2002) in juvenile carps (7.4 ± 2.9 ng/mL pre-stress and 79 ± 14 ng/mL post-stress). The decrease in blood glucose levels after fish had rested for six days could be due to the lack of food and persistent high cortisol which may cause a strong depletion of glycogen. For more precise conclusions repeated tests over long time intervals should be carried out.

The strong exhaustion and glycogen depletion was the probable reason for the lower initial pH and the minimal changes in pH from 0 to 48 h in asphyxia group. These results are in agreement with those reported by Ribas et al. (2007) in Senegal sole. Investigating meat quality, Roth et al. (2007) showed that turbot killed by percussion had more regular postmortem pH compared with fish stunned and killed by other more stressful methods. In our two slaughter methods we obtained initial decrease followed by fast increase in muscle pH in asphyxia group, while the percussion group showed only pH decrease in all measurements (fig.1). This significant increase in muscle pH at 72 h in asphyxia group is a premise to rapid deterioration of the meat. Based on the results we conclude that percussion is a better method for stunning and killing fish because it is less stressful (death occurs almost immediately) than asphyxia (death occurs in 3-3.5 hours) and extends the storage period of the fresh refrigerated fish.

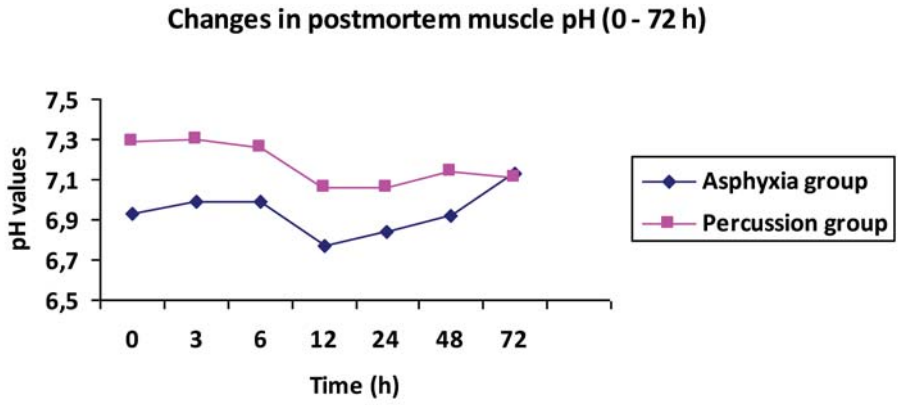


Figure 1.

REFERENCES

1. Barton, B. A. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. INTEG. AND COMP. BIOL., 2002, 42: 517-525.
2. Chandroo, K. P., I. J. H. Duncan & R. D. Moccia. Can fish suffer? Perspectives on sentience, pain, fear and stress. Appl. Anim. Behav. Sci., 2004a, 86, 225-250.
3. Council Regulation (EC) № 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. Official Journal of the European Union 18.11.2009, L 303/2 (11).
4. OIE (2011). Aquatic Animal Health Code. World Organization for Animal Health Paris.
5. Opinion of the Scientific Panel on Animal Health and Welfare on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. The EFSA Journal, 2004, 45, 1-29.
6. Poli, B. M., G. Parisi, F. Scappini & G. Zampacavallo. Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquaculture International, 2005, 13: 29-49.
7. Ribas, L., R. Flos, L. Reig, S. MacKenzie, B. A. Barton & L. Tort. Comparison of methods for anaesthetizing Senegal sole (Solea senegalensis) before slaughter: Stress responses and final product quality. Aquaculture, 2007, 269, 250-258.

8. Rose, J. D. The Neurobehavioral nature of fishes and the question of awareness and pain. Rev. Fish. Sci., 2002, 10, 1-38.
9. Roth, B., A. Imsland, S. Gunnarson, A. Foss & R. Shelvis-Smit. Slaughter quality and rigor contraction in farmed turbot (Scophthalmus maximus); a comparison between different stunning methods. Aquaculture, 2007, 272, 754-761.

ВЛИЈАНИЕ НА ТРАНСПОРТОТ И НАЧИНОТ НА КОЛЕЊЕ ВРЗ РЕАКЦИЈА НА СТРЕС НА ОБИЧНИОТ КРАП (*CYPRINUS CARPIO* L.) ЗА ЧОВЕЧКА ПОТРОШУВАЧКА

Даскалова Александра[1], Павлов Александар[1]

[1]Катедра за хигиена на храна, технологија и контрола, Факултет за ветеринарна медицина, Универзитет во Тракиа, Стара Загора 6000, Бугарија

АПСТРАКТ

Благосостојбата на рибите за време на транспорт, одржувањето на рибите во живот и процесот на колење се многу важни за производство на квалитетн производ за човечка потрошувачка. Целта на овој труд беше да се направи проценка на ефектот на транспортот, чувањето во тенкови во период од 6 дена и методот на колење (со удар во главата веднаш по вадењето од водениот тенк и со асфиксија 3-3,5 часа пред да настапи смрт). Овој труд ја евалуира стрес реакцијата и постморталните промени на pH на мускулите кај обичниот крап (*Cyprinus Carpio* L.). Реакцијата на стресот кај рибите беше одредена преку мерење на нивото на глукоза и кортизол во крвта. Примероци на крв беа земени веданш по транспортот и по шестдневно чување во вода од чешма со постојана аерација. За оценка на постморталните промени во мускулната pH, дел од рибите беа жртвувани со удар во главата, а дел со асфиксија. Постморталната pH вредност беше мерена 0, 3, 6, 12, 24, 48 и 72 часа по колење. Резултатите покажаа сигнификантно зголемување на глукозата во крвта по транспортот и сигнификантно намалување ($P \leq 0,001$) по шестдневно чување во вода од чешма. Нивото на кортизол по транспортот и по одмарање во тенковите не покажа статистички разлики ($P \geq 0,05$). По асфиксија месото од рибите имаше сигнификантно пониска иницијална pH вредност (6,93), споредена со рибите жртвувани со удар во главата (7,29) ($P \leq 0,001$). Нивото на мускулната pH вредност на рибите по асфиксија беше слично по 0 и 48 часа, чувани на 4°C, и покажа сигнификантен пораст по 72 часовно чување ($P \leq 0.05$). Мускулатурата на рибите жртвувани со удар во главата, покажа благо намалување на pH вредноста и по 12 часа чување на 4°C резултатите беа статистички сигнификантни ($P \leq 0.05$), и истите ниски вредности беа презентирани на крајот на експериментот (72 ч).

Добиените резултати укажуваат на можното влијание на стресот од транспорт и асфиксија врз квалитетот на рибиното месо за време на чување во фрижидер.

Клучни зборови: стрес кај риби, обичен крап, pH, рибино месо, колење на риби

UDC: 636.7.066

TYPIFIED THE NERVOUS SYSTEM OF THE DOG IN ORDER TO PROPERLYSOCIALIZATION AND MODELING OF CERTAIN BEHAVIORS

Uzunova Krasimira[1], Todoroska Marina[3], Binev Rumen[2], Miteva Chonka[2], Yuri Mitev[2]

[1]Faculty of Veterinary medicine, Trakia University – Stara Zagora, Bulgaria
[2]Agricultural Faculty, Trakia University – Stara Zagora, Bulgaria
[3]Department of Biology, University of Vienna- Vienna, Austria

ABSTRACT
Behaviors were examined on 27 puppies of breeds of dogs "German shepherd", "Collie" and "Doberman" through the mirror test of Bretto, to characterize the type of nervous system (temperament) for proper education, training and modeling their behavior. It is proved that the applied test is fast, simple, easy to implement and does not cause stressful feelings in experimental dogs, the test is qualify for welfare.
The obtained results show that the most bold and the most balanced are the representatives of "German Shepherd" followed by "Collie" and "Doberman."
Key words: puppies, behavior, temperament test, socialization

INTRODUCTION

The dog is the most recent pet today and have active presence in human life as a frontier guard, courier, hunter, criminologists, tax collector, tutor on children, help the disabled, healer, dog is perfect revealer of human emotional states and because that successfully detects psychosis (2, 3, 4).

Typified of the nervous system (temperament) is a topic on which is working in recent years in Bulgaria. Lot of veterinary colleges from and outside of the European Union isn't aware of the mandatory need for such a manipulation. The characteristic - temperament of the dog is directly related to integration into the environment after birth and subsequent formation of subsequent behavior (1, 3). The most appropriate age for this for us compulsory manipulation is between 3 - weeks and 3 - months of age (7, 8). It is proven that the most accurate and reliable results from a statistical point of view are the results in the course of study during this period (8, 9). According to this (8, 9, 10) indirect link exists between typified the dog's temperament and modeling on behavior. If the owner kept the dog according to the hygienic and biological requirements and have a knowledge of dog's temperament, he will have the right approach to it, the training and education will run smoothly and the dog will develop the desired behavior, without appear on behavioral disorders which sometimes can be from irreversible pathological nature - severe depression, tics, obsessions, anorexia, etc., accompanied by unpleasant behavioral activities of the sick dog: howls, constantly whining, unreasonably barking and so on (1, 10).

Behavioral pathology, however, is not only due to the incorrect approach of man to the animal, that is not only ethological nature (8, 10). It occurs less frequently when the dog is not kept in microclimatic conditions consistent with its biological requirements. However, proper socialization, successful adaptation on animal to the environment in which lives depends mainly from proper behavior of the owner towards animal. This happens when the owner known temperament of the dog which means a successful approach to its seamlessly socialization and the formation of a behavioral model (9).

Is proven that the dog has 4 types of nervous system (temperament):

1. Type L-sanguine - strong, balanced, leadership type of nervous system;
2. Type F-choleric - strong receptive, but flighty and excitable temperament;
3. Type G-phlegmatic - slower and passive type of nervous system;
4. Type A - melancholic - weak, unstable, indifferent, apathetic, but sometimes prone to wicked type of nervous system.

Most common are choleric and sanguine temperaments (1, 7, 8, 9). Some authors have found that quickly and easily socialize dogs are those with sanguine and choleric temperament, followed by phlegmatic type (4, 5, 6).

Typified the nervous system of the dog is an act by which it is possible to avoid increasing the homeless dog's population because if the owner knows the animal's temperament, he will have an appropriate approach to it, and the socialization will progress properly. The dog will not develop behavioral disorders and intolerable behavior which is the main reason for dog to be abandoned from its owner. Indeed, what will do the owner of animal with behavioral disorders? This depends on the value system, but research has shown that the most dogs are abandoned (8, 9, 10).

Determination of the nervous system of the dog is indicator of its welfare, because that animal is intimidated by the possible failure of man to approach it (2, 4, 5).

Today are apply different test methods for typified the nervous system of the dog. The publicized research data show that one of the most used methods for is the test with mirror on Bretto. In this regard, our goal is to characterize temperament and its influence in the socialization period through the ethological manifestations of puppies observed by use of this test, in order to conduct proper upbringing, socialization and the formation of future behavior (10).

MATERIALS AND METHODS

The experimental work was conducted in October 2011 with duration of one week. Studied were 27 puppies (private ownership) on age of 8 weeks. The puppies are representatives of three breeds: "German Shepherd", "Collie" and "Doberman." We chose those breeds because they are the most common in Bulgaria (3, 4, 5).

Each puppy was examined individually and twice in duration of 30 minutes. Every puppy was separated from the mother and other dogs and after research come back to them. According to requirements of the test with mirror on Bretto, the behavior of animals was performed in an empty room (10 m^2). We used a mirror big enough so that puppies can be seen in full size. Both tests were made immediately one after another and were carried by ethologist, person completely unknown to the animals.

Examined were followed behavioral activities - fear, irritability, aggression, vocalization, curiosity by the methods of observation and chronometry.

Assessment on these activities was based on behavioral criteria for determining the intelligence of the dog with use on point system (3).

- In the absence of the ethological event (ex. curiosity) dog gets 0 points - absence or low behavioral activity;
- Upon satisfactory or incomplete expression, 0.5 points – middle grade activity;
- For behavioral activity is on high grade dog gets 1 point

We focus mainly on the breed, not the gender of the puppies, so we don't mark the gender. We used numbering on animals as follows:

- Breed "German Shepherd" - № 1, 2, 3, 4, 5, 6, 7, 8, 9;
- Breed "Collie" - № 10, 11, 12, 13, 14, 15, 16, 17, 18;
- Breed "Doberman" - № 19, 20, 21, 22, 23, 24, 25, 26, 27.

The results are presented tabular rather than ethogram because the ethogram requires behavioral observation at least 48 h, and test of Bretto does not allow such duration of the study.

The entire experiment was consistent with the statutory requirements for the welfare of experimental animals as well as hygiene standards established for these breeds and categories on puppies.

RESULTS AND DISCUSSION

Breed "German Shepherd"

- Puppies № 1, 2, 4, 6, 8 at double study showed a uniform and stable behavior. The five puppies in the beginning of study when saw the mirror demonstrated balance and curiosity. They come nearer to the mirror without concern (calm) and even went behind him (curiosity), without fear and irritability, with big confidence. They were circling around the object interesting for them. We noted vocalization - quiet and joyful whining. These ethological activities gave us reason to allot them to L-type nervous system ie sanguine. Animals were balanced, calm, curious, friendly, real leaders. They weren't fearful, and therefore are not aggressive, because according to publicized research data the fear creates aggression. These dogs are very suitable for guards and for companion. Their training will take place quickly, easily and successfully. They adapt very quickly to the environment in which they live (2, 3, 4).

The percentage of dogs from sanguine type on temperament from breed "German Shepherd" is 55,6% (total 9 representatives of the breed).

- Puppies № 3, 5 and 7 at the beginning of study (first 3 minutes) came near to the mirror, but they also go away from it showing low degree on balance and slightly fear. Over the next few minutes of study they reached the mirror and began to sniffing it (courage and curiosity). We noted middle degree of vocalization - quiet barking. It continued the next 10 minutes before the animal tried to bite the mirror (low-grade aggression), but unsuccessfully. The puppies than started walk away from mirror, went behind the mirror or in front of it until the end of the study period. But in the last two minutes were completely relaxed, there was no fear or great interest for this object. They were just moved around it and sniffing it.

Accordingly observed behaviors we can note that these puppies are with low degree of instability, but in certain situations they manifested serenity, courage and curiosity. Interesting is that puppies demonstrated aggression even weren't fearful. According to shown behavior these animals should be attributed to F-choleric type of nervous system, representing 33,3% of the observed group.

They are strong, brave, curious, fast, but unbalanced and sometimes irritable. Demonstration of aggression is not evidence of wicked animals. It can be avoided if the dog is taught properly. The process of Socialization goes smoothly, but not so easy as with sanguine type of animals who are more balanced. Irritability of the choleric type is the only reason which makes them less preferred by L- type of nervous system. However, these animals can be trained easily and successfully.

- The dog № 9 at the beginning of the experiment didn't come near to the mirror and it was screaming (vocalization and lack of interest first 7 minutes). It stood, was moving slightly, and then approached the object slowly encircling and sniffing it (low-level of curiosity -10 minutes). During the rest observed period did not show adequate behavior. Too quiet, sometimes uneasily, but not aggressive (was not biting the mirror). The puppy did not closely approaching the object, always remained a certain distance (around 30-40 cm) from it. It showed prudence and delayed behavioral activities. Therefore, this animal had to be attributed to a G- phlegmatic type of nervous system, which representing 11, 1% from the observed group. Puppies with this temperament are generally calm, but more slowly socialize. They are sometimes curious, sometimes not. These animals can successfully socialize and develop desired human behavior, but with more tenacity of their owners.

We haven't noted puppies with mixed temperament from this breed.

Breed "Collie"

- Puppies № 10, 11, 12 and 17 showed typical behavior for the L- sanguine type of nervous system. High degree of calm, confidence, courage and curiosity (very soon come near the mirror, reached it and stared at it. Absence of irritability and aggression (they didn't bite mirror). We have noted middle level of vocalization as quieter barking when they for the first time saw the object (5 minute).These puppies represented 44 4% of the observed group. Training on these dogs takes place successfully; they can quickly become socialized with desired behavior.

- Puppies with № 13 and 14 showed behavior typi-

cal for the F- choleric type of nervous system, because from the outset with headlong rush to the mirror (curiosity) and then immediately go away from it (fear). We registered middle level of whining, mixed with barking - 6 minutes, then occurrence on calm by the end of this double study. This initial precipitance followed by brief fear and absence of aggression they weren't biting the mirror, just sniffing it) indicate on some degree of instability. But subsequent calm (20 minutes) was evidence that dogs were choleric - 22.2% of the studied group.

- Puppies with № 15 and 18 have phlegmatic behavior. They were showed delayed behavioral activities, lack of fear, calm, curiosity (sniffing the mirror after 15 minutes from the beginning of observation) and absence of aggression. We noted middle degree of vocalization / yelp -3 minutes to the end of the experiment. They show lack of irritability. This gave us reason to type these puppies in the G- phlegmatic type of nervous system. These puppies represented 22,2% of the studied group. They will need a long and hard training to have a successful socialization and normal behavior.

Dog №16 was characterized by a particularly high grade behavior, fear and irritability, lack of calm, poise, aggression, curiosity, middle level of vocalization. Because of these behavioral characteristics we typified them as A-type of nervous system, melancholic temperament or asocial type. Dogs that represent this type of nervous system are difficult to socialize. It takes a lot of effort by the people for training and education of these animals. They are melancholic, fearful and prone to aggression.

We haven't noted animals with a mixed temperament from this group.

Breed "Doberman"

-Dog № 21 had a typical behavior as L-sanguine type of nervous system. We noted high grade on calm, absence of fear, sanity and curiosity. The puppy has shown middle level on vocalization and lack of aggression. Sanguine type on puppies "Doberman" were 11, 1% of all puppies, representatives of this breed in our experiment.

- Puppies with № 19, 20, 22 and 23 showed typical behavior for type F choleric temperament. Calm, instability, high curiosity and middle level of vocalization. They are 44, 4% of the observed group from "Doberman."

- Puppies № 24 and 25 showed phlegmatic behavioral manifestations characteristically for type G temperament. They have showed middle level of fear, lack of irritability, aggression and curiosity, middle level on vocalization. We observed delayed behavioral activities which are typical for phlegmatic type. These puppies were 22 2% of the representatives of breed "Doberman.

- Dog № 27 was a melancholic temperament. Typically antisocial behavior, accompanied by difficulty socializing and modeling of desired behavioral activities. It represents 11, 1% of the observed group.

- Dog № 26 was mixed temperament - sanguine-choleric, because it showed middle level of fear, irritability, aggression, vocalization and excessive curiosity. It represent 11, 1% of study group from breed "Doberman".

Table 1. *Allotment of the dogs according to their temperament*

Puppy №	L	F	G	A	mixed
1	+				
2	+				
3		+			
4	+				
5		+			
6	+				
7		+			
8	+				
9			+		
10	+				
11	+				
12	+				
13		+			
14		+			
15			+		
16				+	
17	+				
18			+		
19		+			
20		+			
21	+				
22		+			
23		+			
24			+		
25			+		
26					+
27				+	

Analyzing the results we come to the conclusion that the most common types of nervous system on the dog are sanguine and choleric which is represent with 70, 3% of all experimental dogs. This confirms the reasoning of publicized scientific data (7, 9, 10).

Regarding mixed temperament, we can say that we neither confirm it, or reject it (6, 7). In this study only one dog breed "Doberman" demonstrates characteristics typical for two types of temperaments. Because the percentage is too small, only 3, 7% of all puppies, we couldn't assert that this type of nervous system exists in dogs. Certainly with further extensive research in this direction could get to categorization.

Table 2. *Degree of manifestation of the observed behavioral activities*

Puppy №	Fear	Irritability	Aggression	Vocalization	Curiosity
1	0	0	0	0, 5	1
2	0	0	0	0, 5	1
3		0, 5	0, 5	0, 5	1
4	0	0	0	0, 5	1
5		0, 5	0, 5	0, 5	0, 5
6	0	0	0	0, 5	1
7		0, 5	0, 5	0, 5	0, 5
8	0	0	0	0, 5	1
9	0, 5	0	0	0, 5	0, 5
10	0	0	0	0, 5	1
11	0	0	0	0	1
12	0	0	0	0	1
13	0	1	0, 5	0, 5	1
14	0	0, 5	0	0, 5	0, 5
15	0, 5	0	0	0, 5	0
16	1	1	0	0, 5	0
17	0	0	0	0,5	0, 5
18	0	0	0	0	0
19	0	1	0, 5	0, 5	1
20	0	0, 5	0	0, 5	1
21	0	0	0	0, 5	1
22	0	0, 5	0	0, 5	1
23	0	0, 5	0, 5	0, 5	1
24	0, 5	0	0	0, 5	0, 5
25	0, 5	0	0	0, 5	0
26	0, 5	0, 5	0, 5	0, 5	1
27	1	1	0	0, 5	0

Legend:0 points - lack of appropriate behavior activity; 0.5 points - middle activity; 1 point - high-activity.

Both tables 1 and 2 shows that in terms of socialization and modeling the behavior on the first place is breed "German Shepherd" with the most stability types of nervous system 29, 6% sanguine and choleric , which coincides with the scientific assertion that these dogs are smarter (3, 5, 6). But some authors, said that breed "Collie" is not inferior according to breed "German Shepherd" based on process of socialization and the formation of certain behavior (4). Our results reject this reasoning because according to them, "Collie" is after breed "Doberman" with 22, 2% representatives of choleric type and sanguine type of temperament . The difference is very small, but is in favor of breed "German Shepherd. In both species was not found mixed temperament. But the breed "German Shepherd" have 11, 1% puppies with phlegmatic type on nervous system, stronger, but slower in learning and socialization, in relation to breed "Collie" which have 22, 2% representatives on this type of temperament. This fact was crucial to setting breed "Collie" in second place.

Based on socialization process and behavioral model from the three studied breeds, we noted that on third place is breed "Doberman" in which we found only 11, 1% presence on sanguine type. Moreover, only here we found the presence of asocial, melancholic temperament ie weaker and unbalanced puppies. These results coincide with the conclusions of publicized research data, that representatives of breed "Doberman" are unbalanced, wicked and aggressive dogs compared to the breed "German Shepherd" and breed "Collie" (2 , 6, 7, 9). Therefore, the most quickly and most successfully socialize and form certain behaviors puppies of breed "German Shepherd" followed by "Collie" and "Doberman."

CONCLUSION

Temperament is the general attitude a dog displays towards people and other animals. it is the combined inherited and acquired physical and mental traits that influence the dog's behavior. Exists indirect link between typified the dog's temperament and modeling on behavior. Temperament testing evaluates an individual dog's

temperament through a series of tests that measure traits including stability, confidence, shyness, friendliness, aggressiveness etc. To typify the nervous system on dog in our study we used the test with mirror of Bretto. This applied test was perceived very well from the observed dogs. They weren't stressed which is proved by their appropriate behavior during and after experiment. The test is carried out easy and quickly, and do not require special conditions for its implementation.

So while temperament testing can be extremely valuable, it is important to remember that while we may not be able to change an individual dog's genetic history, we can still help shape his attitude towards people, animals, things and places that he will encounter in life, in addition to managing the dog's behavior.

The temperament of dogs is very important for their socialization. Quickly and easily socialize dogs are those with sanguine and choleric temperament, followed by phlegmatic type. Obtained results in our study show that in terms of socialization and modeling the behavior on the first place is breed "German Shepherd", followed by "Collie" and "Doberman" which coincides with the scientific assertion (3, 5, 6).

Suitable comparative studies in scientific field of ethology and cynology are little. Their appearance necessarily have to increase because only on this way it is possible to characterize the correct type of nervous system of the dog, with a view to successful socialization and the formation of appropriate behavior. Knowing the temperament of the animal the owner will have the adequate approach to it, training will be correct, will shape the appropriate behavior as a result of which will reduce appear on behavioral abnormalities and will reduce the number of abandoned dogs.

REFERENCES

1. Мицулов, Андрей, 1993. Породите кучета в България, Rossyx Canning, The Dog Care Departement.
2. Узунова, Красимира, 2006. Изследване върху някои хигиеннотехнологичин показатели на животновъдни обекти за кучета, 32- 35.
3. Compan, R., Scapini, F., 2002. Les causes proximales du comportement. In: Ethologie. Approche systematique du compertement, 35- 98.
4. Haverbecke, A., Messaudi, F., et al., 2009. Efficiency of working dogs undergoing a new Human Familiriatization and Training Program. J. Vet. Behavior.
6. Keeling, L., Jensen, 2002. Behavioural distourbances, stress and welfare. In: The Ethologie of domestiques animals, an Introductory text. Coords: Jensen P. Cab Publichin, 79- 98.
7. Paris, Thierry, 1996. Compertement social du chien, le Chien farouche et mordeur tome 1, 44 Seminaire de SFC, Toulouse, p. 222.
8. Queinnec, Bernadette, 1998. Le chien dans la societe, Appreciation des aptitudes chez le chien,Seminaire de SFC, Toulouse, p. 312.
9. Renaud, S., 1996. Test utilizes pour l, analyse du caractere. Le comportement social du chien, Tome 2, Annexes, Seminaire de SFC, Lyon, 30.
10. Vastrade, Francoise, 1994. Le comportement social du chien. La socialization du chiot et son evaluation, SFC, 1994, p. 371.

КАРАКТЕРИЗАЦИЈА НА НЕРВНИОТ СИСТЕМ КАЈ КУЧИЊАТА ЗА ПРАВИЛНА СОЦИЈАЛИЗАЦИЈА И НАСОЧУВАЊЕ КОН ОДРЕДЕНО ОДНЕСУВАЊЕ

Узунова Красимира[1], Тодороска Марина[3], Бинев Румен[2], Митева Чонка[2], Јури Митев[2]

[1]*Факултет за Ветеринарна Медицина, Тракиски Универзитет Стара Загора, Бугарија*
[2]*Земјоделски Факултет, Тракиски Универзитет Стара Загора, Бугарија*
[3]*Катедра за Биологија, Виенски Универзитет, Виена, Австрија*

АПСТРАКТ

Однесувањето беше проучувано кај 27 кученца од расата Германски овчар, Коли и Доберман преку тестот со огледало по Брето, за да се карактеризира типот на темперамент за правилна едукација, тренинг и насочување на однесувањето. Беше докажано дека применетиот тест е брз, едноставен, лесен за имплементација и не предизвикува стрес кај експерименталните кучиња и е применлив во благосостојаба на животните.

Добиените резултати покажуваат дека најхрабри и нај урамнотежени се претставниците на Германскиот овчар, а по нив следува расата Коли и Доберман.

Клучни зборови: кученца, однесување, темперамент, социјализација

UDC: 636.52/.58.083/.084

THE EFFECT OF FIRST FREEDOM RESTRICTION ON BROILER WELFARE

Blagoevska Katerina[1], Dodovski Aleksandar[1], Blagoevski Andreja[2], Radeski Miroslav[1], Popovska-Percinik Florina[1], Ilieski Vlatko[1], Stojkovski Velimir[1], Mickov Ljupco[1], Esmerov Igor[1]

[1]Animal Welfare Center, Faculty of Veterinary medicine – Skopje, University "St. Cyril and Methodius", Skopje, R. Macedonia
[2]GFA DOOEL, Skopje, R. Macedonia

ABSTRACT
Animal's physical state, sense of well-being and protection from unnecessary suffering are provided through the "Five freedoms" concept for Animal welfare. The first freedom focuses on animal's free and continuous access to drinking water and ad-libitum access to feed during all daylight hours in order to meet the nutritional needs for their health. The aim of this paper was to estimate the restriction of one or both factors of the first freedom, upon animal outputs, in the first days post hatch of broiler chicks.
In the experiment we used a total of 120 birds, randomly distributed into four different treatments: 1) access to feed and water ad libitum, F+W (control group); 2) deprived from water, F-W; 3) deprived from feed, W-F; and 4) deprived from both feed and water,-FW. The duration of the experiment was from the second till the fifth day after hatch of the chicks. Feed, water and feed and water, were restrained from the experimental groups respectively, in the afternoon on the day of housing. Birds in control group behaved full of temperament, energetic, moving freely, taking feed and water, with clean, bright and smooth well laid feathers, timeliness growth of wing and tail feathers, and feces characteristic for the first days of life. In all experimental groups, birds showed different behavior, which was accompanied with apathy, twittering, closed eye lids, folded wings and bristling feathers, slurry and sloppy diarrhea with dark brown color. Body weight, on the control group increased for 108% of the initial body weight, while in all experimental groups, the body weight decreases significantly ($p<0.05$).
From the results, we can conclude that maintaining of the first freedom in the first days after hatch of broiler chicks is very important for a good start of broiler breeding and production.
Key words: animal welfare, restriction, first freedom, broiler performance

INTRODUCTION

Animal's physical state, sense of well-being and protection from unnecessary suffering are provided through the "Five freedoms" concept for Animal welfare. This concept is perceived whether the animal is on farm, in transit, at market or at a place of slaughter.

The first freedom focuses on animal's free and continuous access to drinking water and ad-libitum access to feed during all daylight hours in order to meet the nutritional needs for their health.

When talking about nutritional (feed and water needs) needs of neonatal chicks, they must be provided within few hours after hatch, including transportation and accommodation in hatcheries (1). Water restriction is related to a typical stress behavior, with muscular motility coordination, modulated directly or not by the neuroendocrine system (2). Limited water consumption influences feed intake as well (3). When water is supplied *ad libitum*, the birds develop a very characteristic feed intake behavior and eat during short periods in a day. On the other hand, this behavior may vary according to water availability and management (4). When chicks are submitted to feed restriction, water consumption peaks when feed is supplied, because water intake is highly related to feed intake (5). Any factor influencing feed intake will also affect water consumption and vice-versa (4). Whether the nutrition is non-sufficient, non-regular or completely restricted, the birds exhibit behavioral symptoms of frustration (5,6), often accompanied with frequent feather plucking.

When water is restricted chicks manifest following changes: depressed growth decreased feed need and glucogen deposit, oliguria and increased mortality.

Long term water deprivation provokes pathological changes such as: necrosis of beak's mucosa membrane, crop's constipation, petechial hemorrhages and erosions of the gizzard, necrosis of the trachea, brain edema, nephrosis, ovarian follicles necrosis, glued eyelids, rough skin and death as a result of autointoxication (7,8).

This paper will focus on the effect of the first freedom absence, "freedom from hunger and thirst", in the first days post hatch, upon animal outputs.

MATERIAL AND METHODS

In the experiment we used a total of 120 birds, randomly distributed into four different treatments: 1) ad libitum access to feed and water, F+W (control group); 2) deprived from water, F-W; 3) deprived from feed, W-F; and 4) deprived from both feed and water,-FW. The duration of the experiment was from the second till the fifth day after hatch of the chicks. Feed, water and feed and water, were restrained from the experimental groups respectively, in the afternoon on the day of housing. All birds were reared in the same environment, with light and controlled environmental temperature, and nutritional requirements according to the specifications of the Ross Genetic Line Manual (9).

The welfare assessment of broilers was done daily, according to the Welfare Quality assessment protocol for poultry (10). Daily body weight gain and mortality rate were registered.

Means were calculated using LSMEANS, and were

compared by probability of difference (PDIFF) by the Student t-test at $p<0.05$ significance level.

RESULTS

On the first day of the experiment (day of housing), birds in all four groups showed normal behavior: they were full of temperament, energetic, moving freely, taking feed and water, with clean, bright and smooth well laid feathers, timeliness growth of wing and tail feathers, and feces characteristic for the first days of life. The mean body weight for all groups was 41,5 gr. All parameters leaded to a conclusion, that the birds on the day of the hatch were free of any impairments and disturbances.

The chicks in the control group, F+W, during the whole duration of the experiment had the same behavior as on the day of hatch. Feed and water consumption were ad libitum, and their daily body weight gain followed the expected weight according to the hybrid's genetic line manual. On the final day of the experiment the mean value of the chick's body weight in the control group was 86,5 gr (Fig. 1). No mortality was observed in this group.

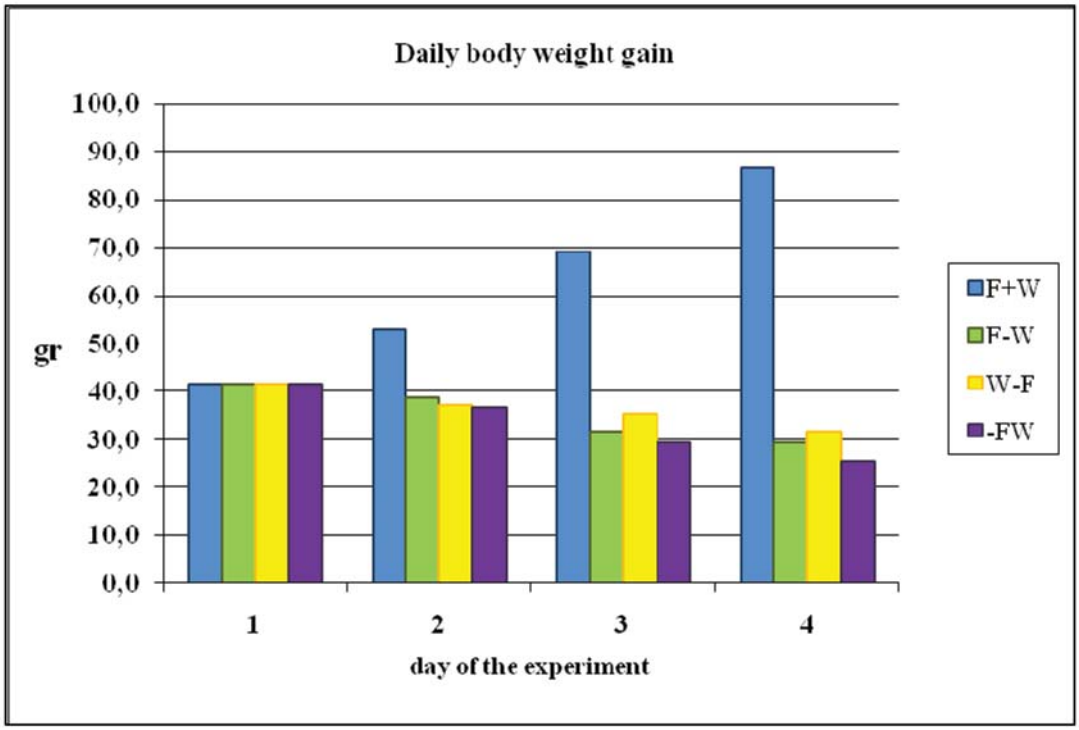
Birds in groups F-W and W-F, on the first day of the experiment behaved anxious and frustrated, with feather plucking. The last day, they developed signs of apathy, twittering, closed eye lids, folded wings and bristling feathers. Some birds from group W-F, had slurry and sloppy diarrhea with dark brown color, while birds in group F-W, showed reduced feed intake, due to the water restriction. Birds in both groups showed growth lags. The body weight significantly ($p<0.05$) decreased for 28,9 and 23,5 %, respectively in groups F-W and W-F (Fig. 1). By the end of the experiment, the percentage of dead chicks was 9,27 and 22, respectively (Fig. 2)

Table 1. *Body weight in control and experimental birds*

	Groups			
Day of experiment	F+W, gr	F-W, gr	W-F, gr	-FW, gr
1	41,5±0,93	41,5±0,93	41,5±0,93	41,5±0,93
2	53±0,88	38,75±1,34	37,15±1,97	36,75±2,27
3	69±1,21	31,68±2,25*	35,27±2,45*	29,3±2,78*
4	86,5±1,16	29,25±1,78*	31,75±2,67*	25,5±2,43*

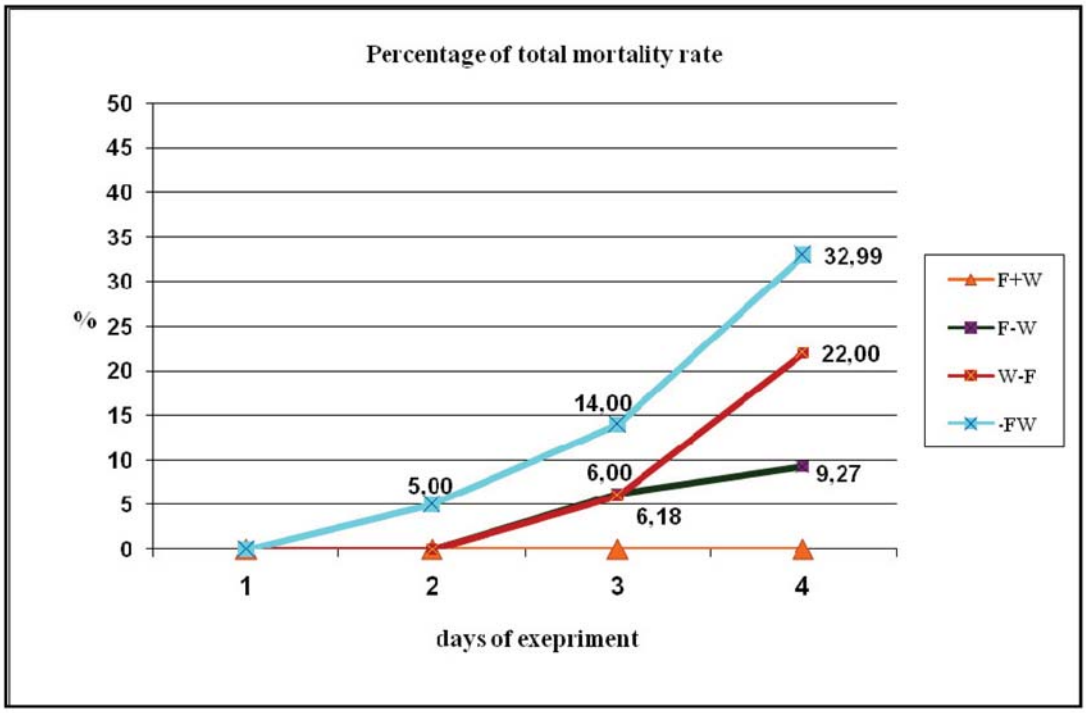
*The values are the means ± SD (n=7/group). * $p<0.05$ vs. control*

Figure 1. *Daily body weight gain in birds subjected to feed, water, feed and water restriction*



In -FW te manifestation of behavioral changes was the most intensed, especially on the last day of the experiment. During the whole duration of the expeirment, they were twittering, slumbering with closed eye lids, with head down under, folded wings and bristling feathers. Due to lack of feed and water, the chicks were eating the litter. In 90 % of the birds, slurry and sloppy diarrhea with dark brown color was noticed. The body weight significantly ($p<0.05$) decreased for 38.5 % on the last day of the experiment (Fig.10 . Total mortality percentage in this group was 32.9 (Fig.2).

Figure 2. *Mortality in birds subjected to feed, water, feed and water restriction*



CONCLUSIONS

Behavioral changes demonstrated in the experimental groups, as a good indicator for the assessment of the freedom for expressing normal behavior, could be used as a sign for physiological disorders resulting from impairment of the first freedom. Presented results are showing that there is a significant difference of total mortality rates between different test groups, compared to the control group. Therefore, the percentage of total mortality rate could be used as an output indicator for the disturbance of the firs freedom of broilers. In general, it could be stated that chicks in the first week post hatch are more susceptible to water deficiency, than feed.

Overall, enabling free access of feed and water in the first days after hatch of broiler chicks is very important for a good start of broiler breeding and production.

REFERENCES

1. Dibner, J. J., Knight, C. D., Ivey F. J.(1998): The Feeding of Neonatal Poultry. World Poultry 14(5):36-42.
2. Zulkilfi, I.; Siegel, P.B. The good and the bad of stress. Arbor Acress: Glastonbury, 1995. p.18-23. (Service Buletin).
3. Larbier M, Leclercq B. Nutrition and feeding of poultry: intake of food and water. Nottingham: Nottingham University Press; 1994. p.7-14.
4. Macari M. Metabolismo hídrico da poedeira comercial. In: V Simpósio Técnico de Produção de Ovos; 1995; Jaboticabal. Resumos... Jaboticabal: APA; 1995. p.109-131
5. Leeson S. Summers JDV. Broiler and breeder production: nutrition and feeding. Guelph: University Books; 2000. p.136-217.
6. Kostal, L., C.J. Savory and B.O. Hughes, 1992. Diurnal and individual variation in behavior of restricted-fed broiler breeds. Appl. Anim. Beh. Sci. , 32: 361-374.
7. Arad, Z., Marder, J., Eylath, U. (1983): Serum electrolyte and enzyme response to heat stress and dehydtratation in the fowl (Gallus domesticus). Comp.Biochem.Physiol.A. 74(2):449-53.
8. Savory, C.J. and K. Maros, 1993. Influence of degree of food restriction, age and time of day on behavior of broiler breeder chickens. Beh. Processes, 29: 179-190.
9. Ross broiler management manual (2009). http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross_Broiler_Manual_09.pdf
10. Welfare Quality, 2009. The Welfare Quality assessment protocol for poultry (broilers, laying hens). The Welfare Quality Consortium, Lelystad

ЕФЕКТОТ НА РЕСТРИКЦИЈА НА ПРВАТА СЛОБОДА ВРЗ БЛАГОСОСТОЈБАТА НА БРОЈЛЕРСКИ ПИЛИЊА

Благоевска Катерина[1], Додовски Александар[1], Благоевски Андреја[2], Радески Мирослав[1], Поповска-Перчиниќ Флорина[1], Илиески Влатко[1], Стојковски Велимир[1], Мицков Љупчо[1], Есмеров Игор[1]

[1]Центар за благосостојба, Факултет за ветеринарна медицина – Скопје, Универзитет "Св. Кирил и Методиј", Скопје, Р. Македонија
[2]ГФА ДООЕЛ, Скопеје, Р. Македонија

АПСТРАКТ

Физичката состојба на животните, чувството на благосостојба и заштитата од непотребни страдања се пропишани со концептот на "Петте слободи„ од Благосотојбата на животните. Првата слобода се однесува на слободен и континуиран пристап до вода и *ad libitum* пристап до храна во текот на денот, се со цел да се задоволат хранливите потреби неопходни за здравјето на животните. Целта на овој труд е да се направи проценка на рестрикцијата на еден или двата услови на првата слобода, врз производните капацитети на пилињата во првата недела по ведењето.

Во експериментот се користени вкупно 120 пилња, кои по случаен избор беа изложени на 4 различни третмани: 1) пристап до храна и вода, *ad libitum* (Х+В); 2) без вода, Х-В; 3) без храна, В-Х; и 4) без храна и вода –ХВ. Времетраењето на експериментот беше од вториот до петтиот ден по ведење. Храната, водата и храната и водата беа отстранети од експерименталните групи, последователно, во попладневните часови на денот на вселување на пилињата во објектот. Однесувањето на пилињата од контролната група беше карактеристично за прв ден по ведење. Тие беа темпераментни, слободнио се движеа во боксот, примаа храна и вода, пердувите им беа чисти, сјајни и мазни, добро полегнати, со навремен раст на крилни и опашни пердуви и измет карактеристичен за правата недела од животот. Кај сите експериментални групи пилињата покажаа различно однесување, кое беше пропратено со апатија, цвилење, затворени очни капаци, спуштени крила и накострешени пердуви, кашеста и ретка дијареа со темно кафеава боја. Телесната маса кај контролната група се зголеми за 108% од почетната, додека кај сите експериментални групи беше забележано сигнификантно намалување на телесната маса ($p<0,05$).

Од добиените резултати може да се заклучи дека одржувањето на првата слобода во првите денови од животот на пилињата е многу важна за добар почеток во бројлерското производтво и одгледување.

Клучни зборови: благосостојба, рестрикција, прва слобода, бројлери, перформанси

UDC: 636.2.034.083

ASSESSING THE WELFARE OF DAIRY CATTLE USING OUTCOME BASED MEASURES AND HUMAN – ANIMAL RELATIONSHIP IN DIFFERENT HOUSING SYSTEMS

Radeski Miroslav, Ilieski Vlatko

Animal Welfare Center, Faculty of Veterinary Medicine,
University "Ss. Cyril and Methodius", Skopje, R. Macedonia

ABSTRACT
Various types of housing systems for dairy cattle have different impact on their welfare which could be assessed using outcome based measures. In addition, establishinggood relationship between stockman and animals is essential for good animal welfare. In R. Macedonia most of the dairy cattle production is on extensive and intensive tie-stall farms. Therefore, the main objective of this paper is to assess the welfare of dairy cattle in these housing systems using animal based measures and to determine the level of human – animal relationship. This research was conducted on six dairy farms in Macedonia, where one was with intensive tie-stall system, involving 85 animals tested, out of 111. The performed welfare assessment was designed in accordance with the established welfare principles, criteria and measures by the Welfare Quality® Project, used nine defined welfare criteria, measured with 23 developed measures. According with the welfare measures, a Welfare assessment protocol for dairy cattle was created and avoidance – distance test was conducted. The intensive tie-stall system shows lowest score regarding absence of prolonged hunger influencing to the overall score of Good feeding principle. Regarding cleanliness of the lower leg, upper leg/flank and udder, all farms have categorized as farms with "serious problem" on this issue, emphasizing the hygiene conditions as a crucial factor for better animal welfare. Lowest scores in the avoidance distance test for determining the human-animal relationship are present at the intensive tie-stall system and one of the extensive ones. These scores are due to lower interest, motivation and knowledge of the farm's workers, vital for good welfare of dairy cattle. In order to gain relevant data and measures, it is necessary to involve more dairy farms and different housing systems. Thus, creating a reliable baseline study for animal welfare of dairy cattle for the existing housing systems.
Key words: welfare assessment, dairy cattle, human–animal relationship, animal based measures

INTRODUCTION

The maintenance of good animal welfare for dairy cattle is an essential part in the dairy operation systems. Compliance with the international animal welfare regulations (European Commission Directives) and national legislation; as well as, respecting the general attitudes of consumers towards implementing animal welfare standards in food industry [1]; and of most significance, fulfilling physiological and behavioral needs of the animal in context of good health, production and reproduction, are foundation for implementing animal welfare standards in the daily routine of dairy cattle management. Therefore, the primary objective of farmers should be strong commitment to animal welfare.

There are various types of housing systems for dairy cattle ranging from free range on pasture to intensive tie stall farming. Each type of housing has different impact on the welfare of dairy cattle. The selection of the housing system which will provide the highest level of animal welfare is determined by the breed preferences, geographical and climate conditions. On the other hand, the various housing systems and herd size determine the herdsman's dedication and relation with the animals which is crucial to their welfare.

Establishing good relationship between stockman and animals is essential for good animal welfare. This human – animal relationship (HAR) represents their mutual perception [2]. Many studies confirmed that negative animals' handling experiences results with higher level of fear of man and negative effects on production, reproduction, welfare and increasing the risk of injuries for both, animal and man [3,4,5]. Otherwise, positive handling might improve the welfare [6], resulting with good animal health, performance and stockman's confidence in animal.

Implementation of welfare standards must be accompanied with proper assessment. The assessment must be based on reliable scientific systems for assessing animals' welfare status. The recently adopted EU Strategy for the Protection and Welfare of Animals 2012–2015 highlights that the possibility of using scientifically validated outcome-based indicators complementing prescriptive requirements in EU legislation will be considered when necessary [7]. The factors that affect an animal's welfare include the physical environment, resources available to the animal and the management practices of the farm. Depending on its characteristics (breed, sex, age, etc.) the animal will respond to these inputs and the animal's responses are assessed using *animal-based measures*. Animal-based measures are evaluative, obtained in a precise way and usually quantitative. They give an indication of an animal's welfare, but a set of measures is normally needed to provide a good assessment of welfare. Animal-based measures can be collected on-farm either by observation or inspection of the animal or by assessing the effects of a response on the environment. Animal-based measures have usually been used to identify animals whose welfare is poor, but also are useful for identifying improvements in welfare in order to maximize benefits [8].

The European Welfare Quality® Project was set out to develop scientifically based tools to assess ani-

mal welfare. The acquired data provides feedback to animal unit managers about the welfare status of their animals and is translated into accessible and understandable information on the welfare status of food producing animals, including dairy cattle [9]. This paper uses the outcome based methodology for assessment of animal welfare designed by this project.

In Republic of Macedonia the two major housing systems for dairy cattle production are extensive tie-stall systems with around 10 dairy cows (individual household farmers) and intensive tie-stall systems with more than 50 animals. In most of the tie-stall systems cattle are kept indoors during the whole year. The aim of this paper is to assess the welfare of dairy cattle in the present housing systems using outcome (animal) based measures and to determine the level of human – animal relationship, as well as to address the key issues regarding animal welfare at these housing facilities.

MATERIALS AND METHODS

This research was conducted on six dairy farms in Macedonia, one farm (Farm A) with intensive tie stall system and the remaining five (Farm B - E) individual household farms with extensive tie-stall system. For assessing HAR one additional individual household farm (Farm F) was processed. The number of tested animals and main profile of each farm is presented in Table 1. The sampling procedure for the intensive farm system was performed according to the procedure suggested in the Assessment protocol for Cattle [9], while at the household farms with around ten dairy cattle, all animals were tested.

Table 1. *Profile and number of dairy farms and assessed animals*

No.	Farm	Housing system	No. milking cows	No. dry cows	No. heifers	Total no. of animals	No. animal tested
1	**A**	Intensive tie-stall system	43	0	17	60	**37**
2	**B**	Household extensive tie-stall system	8	3	0	11	**11**
3	**C**	Household extensive tie-stall system	5	0	2	10	**7**
4	**D**	Household extensive tie-stall system	11	1	6	18	**18**
5	**E**	Household extensive tie-stall system	3	1	1	5	**5**
6	**F**	Household extensive tie-stall system	5	0	2	7	**7**
Total number:		**6**	**75**	**5**	**28**	**111**	**85**

The animal welfare assessment of dairy cattle was based on the Welfare Quality Assessment protocol for cattle [9].The performed welfare assessment was designed in accordance with the established welfare principles, criteria and measures by the Welfare Quality® Project. This assessment uses nine out of twelve defined welfare criteria, measured with 23 developed measures for checking the criteria. Welfare principles, criteria and measures applied in this research are summarized in Table 2. According with the welfare measures, a Welfare assessment protocol for dairy cattle was created. This protocol consists of forms for recording the specific data for each animal based measure. The assessment forms were filled on the farm during an observation of each animal in the sample.

Table 2. *Welfare principles, criteria and measures applied in this research*

Welfare principle	Welfare Criteria	Measures
1. Good feeding	1.1 Absence of prolonged hunger	1.1.1 BCS
	1.2 Absence of prolonged thirst	1.2.1 Water provision
		1.2.2 Cleanliness of water points
		1.2.3 Water flow
2. Good housing	2.1 Comfort around resting	2.1.1 Cleanliness of udders
		2.1.2 Cleanliness of flank/upper legs
		2.1.3 Cleanliness of lower legs
	2.2 Ease of movement	2.2.1 Presence of tethering
		2.2.2Access to outdoor loafing area or pasture
3. Good health	3.1 Absence of injuries	3.1.1. Lameness
		3.1.2 Integument alterations
	3.2 Absence of disease	3.2.1 Nasal discharge
		3.2.2 Ocular discharge
		3.2.3 Hampered respiration
		3.2.3 Diarrhea
		3.2.4 Vulvar discharge
		3.2.5 Mortality
		3.2.6 Dystocia
		3.2.7 Downer cows
	3.3 Absence of pain induced by management procedures	3.3.1 Disbudding/Dehorning
		3.3.2 Tail docking
4. Appropriate behavior	4.1 Expression of other behaviors	4.1.1 Access to pasture
	4.2 Good human - animal relationship	4.2.1 Avoidance distance

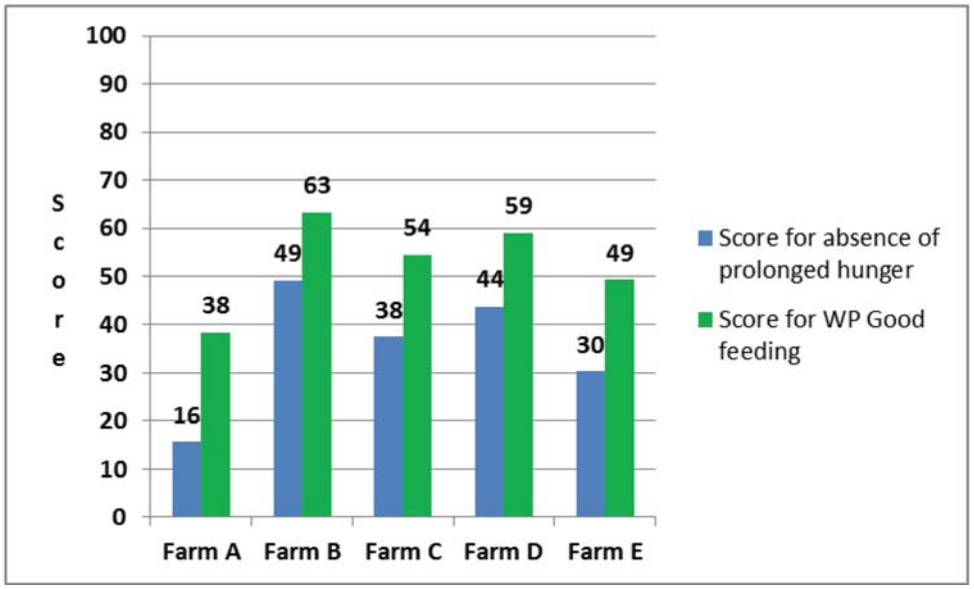
Avoidance distance test was used for assessing the human – animal relationship welfare criteria. This test was performed considering the suggestions of Windschnurer et al. as part of the Welfare Quality® [10]. Each animal from the sample was tested two times with the assessor placed in front of the animal. Since the housing facilities did not allow the appropriate distance of 2m in front of the animal, the alternative method was used, with approaching the animal from the 45^0 angle at distance of 2.5m. The assessor approached tested animals with one arm held in an angle of 45^0 in front of the body, with a speed of one step per second. When the animal reacted by showing clear signs of avoidance or withdrawal (turning the head aside or stepping back) the distance between the assessor's hand and the muzzle was measured in cm, with the resolution of 10cm. If the cow didn't show any signs of avoidance, the assessor continues to approach until he touched the animal's muzzle. According the Assessment Protocol for cattle obtained measures are categorized in four categories: animals that can be touched; animals that can be approached 50cm but not touched; animals that can be approached as closely as 100 to 50cm and animals that cannot be approached as closely as 100cm [9].

The gathered data from the Assessment protocol were analyzed using the scoring system described in the Welfare Quality Assessment protocol for cattle and appropriate software http://www1.clermont.inra.fr/wq. In addition the data were statistically analyzed by MS Excel and SPSS Statistics 17.0.

RESULTS

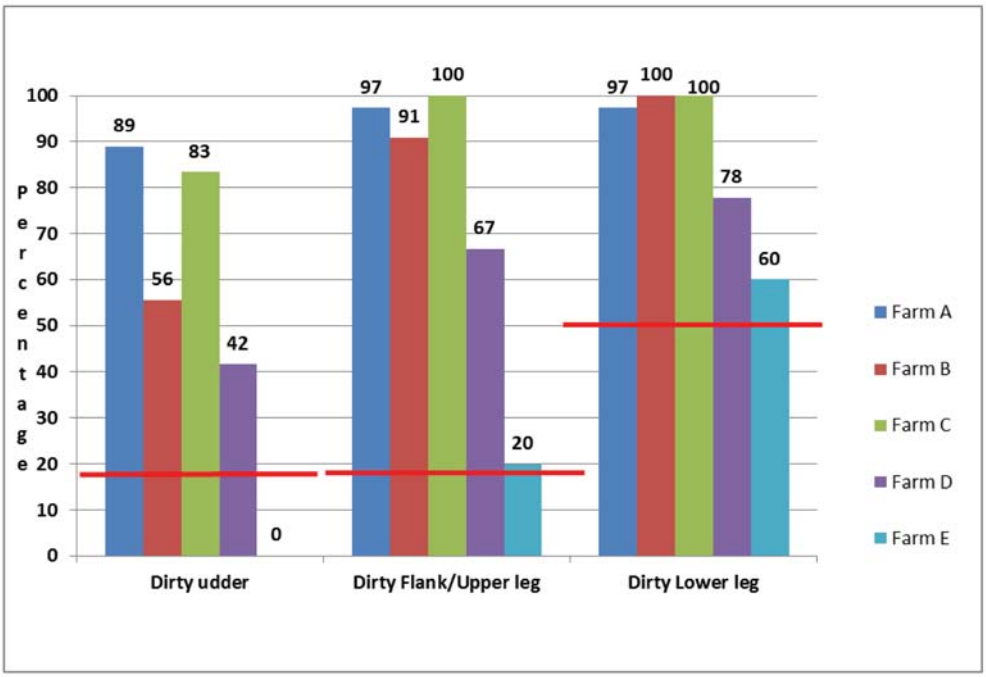
1. Regarding the Principle – *Good feeding* – in all farms the measures suggested that there is absence of prolonged thirst in the animals. In fact, all tested animals have constant water provision and there are 2 animals per bowl as type of water supply. The bowls mostly are clean and there is sufficient water flow (above 10L/min). This was not the case regarding absence of prolonged hunger, which was measured using body condition scoring (BCS) on each of the sample animals. This measure is presented through the scoring system of the Welfare Quality Assessment protocol, Figure 1. The intensive tie-stall system (Farm A) shows lowest score regarding this measure which influences the overall score of this principle.

Figure 1. *Scores for animal welfare principal (WP) Good feeding and Absence of prolonged hunger*



2. In the *Good Housing* principle two of the welfare criteria were tested. The criteria ease of movement cannot be classified because all tested farms were using tie-stall system and are not providing access to pasture and outdoor runs throughout the year. This considerably decreases the overall animal welfare. In addition, the criteria comfort around resting likewise has shown poor results, Figure 2. In these criteria three measures were assessed: cleanliness of lower leg, upper leg/flank and udder. According the Welfare Quality Assessment protocol all farms have categorized as farms with "serious problem" regarding this issue.

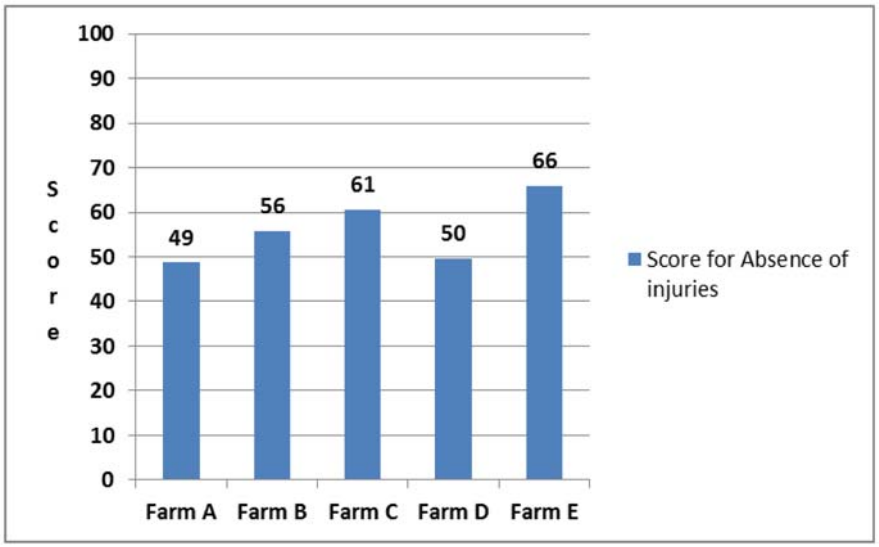
Figure 2. *Percentage of dirty lower leg, upper leg and udder in tested animals. Red lines are showing the upper limit from where cleanliness is considered as "serious problem" according Welfare Quality Assessment protocol. The Figure shows that only Farm E has no problem with cleanliness on the udder, but not for the other two categories.*



3. Three welfare criteria were assessed concerning the welfare principle *Good Health.* The criteria absence of pain during management procedures was assessed regarding the percentage of animals submitted to the procedures of disbudding/dehorning and tail docking. Only on one farm (Farm D) disbudding procedure was performed on 50% of the animals, at the age of 4 weeks, with caustic paste and without any analgesia or anesthesia on the animals. The measurements taken concerning Absence of disease criteria have shown that Dystocia (17% of all animals in the last twelve months) at the Farm A and Diarrhea (28% of tested animals) at Farm D

have passed the defined Alarm threshold, while the other disorders are not showing significant percentage of animal suffering. The Absence of injuries criteria measured by the presence of lameness and integument alterations has presented acceptable and enhanced welfare categories, Figure 3.

Figure 3. *Scores for Absence of injuries according Welfare Quality Assessment protocol.*



4. Considering the *Appropriate behavior* principle the main measurement was Avoidance distance test as part of the Human-animal relationship. The obtained results are presented as scores developed by the suggested I-spline function in the Welfare Quality Assessment protocol, Figure 4. Analysis of this measurement is based on the percentage of animals belonging to one of the four categories (animals that can be touched; up to 50cm; up to 100cm and above 100 cm), Table 3. Presented results are showing that the intensive tie-stall farm (Farm A) and one of the extensive household farms (Farm B) have the lowest score regarding HAR.

Figure 4. *Scores for Avoidance distance test. Farm E shows the best HAR, in contrast to Farm B and A.*

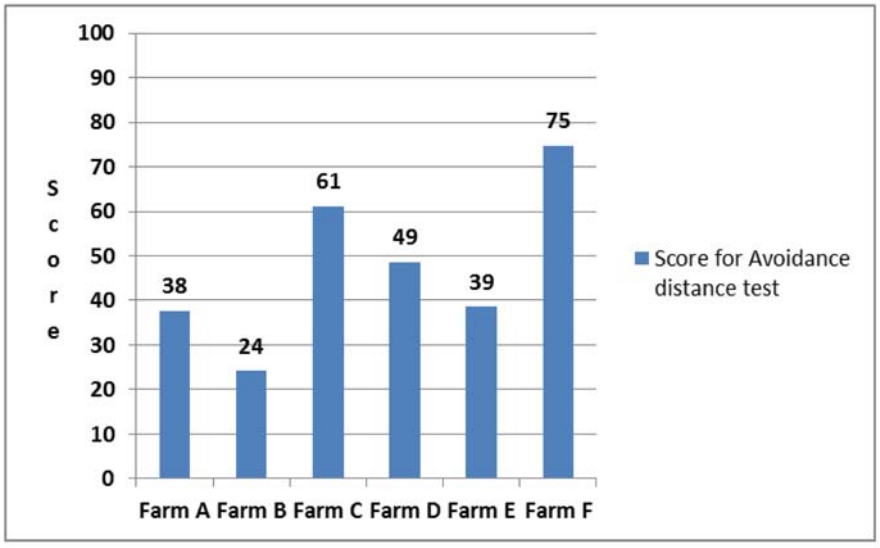


Table 3. *Distribution of avoidance distance test results by the four categories.*

FARM	Cows can be touched	up to 50cm	50cm – 100cm	Above 100cm	Tested animals	Mean cm	SEM cm
Farm A	21,62	37,84	35,14	5,41	37	45,00	5,43
Farm B	18,18	18,18	27,27	36,36	11	68,64	14,22
Farm C	14,29	85,71	0,00	0,00	7	24,29	6,94
Farm D	44,44	44,44	0,00	11,11	18	25,83	8,22
Farm E	0,00	60,00	40,00	0,00	5	54,00	7,31
Farm F	57,14	42,86	0,00	0,00	7	40,00	16,9

CONCLUSIONS

This research has shown that animal – based measures are good ground for assessing the animal welfare of dairy cattle and should be implemented in the routine assessments. The obtained results are showing that in the Welfare principle Good feeding there is a significant low score for absence of prolonged hunger in the farm with the intensive tie-stall housing in comparison with the extensive farms. The most distinctive disturbance of animal welfare standards are presented in the Good housing principle, where all farms have a serious problem with the cleanliness of the animals and good hygiene practices. In addition, all the farms are not providing access to pasture or outdoor runs throughout the year, which considerably disrupts the welfare of the animals. On this principle, the dairy cattle managers should put more efforts in respecting the animal welfare standards in the daily routine. Regarding Good Health more detailed analysis should be performed (especially on milk somatic cell count) in order to obtained relevant data. Although, present results suggested that this principle is not at risk, but in the intensive tie –stall farming dystocia has an important role in animal's suffering. This is in a relation with the used breeding material and human dedication in the managing process, which can be considered as minor issue in the extensive systems. Likewise, the human-animal relationship is better in the extensive household farms, showed through the avoidance distance test, in comparison with the intensive tie-stall systems. However, there are cases in the extensive systems of lower scores for HAR, which depends of the stockman's knowledge on managing and manipulating the animals. The intensive tie-stall system is showing lower HAR results as a consequence of lower interest, motivation and knowledge of the farm's personnel.

This research shows only the initial results and it is necessary to continue by involving more dairy farms and different housing systems in Macedonia. Thus, a baseline study for animal welfare of dairy cattle could be created in order to point out the key areas for improvement in dairy cattle production.

REFERENCES

1. European Commission (2007). *Attitudes of EU citizens towards Animal Welfare.* Eurobarometer, Brussels, 82
2. Estep, D.Q., Hetts, S., (1992). *Interactions, relationships, and bonds: the conceptual basis for scientist–animal relations*. In: Davis, H., Balfour, A.D. (Eds.), The Inevitable Bond—Examining Scientist–Animal Interactions. CAB International, Cambridge, pp. 6–26
3. Seabrook, M.F., (1972). *A study to determine the influence of the herdsmans personality on milk yield*. J. Agric. Labour Sci. 1, 45–59.
4. Hemsworth, P.H., Coleman, G.J., (1998). *Human–Livestock Interactions, The Stockperson and the Productivity of Intensively Farmed Animals*. CAB International, Wallingford
5. Waiblinger, S., Menke, C., Coleman, G., (2002). *The relationship between attitudes, personal characteristics and behavior of stock people and subsequent behavior and production of dairy cows*. Appl. Anim. Behav. Sci. 79, 195–219
6. Waiblinger, S., Menke, C., Fцlsch, D.W., (2003). *Influences on the avoidance and approach behaviour of dairy cows towards humans on 35 farms*. Appl. Anim. Behav. Sci. 84, 23–39.
7. European Commission (2012). Communication from the commission to the European Parliament, the council and the European economic and social committee on the *European Union Strategy for the Protection and Welfare of Animals 2012-2015*, Brussels
8. European Food Safety Authority, (2012). *Scientific opinion, Statement on the use of animal-based measures to assess the welfare of animals*. EFSA Journal 2012;10(6):2767
9. Welfare Quality®, (2009). *Welfare Quality® assessment protocol for cattle*. Welfare Quality® Consortium, Lelystad, Netherlands
10. Windschnurer, I., Schmied, C., Boivin, X., Waiblinger, S., (2009). *Assessment of animal welfare measures for dairy Cattle, Beef Bulls and Veal Calves*. Welfare Quality Reports No.11,Upsala, Sweden, 137-152

ПРОЦЕНКА НА БЛАГОСОСТОЈБАТА КАЈ МЛЕЧНИ ГОВЕДА КОРИСТЕЈЌИ МЕРКИ БАЗИРАНИ НА ПОСЛЕДИЦИТЕ И ВРСКАТА ЧОВЕК – ЖИВОТНО КАЈ РАЗЛИЧНИ СИСТЕМИ НА ОДГЛЕДУВАЊЕ

Радески Мирослав, Илиески Влатко

Центар за благосостојба на животни, Факултет за Ветеринарна Медицина, Универзитет „Св. Кирил и Методиј“, Скопје, Македонија

АПСТРАКТ

Различни системи на одгледување кај млечните говеда имаат различно влијание врз нивната благосостојба која може да се процени користејќи мерки базирани на последиците. Дополнително, воспоставувањето на добар однос помеѓу одгледувачот и животните е есенцијално за благосостојбата на животните. Во Р. Македонија повеќето системи на одгледување на млечни говеда се во екстензивни и интензивни системи со врзано држење на говедата. Затоа, главната цел на овој труд е да ја процени благосостојбата на млечните говеда во овие системи на одгледување користејќи мерки базирани на последиците и да го одреди степенот на врската човек – животно. Ова истражување беше спроведено на шест фарми за млечни говеда во Македонија, од кои едната беше со интензивен систем со врзано држење, каде од вкупно 111 говеда, 85 беа тестирани. Извршената проценка на благосостојбата беше изработена според воспоставените принципи, критериуми и мерки за благосостојба на Проектот Welfare Quality®, користејќи девет дефинирани критериуми за благосостојба, мерени според 23 развиени мерки. Според мерките за благосостојба беше изработен Протокол за проценка на благосостојбата, а спроведен беше и тестот на избегнување.Интензивниот систем со врзано држење доби најниски бодови во однос на пролонгиран глад кај животните, што влијаеше на целокупната оценка за принципот Добра исхрана. Во однос на чистотата на долните делови на нозете, горните делови на нозете/препоните и вимето, сите фарми беа категоризирани како фарми со „сериозен проблем“, истакнувајќи ги хигиенските услови како круцијален фактор за подобра благосостојба на животните. Најниски бодови во тестот на избегнување, кој се употреби за одредување на врската човек – животно, доби системот со интензивно врзано држење и една фарма од екстензивниот систем. Овие бодови се должат на нискиот интерес, мотивација и знаење на работниците во фармата, што е витално за благосостојбата на млечните говеда.Со цел да се добијат релевантни податоци и мерки, потребно е инволвирање на повеќе фарми за млечни говеда и различни системи на одгледување. На тој начин ќе се изработи релевантна основна студија за благосостојбата на млечните говеда во постојните системи на одгледување.

Клучни зборови:проценка на благосостојбата, млечни говеда, врска човек – животно, мерки базирани на животните

UDC: 591.481.2:591.128.044]:599.323.41

ACTH CELLS AFTER CHRONIC EXPOSURE OF RATS TO HIGH AMBIENT TEMPERATURE: MORPHOLOGICAL STUDY

Popovska-Percinic Florina[1], Ajdzanovic Vladimir[2], Trifunovic Svetlana[2], Ilieski Vlatko[1], Pendovski Lazo[1], Blagoevska Katerina[3], Milosevic Verica[2]

[1]Department of functional morphology, Faculty of Veterinary Medicine – Skopje, University "Ss. Cyril and Methodius", Skopje, R. Macedonia
[2]Department of cytology, Institute for Biological Research, "Siniša Stanković", University of Belgrade, Belgrade, Serbia
[3] Department of biochemistry and cell biology, Faculty of Veterinary Medicine – Skopje, University "Ss. Cyril and Methodius", Skopje, R. Macedonia

ABSTRACT
The aim of this study was to examine the effect of chronic exposure to elevated ambient temperature on the morphological characteristics of immunopositive ACTH cells in adult male Wistar rats. The animals were continually kept at 35±1°C for 7 days (experimental group), while the control group was kept at room temperature (20±2°C). The ACTH-producing cells were visualized using the peroxidase-antiperoxidase (PAP) immunohistochemical procedure. The analyses have shown that in the experimental group, there was a dominance of oval over the stellate cellular form, whereas the localisation of the ACTH cells was not changed, compared with the controls. There was a significant decrease ($p<0.05$) of the body weight, the cellular and nuclear volume as well as the volume density of ACTH cells by 22.6%, 18.1%, 14.8% and 40,0%, respectively, in comparison with the controls. These findings suggest that 7 day exposure of adult male rats to elevated ambient temperature has the inhibitory effect on morphological characteristics of ACTH cells.
Key worlds: ACTH cells, chronic exposure to heat,_ morphological characteristics , rats

INTRODUCTION

All living organisms are chronicaly exposed to elevated ambient temperature during summer time in regions with moderate continental climate. Exposure to high ambient temperature is one of the strongest types of physical stressors (1), affecting the activity of HPA axis (2) and ACTH cells (3,4). Previous studies have shown increase, decrease or no changes (5,6,7,8) of plasma ACTH level during long term exposure to elevated ambient temperature. Taking into consideration that there are no morphological studies of pituitary ACTH cells after prolonged animal exposure to heat stress, the aim of this study was to examine the effect of 7 day continuous exposure of rats to 35±1°C on morphological characteristics of ACTH cells.

MATERIAL AND METHODS

The experimental animals, adult male Wistar rats (n=7), were exposed for 7 days to high ambient temperature (35±1°C). The control group (n=7) was kept at room temperature (20±2°C). The pituitary glands were fixed in Bouin's solution, serially cut and the ACTH cells were identified by peroxidase-antiperoxidase (PAP) immunohistochemical procedure. The volume of pituitary ACTH cells and their nuclei were estimated using the multipurpose test system M_{42}.

RESULTS AND DISCUSSION

The body weight in experimental group was significantly decreased ($p<0.05$) by 22.6%, compared to the controls, while there were no changes of the absolute and relative pituitary weight (Tab.1). In experimental group, the dominance of oval over the stellate cellular form was observed, whereas the localisation of the ACTH cells was not changed (Fig. 1A, B). Stereological investigation revealed significant decrease of the cellular, nuclear as well as volume density of ACTH cells ($p<0.05$) by 18.1%, 14.8% and 40.0%, respectively, in comparison with the controls (Fig. 2A, B). The obtained results in this study point toward decreased activity of pituitary ACTH cells. This is in coordinance with the findings that prolonged or repeated stress in rats results in a reduction (by 20–50%) of CRH binding in the anterior pituitary (9). It was also reported that during chronic stress there is a reduction of the CRH-induced ACTH secretory response in rats (10).

Table 1. Body weight, absolute and relative pituitary weight in control and experimental rats

Experimental group	Body weight(g)	Absolute weight (mg)	Relative weight (mg%)
Control	337.5 ± 26.9	6.5 ± 0.6	2.5 ± 0.1
7.DAY	261.2 ±21.8* (-22.6%)	6.3 ± 0.5 (n.s.)	2.4 ± 0.2 (n.s.)

*The values are the means ± SD (n=7/group). * $p<0.05$ vs. control*

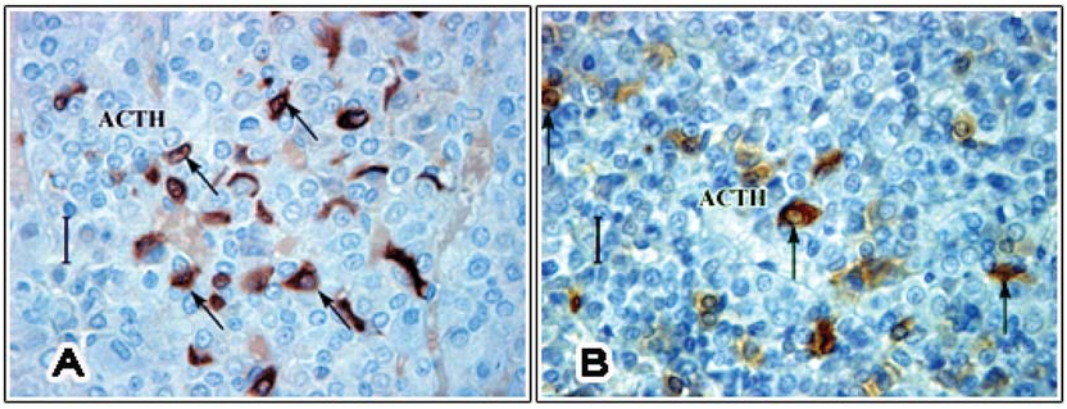


Figure 1. *Immunocytochemically labeled ACTH cells in: control (A) and animals exposed 7 days to high temperature (35 ± 1°C) (B). (PAP; 63x; bar 16µm)*

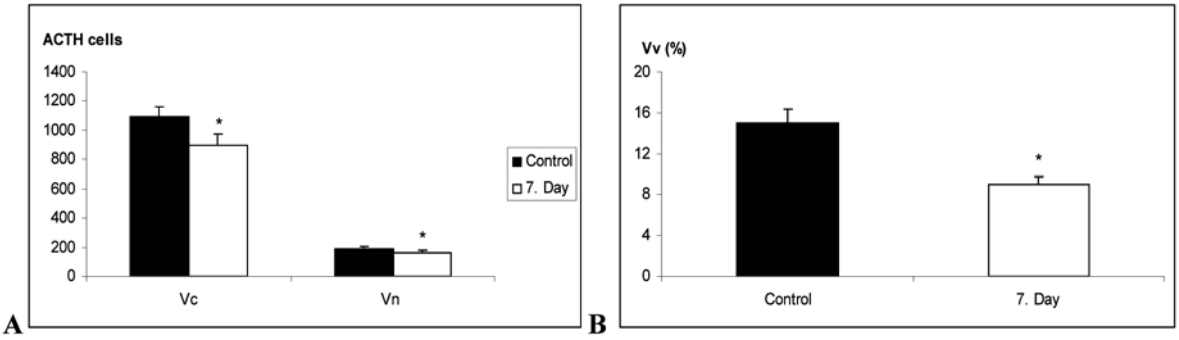


Figure 2. A*, Cellular volume (Vc; µm³) and nuclear volume (Vv; µm³) of the immunoreactive ACTH cells;* ***B****, Relative volume density (Vv; %) of ACTH cells expressed as percentages of total gland tissue. All values are the means ± SD (n=7/group). * p<0.05 vs. control.*

CONCLUSION

These findings suggest that 7 day exposure of adult male rats to elevated ambient temperature has the inhibitory effect on morphological characteristics of ACTH cells.

REFERENCES

1. Djordjevic J., Cvijic G., Davidovic G. (2003): Different activation of ACTH and corticosterone release in response to various stressors in rats.Physiol. Res., 52. 67-72.
2. Svirid, VD. (2002): Effect of environmental thermal factor on synthesis of specific proteins in albino mice. Bull Exp Biol Med.,133(3), 285-289.
3. Pacak K., Palkovits M., Kopin IJ., Goldstein DS. (1995): Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and symphatoadrenal activity: in vivo microdialyses studies. Front Neuroendocrinol., 16, 89-150.
4. Popovska-Perčinić, F., Ajdžanović, V., Dinevska-Kofkarovska, S., Jordanova, M., Trifunović, S., Šošić-Jurjević, B., Milošević, V. (2011): Morphofunctional characteristics od pituitary corticotropes in an animal model of heat stress. J. Med. Biochem., 30, 4, 287-292.
5. Gjuladin, T. (2004): The effect of high ambient temperature on the hormonal status in diabetic rats. Master thesis, Skopje
6. Mitev (1983): The effect of hyperthermic environment and some endocrine factors on the glycogen amount in liver and other parameters in white rat. Doctoral thesis, Skopje
7. Brandenberger, G., Follenius, M., Di Nisi, J., Libert, JP., Simon, C. (1989): Amplification of nocturnal oscillations in PRA and aldosterone during continuous heat exposure. J Appl Physiol., Mar;66(3), 1280-1286.
8. Saini, J., Brandenberger, G., Libert, J. P., Follenius, M. (1993): Nocturnal pituitary hormone and renin profiles during chronic heat exposure. J Appl Physiol. Jul;75(1), 294-300.
9. Rabadan-Diehl, C., Kiss, A., Camacho, C., Aguilera, G. (1996): Regulation of messenger ribonucleic acid for corticotrophin releasing hormone receptor in the pituitary during stress. Endocrinology, 137, 3808–3814.
10. Odio MR, Brodish A. (1990): Effects of chronic stress on in vivo pituitary-adrenocortical responses to corticotropin releasing hormone. Neuropeptides. Mar;15(3):143-52.

АСТН КЛЕТКИ ПО ХРОНИЧНА ЕКСПОЗИЦИЈА НА СТАОРЦИ НА ВИСОКА АМБИЕНТАЛНА ТЕМПЕРАТУРА: МОРФОЛОШКА СТУДИЈА

Поповска-Перчиниќ Флорина[1], Ајџановиќ Владимир[2], Трифуновиќ Светлана[2], Илиески Влатко[1], Пендовски Лазо[1], Благоевска Катерина[3], Милошевиќ Верица[2]

[1]Катедра за функционална морфологија, Факултет за ветеринарна медицина-Скопје, Универзитет „Св. Кирил и Методиј“, Скопје, Македонија
[2]Одделение за цитологија, Институт за биолошки истражувања „Синиша Станковиќ“, Универзитет во Белград, Белград, Србија
[3]Катедра за биохемија и биологија на клетка, Факултет за ветеринарна медицина-Скопје, Универзитет „Св. Кирил и Методиј“, Скопје, Македонија

АПСТРАКТ
Целта на оваа студија беше да се испита ефектот на хроничната изложеност на зголемена амбиентална температура врз морфолошките карактеристики на имунопозитивните АСТН-клетки кај адултни Wistar стаорци од машки пол. Животните беа континуирано изложени на температура од 35±1°C за време од 7 дена (експериментална група), додека контролната група беше чувана на собна температура (20±2°C). АСТН-клетките беа визуелизирани со користење на пероксидаза-антипероксидаза (ПАП) имунохистохемискиот метод. Анализите покажаа дека во споредба со контролите, кај експерименталната група доминираа овални во однос на sвездолики клетки, додека немаше промена во нивната локацијата. Беше евидентирано значително намалување ($p<0.05$) на телесната тежина, клеточниот и јадрениот волумен како и релативната густина на АСТН-клетки за 22,6%, 18,1%, 14,8% и 40,0%, соодветно, во споредба со контролите. Овие наоди укажуваат на тоа дека седумдневната изложеност на адултни машки стаорци на покачена амбиентална температура има инхибиторен ефект врз морфолошките карактеристики на АСТН-клетките.
Клучни зборови: АСТН-клетки, хронична изложеност на топло, морфолошки карактеристики, стаорец

UDC: 636.35.082:575.22

MULTI-DIMENSIONAL SCALING ANALYSIS OF GENOME-WIDE SNP DATA ON ITALIAN SHEEP BREEDS REVEALS A STRONG PHYLOGEOGRAPHIC GRADIENT

Ciani Elena[1], D'andrea Mariasilvia[2], Lasagna Emiliano[3], Napolitano Francesco[4], Carta Antonello[5], Matassino Donato[6], Crepaldi Paola[7], Ciampolini Roberta[8], Bordonaro Salvatore[9], Modesto Paola[10], Macciotta Nicolò p.p. [11], Ajmone Marsan Paolo[12], Portolano Baldassarre[13], Kompan Drago[14], Consorzio Biovit[14]

[1]Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università degli Studi di Bari, Italia; [2]Dipartimento di Scienze Animali Vegetali e dell'Ambiente, Università degli Studi del Molise, Campobasso, Italia; [3]Dipartimento di Biologia Applicata, Università degli Studi di Perugia, Italia; [4]CRA-Centro di ricerca per la produzione delle carni e il miglioramento genetico, Monterotondo, Roma, Italia; [5]Dipartimento per la Ricerca nelle Produzioni Animali - AGRIS Sardegna, Sassari, Italia; [6]ConSDABI - Consorzio per la Sperimentazione, Divulgazione e Applicazione di Biotecniche Innovative, Benevento, Italia; [7]Dipartimento di Scienze veterinarie e Sanità pubblica, Università degli Studi di Milano, Italia; [8]Dipartimento di Patologia Animale, Profilassi e Igiene Alimentare, Università degli Studi di Pisa, Italy; [9]Dipartimento di Scienze delle Produzioni Agrarie e Alimentari, Università degli Studi di Catania, Italia; [10]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italia; [11]Dipartimento di Scienze Zootecniche, Università degli Studi di Sassari, Italia; [12]Istituto di Zootecnica, Università Cattolica del Sacro Cuore, Piacenza, Italia; [13]Dipartimento DEMETRA, Università degli Studi di Palermo, Italia; [14]Biotechnical Faculty, University of Ljubljana, Slovenia.

*Corresponding author: elena.ciani@uniba.it

ABSTRACT

In Italy several local sheep breeds and populations are reared, though most of them are endangered or in very critical conditions and, therefore, in need of conservation actions. This paper aims to outline the genetic relationship among nineteen Italian local and country-wide sheep breeds by the means of a multidimensional scaling analysis conducted over the allele sharing distance estimated from the genotypic data at the Illumina OvineSNP50 BeadChip. The analysis highlighted the presence of a strong geographical gradient in the distribution of the genetic variability of the considered breeds, with a North to South main gradient, along which almost all the breeds were positioned, followed by a minor East to West gradient, discriminating insular breeds from continental breeds. The results suggest that historical events of admixture and gene flow among contiguous areas played a significant role in determining the current pattern of genetic diversity among the Italian breeds, consistently with traditional sheep management practices (extensive rearing and transhumance) characterizing the history of sheep breeding in the Italian peninsula.

Key words: Single Nucleotide Polymorphisms; sheep breeds; genetic variability; multidimensional scaling analysis.

INTRODUCTION

Despite the dramatic structural and socio-economic changes occurred in the last fifty years to the Italian rural scenario, the country is still rich of several sheep breeds and populations, with some sixty local breeds officially registered. However, most of them are endangered or in very critical conditions and survive only thanks to the passionate actions of some devoted farmers. The safeguard and valorization of these breeds represent today a possible strategic option, in view of a general re-thinking of the primary production systems as oriented to a higher social and environmental sustainability. One of the first step in genetic conservation is the monitoring of the current level of genetic variability within breeds and the study of the genetic relationships among them. Several works have been carried out previously on Italian sheep breeds, though mainly with sparse STR markers and at a regional scale (1-5); Italian breeds have been also included in some international studies conducted by STR and SNP markers (6-8) though very few in number so far. For these reasons, we decided to address the analysis of the genetic variability of the Italian sheep breeds at a national-scale and with a dense SNP panel.

MATERIALS AND METHODS

A total of 500 blood samples, representative of 20 Italian sheep breeds, have been collected and analyzed at more than 50K SNP loci by using the OvineSNP50 BeadChip (Illumina®). Genotypic data have been edited adopting the following parameters: removal of allosomic loci, removal of individual with "individual missingness" > 0.10, removal of loci with "call rate" <0.01 and MAF (Minor Allele Frequency) <0.01. After editing, 44.006 loci and 496 animals were left. Statistical analyses were carried out using PLINK v 1.01 (9). In particular, the average proportion of alleles shared was calculated as (IBS2+0.5*IBS1)/N, where IBS1 and IBS2 are the number of loci which share either 1 or 2 alleles identical by state (IBS), respectively, and N is the number of loci tested. An IBS matrix of distance (D = 1-IBS) was then constructed containing each pair-wise combination of all 496 individuals and a multidimensional scaling (MDS) analysis was performed on these data.

RESULTS

The Illumina OvineSNP50 BeadChip revealed a high level of polymorphism in our population sample, with,

on average, less than 3% of loci being monomorphic within breeds (data not shown). In order to reconstruct genetic relationships among the Italian sheep breeds considered in the study, a multidimensional scaling (MDS) approach has been adopted, based on the calculation of the distance D = 1-IBS among all the possible pairs of animals. The obtained results are visualized in the MDS plot presented in Figure 1, were animals from the Altamurana breed have been removed since having being detected as outliers.



Figure 1. *Plot of the first two components (C1 and C2, respectively) obtained from the multidimensional scaling analysis of the pair-wise D distance matrix among the 496 considered animals. SAB, Sardinian Ancestral Black; SW, Sardinian White.*

As can be clearly observed, the distribution of the genetic variability of the considered Italian sheep breeds strongly reflects their geographical distribution, with a North to South main gradient, along which almost all the breeds are positioned, followed by a minor East to West gradient, that discriminates insular breeds (from Sardinia and Sicily) from the other continental breeds. These results suggest that historical events of admixture and gene flow among contiguous areas played a significant role in determining the current pattern of genetic diversity among the Italian breeds. This hypothesis is also consistent with the traditional sheep management practices (extensive rearing and transhumance) that have been characterized the more and less recent history of sheep breeding in the Italian peninsula. Despite the presence of such a genetic gradient, other analyses, like those conducted using the software STRUCTURE (10), highlight for most Italian breeds an original genetic background (data not shown), that deserves attention and protection efforts.

CONCLUSIONS

The study, conducted on twenty local and country-wide Italian sheep breeds, revealed the presence of a strong phylogeographic gradient, thus suggesting the importance of past and recent gene flow in shaping modern genetic structure. The wide amount of genotypic data produced by typing animals at the Illumina OvineSNP50 BeadChip will be further analyzed in order to (i) deepen our knowledge on the genetic relationships among specific breeds or breed groups, also including foreign breeds from the large collaborative Sheep Hapmap Project (www.sheephapmap.org), such as Merinos and Merinos-type breeds, (ii) identify functional genomic regions under differential selection pressure (selection signatures) and (iii) select the most informative SNP panels for breed authentication of sheep products.

REFERENCES

1. Bozzi R., Degl'Innocenti P., Rivera Diaz P., Nardi L. Crovetti, A., Sargentini C., Giorgetti A. (2009). Genetic characterization and breed assignment in five Italian sheep breeds using microsatellite markers. Small Rumin. Res. 85(1):50-57.
2. Dalvit C., Saccà E., Cassandro M., Gervaso M., Pastore E., Piasentier E. (2008). Genetic diversity and variability in Alpine sheep breeds. Small Rumin. Res. 80(1-3):45-51.
3. d'Angelo F., Albenzio M., Sevi A., Ciampolini R., Cecchi F., Ciani E., Muscio A. (2009). Genetic variability of the Gentile di Puglia sheep breed based on microsatellite polymorphism. J. Anim. Sci. 87(4):1205-1209.
4. Lasagna E., Bianchi M., Ceccobelli S., Landi V., Martínez

Martínez A., Vega Pla J.L., Panella F., Delgado Bermejo J.V., Sarti F.M. (2011). Genetic relationships and population structure in three Italian Merino-derived sheep breeds. Small Ruminant Res. 96:111-119.
5. Moioli B., Napolitano F., Orrù L., Catillo G. (2006). Analysis of the genetic diversity between Gentile di Puglia, Sopravissana and Sarda sheep breeds using microsatellite markers. Ital. J. Anim. Sci. 5(1):73-78.
6. Kijas J.W., Lenstra J.A., Hayes B., Boitard S., Porto Neto L.R., San Cristobal M., Servin B., McCulloch R., Whan V., Gietzen K., Paiva S., Barendse W., Ciani E., Raadsma H., McEwan J., Dalrymple B., other members of the International Sheep Genomics Consortium (2012). Genome-wide analysis of the world's sheep breeds reveals high levels of historic mixture and strong recent selection. PLoS Biol 10(2): e1001258. doi:10.1371/journal.pbio.1001258.
7. Lawson Handley L.J., Byrne K., Santucci F., Townsend S., Taylor M., Bruford M.W., Hewitt G.M. (2007). Genetic structure of European sheep breeds. Heredity. 99(6):620-631.
8. Peter C., Bruford M., Perez T., Dalamitra S., Hewitt G., Erhardt G. (2007). Genetic diversity and subdivision of 57 European and Middle-Eastern sheep breeds. Anim. Genet. 38(1):37-44.
9. Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira M.A., Bender D., Maller J., Sklar P., de Bakker P.I., Daly M.J., Sham P.C. (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81(3):559-75.
10. Pritchard J.K., Stephens M., Donnelly P. (2000). Inference of population structure using multilocus genotype data. Genetics 155:945–959.

МУЛТИ-ДИМЕНЗИОНАЛНА АНАЛИЗА НА SNP ГЕНОТИПОВИ КАЈ ИТАЛИЈАНСКИ РАСИ НА ОВЦИ ПОКАЖУВА СИЛНО ИЗРАЗЕНА ФИЛОГЕОГРАФСКА НАКЛОНЕТОСТ

Цјани Елена[1], Д'андреа Мариасилва[2], Лазања Емилијано [3], Наполитано Франческо[4], Карта Антонело [5], Матасино Донато [6], Крепалди Паола [7], Цјамполини Роберта[8], Бордонаро Салавторе[9], Модесто Паола[10], Мациота Николо п.п.[11], Ајмон Марсан Паоло[12], Портолано Балдасаре[13], Компан Драго [14], Биовита Конзорциум[14]

[1]Оддел за биологија, биотехнологија и фармакологија, Универзитет во Бари, Италија
[2]Оддел животни, растенија и животна средина, Универзитет во Молизе, Кампобосо, Италија
[3]Оддел за применета биологија на Универзитетот во Перуџа, Италија
[4] CRA-Истражувачки центар за производство на месо и генетско подобрување, Монтеротондо, Рим, Италија
[5] Оддел за истражување на животниско производство – AGRIS Sardegna, Сасари, Италија
[6]ConSDABI - Конзорциум за тестирање, распространетост и примена на иновативни биотехнологии, Беневенто, Италија;
[7]Оддел за ветеринарни науки и јавно здравство, Универзитет во Милано, Италија
[8] Оддел на патологија на животни, профилакса и хигиена на храна, Универзитетот во Пиза, Италија
[9] Оддел на земјоделско-прехранбени науки на Универзитетот во Катанија, Италија
[10] Институт за експериментална зоопрофилакса во Пиемонт, Лигурија и Вале ди Аоста, Торино, Италија
[11] Оддел за зоотехнички науки, Универзитет во Сасари, Италија
[12] Институт на Сточарство, католички универзитет на Сакр Кер, Пјаченца, Италија
[13] Оддел ДЕМЕТРА, Универзитет во Палермо, Италија
[14] Биотехнички факултет, Универзитет во Љубљана, Словенија.

АПСТРАКТ
Во Италија се одгледуваат неколку локални раси и соеви на овци од кои повеќето се загрозени или се во критична состојба со што се наметнува потреба за спроведување на мерки за нивна конзервација. Трудот ја презентира генетската поврзаност помеѓу 19 локални и национални италијански раси на овци врз база на мулти-димензионална анализа на алелните дистанци добиени со генотипизација на грлата со употреба на OvineSNP50 BeadChip (Illumina). Анализата покажа силна географска поврзаност помеѓу генетската варијабилност на истражуваните раси, со главен правец на групирање север-исток, проследен со споредeн правец исток-запад кој овозможува дистинкција на островските од континенталните раси. Од добиените резултати може да се заклучи дека генетската варијабилност која денес постои помеѓу италијанските раси се должи на рекомбинацијата и трансферот на гени кои се одвивале во минатото помеѓу расите одгледувани во соседните географски региони, надополнети со екстензивниот-номадски начин на одгледување, фактори кои заедно го окарактеризирале одгледувањето на овци на Италијанскиот полуостров.
Клучни зборови: SNP маркери, раси на овци, генетска варијабилност, мултидимензионална анализа.

UDC: 599.742.11-115:575.222(497.7)

MICROSATELITE GENOME CHARACTERIZATION OF THE GRAY WOLF IN REPUBLIC OF MACEDONIA

Esmerov Igor[1], Branko Atanasov[2], Nikola Adamov[3],
Katerina Blagoevska[1] Stojkovski Velimir[1]

[1]*Departement of Cell Biology and Biochemistry*
Faculty of Veterinary Medicine, University „St.Cyril and Methodius" Skopje- Republic of Macedonis
[2]*Departement of Reproductio*
Faculty of Veterinary Medicine, University „St.Cyril and Methodius" Skopje- Republic of Macedonis
[3]*Departement of Animal Breeding and Genetics*
Faculty of Veterinary Medicine, University „St.Cyril and Methodius" Skopje- Republic of Macedonis

ABSTRACT

The DNA microsatelites represented co-dominant genetic marker that is widely used in characterizing the biodiversity of certain populations.

The genetic variability of the population of the gray wolf was determined by analysis of 10 DNA microsatelite locus (FH2361, DGN10, FH3287, FH3924, FH3608, FH3023, FH3489, FH3721, FH4027 and FH2141).

The study included 26 samples of gray wolf, which originated from the mountains from western Macedonia. The informative content of the DNA microsatelite locus was determined according to the Polymorhism Informativ Content-PIC as well as the number of detected alleles per locus. All the ten DNA microsatelite locus were highly polymorphic.

In the genome of the gray wolf the highest value for PIC was determined in locus FH4027 (0.907), and the lowest in locus FH3489 (0.692).

The number of detected alleles per locus of the gray wolf varied from 5 in the locus FH3489 to 15 in the locus FH4027.

The intra- population genetic variability was confirmed thorough the number of detected alleles for each DNA microsatelite locus, the average number of alleles for all ten DNA microsatelite markers and the total number of detected alleles characteristic for the given population.

In the population of the gray wolf the number of common alleles was highest in the locus FH2361 at the level of 26, from which 24 were heterozygote and 2 were homozygote.

According to the results of this study can be concluded that there is relatively low genetic variability in terms of the researched parameters in the population of the gray wolf. This finding, in accordance with most similar studies of other authors implicates that the gray wolf is relatively pure kind, which in its reproduction has not undergone cross breed with other similar kinds such as the dog and the shakal.

Key words: dog, wolf, DNA microsatelites, genetic variability

INTRODUCTION

The DNA markers which are used in construction with genetic maps are generally divided in 2 groups (O'Brien et al., 1993). The first group (type 1) consists of DNA markers associated with conservative genetic sequences in the mammals, while the second group (type 2) are highly polimorphic, mostly anonymous DNA sequences.

When genes or genetic markers are located near a chromosome they are not inherited independently, but instead they co-segregate during the meosis division, and the occurrence of this phenomenon is called linkage. When high density of markers occurs on one chromosome they can be organized into a linkage map of the genome. (Botstein, 1980).

The most important momentum in the development of evolutionary studies and population genetics may be emphasized the development of PCR techniques, the invention and the routine usage of the hyper-variable DNA microsatellite loci and the possibility for routine sequencing of the DNA molecule. The development of appropriate software programs for analysis of previously obtained thrusts only additionally defines the role of these research. (Epping et al., 1995.).

The main features of the DNA markers used in this type of study are sensitivity, the ability to be multi locus and single locus, the level of informative for the frequency, of the alleles, and in terms of the origin can be organelles and nuclear (nuclear). (Sunnucks, 2000).

Due to the high informational level of the microsatelite DNA successful attempts are done in the same survey as well as comparison of its variability between different strains, and between representatives of the species with more or less similar phenotype.

Some areas of repeating sequences which show variations in the length, where the various alleles contain different number of repeating units are known as Simple sequence length polymorphisms (SSLPs). SSLPs is most often multiallellic due to the number of the different length variations.

There are two types of SSLPs: minisatelites and microsatelites:

a) The minisatelites are also known as variable number of tandem repeats, where the repeating units reach to 25bp.
b) Microsatelites, or simple tandem repeats (short tandem repeats – STRs) are short sequences of DNA, with size of 1-6 bp which repeat several times in a

row and are characterized with a high informational level. They represent co-dominant genetic marker with wide application in the characterizing of the biodiversity. Most frequent DNA markers which are used in construction of a genetic map are the DNA microsatelites. The DNA microsatelites represent anonymous DNA segments, which is actually their only disadvantage, and due to this can be applied in related species. (Weber and May, 1989).

The microsatelites are more popular DNA markers than the minisatelits due to two reasons:

- Firstly, the minisatelites are mostly located in the telomeric regions at the ends of the chromozomes. The microsatelites are more widespread throug the genome.
- Secondly, the fastest way of determining the polyphormic length is with PCR, but it's common knowledge that the determining with PCR is more accurate when dealing with sequences smaller than 300 bp.

Most of the microsatelites alleles are longer due to the larger number of the repeating units which usually are located in one area, and consequently it is necessary with the PCR method to determine sequences from several kilobases. The typical microsatelites consist of 10-30 copies which are repeating, but which are not longer than 4-6 bp in the length and for this reason are more accessible for analysis with PCR.

In accordance with the analysis of the structures, the frequencies and the density of the DNA microsatelites the informative level differs, and according to the type of the repeating sequence they can be bi, three and tetra nucleotide repeats. The bi-nucleotide repeats CA are represented more in the animals, while the bi-nucleotide repeats TA and GA in the plants. The density of the DNA microsatelite locus of the plants is similar with the one of the animals, with average size of approximately 10 repaets. (Jarne and Lagoda, 1996).

Based on their structure, the DNA microsatelites can be divided in three groups: clear, complex and interspersed.

More than 25% of the identified locus' belong to the group of the complex ore interspersed bi-nucleotide repeats, which are less polymorphic than the clear, interspersed bi-nucleotide repeats. (Jarne and Lagoda, 1996). Approximately 67% of the detected SSR are bi-nucleotide repeats (You-Chun et al,. 2002)

The tri-nucleotide repeats are represented in both the plants and the animals, but however they are best known in the human population, due to the connection with certain diseases. If they are located in the coding part of the genome (exon) they are often associated with occurrence of certain diseases. (You-Chun et al,. 2002)

The frequency and the location of the tetra-nucleotide repeats are depended on the species. In the genome of the human population they are grouped near the sex chromosomes (Jarne and Lagoda, 1996). Most represented tetra-nucleotide repeats are GATA/GACA repeating sequence. All of these repeats are usually happening in the none-coding part of the genome, and their number is significantly variable.

The first hypothesis directs to the positive correlation between the growth of the genome and the number of microsatelite locus, and negative correlation with the microsatelite depression, or the number of the DNA bases which separates the neighbouring microsatelite locus and the size of the genome.

The second hypothesis directs to the class-specific difference between the frequency of the microsatelite locus based on the variation and the frequency of their evolutional precursors. (Neff and Gross, 2011)

The variation which emerges in the DNA regions is a result of the evolutional pressure on the populations expressed through mutations, selection, genetic drift (accidental changes in the population) or re-combinations which occur in the DNA molecule of the species.

The rapidity with which the mutation occurs in the DNA microsatelite locus significantly varies. The rapid mutation of the microsatelite locus can be explained through two mechanisms. According to You-Chun et al (2002), the first mechanism relates to the rapid mutation which occurs as a result of the re-combination of the DNA molecule in case of uneven crossing over. While according to Levinson and Gutman (1987) as a mechanism for the rapid mutation of the microsatelites they include sliped strand misparing (presence of the nucleotide on given position in one DNA chain which is not complementary with the other nucleotide of the appropriate position, SSM) during the DNA replication. (You-Chun et al., 2002).

The more significant function of the DNA microsatelite is defined as an indispensable source of the genetic variation, the way the nature complements the genetic variation in the populations through the genetic drift and the selection. (You-Chun et al., 2002)

Brinkmann B. et al. (1998) points out that in the evolutional studies the microsatelites have certain advantages in terms of the other markers. As more important it is emphasized: the high level of polymorphism, their location in almost all chromosome regions, the small amount of DNA necessary for amplification of the locus, the easy detection with assistance of the PCR method and the sophisticated appliances for their genome- classification.

The main disadvantage is their anonymity in the genome.

As very important factors when using DNA microsatelites in molecular characterization of the animals are recommended FAO's (Food and Agriculture Organisation) criteria for DNA microsatelites:

- The microsatelites need to have the Mendeley;s inheritance
- Each microsatelite plus needs to have at least 4 alleles
- Usage of already pointed microsatelite markers in certain mapping studies
- Usage of microsatelite locus in related species, different races of dogs, wolves and chakals
- Accessibility of the research of the microsatelite locus in published reports

MATERIALS AND METHODS

- Isolation of DNA from reticulocyte
- Decomposition of the cells with proteinase
- Isolation of DNA was done with phenol/ chloroform extraction and precipitation with ethyl alcohol
- Check up of concentration and clearness of DNA
- Amplification of the core DNA microsatelite locus
- Native DNA electrophoresis in polyacrylic gel (DNA-PAGE)

Table 1. *Sequence and localization which are used for PCR amplification and genome-classification*

STR Primer	Forward primer Revers primer	Chromosome and position in the chromosome	Size of the region	PIC
FH 3023 F/R	5'-AAGCTCATGGAGTTTCTCACTC-3' 5'-AGGGAGCAGCTTTGAGTTTC-3'	CFA24 position 4	374 (352-444)	0.92
FH 3287 F/R	5'-AGGAATGCAGCAAAGTGG 3' 5'-AGTCCATGCACACAGAAGG-3'	CFA24 position 51	482 (379-478)	0.82
DGN10 F/R	5'-TCTGCCTATGTCTCTGCCTCTC-3' 5'-GGCACCTATTCTGGGAAACTTT-3'	CFA26 position 34	251 (244-255)	0.83
FH 3924 F/R	5'-CAGCCTACAATTCCAACAGC-3' 5'-GCTGTGCTCAACATAGTAATTGG-3'	CFA 27 position 48	390 (353-397)	0.85
FH 3489 F/R	5'-GGCCACGAATGCTATTTACC-3' 5'-TCCTCATATGTCCTCAACTGC-3'	CFA30 position 3	278 (242-270)	0.8
FH2361 F/R	5'-GCTTGGAAGGTGAGACTGAATG-3' 5'-AGCACTTAGAATGTACCAGGCAC-3'	CFA32 position 22	370 (344-436)	0.75
FH 3608 F/R	5'-GACCACAAAGAAATCACTTGC-3' 5'-GACTTGCGGGATGAAAGG-3'	CFA 33 position 10	400 (388-421)	0.81
FH 2141 F/R	5' - CAATGCTCCTTAGATTGCAGG - 3' 5' - ACCCAAGGAAACTAGAAGGAAG - 3'	CFA 25 position 42	550 (522-623)	0.89
Fh 4027 F/R	5' - CGTTCTTTCTTCAAAAGAGTGC - 3' 5' - GCTTGGTCAGTAGAGCATGAG - 3'	CFA 25 position 63	450 (370-554)	0.90
FH3721 F/R	5'-GTTGGCAAATTGAACACCAA-3' 5'-ATTACTTTCAACAACCCACTCC-3'	CFI34 position 1	201 (201-245)	0.86

RESULTS AND DISCUSSION

On the locus DGN10 of the grey wolf it were detected 9 alleles with different sizes. The observed heterozygosity was 0.846, while the expected heterozygosity was 0.816. Most present was the alleles with size of 224 bp and with allelic frequency of 32,69%, and least present was the alleles with size of 264 bp and with allelic frequency of 1.92%

On the locus FH3023 were detected 7 alleles with different sizes. The observed heterozygosity was 0.769, while the expected heterozygosity was 0.828. Most present was the alleles with size of 370 bp and with allelic frequency of 32,69%, and least present was the alleles with size of 386 bp and with allelic frequency of 7,69 %. The number of detected alleles with different size is 8.

The largest allelic frequency in the genome of the grey wolf for the locus FH3272 is 30.77% for the alleles with size of 220 bp, and the smallest allelic frequency of 3.85% showed the alleles with size of 232 bp. The observed heterozygosity was 0.769, while the expected

heterozygosity was 0.810.

On the locus FH3287 were detected 10 alleles with different sizes. The observed heterozygosity was 0.692, while the expected heterozygosity was 0.802. Most present was the alleles with size of 374 bp and with allelic frequency of 38,46%, and least present was the alleles with size of 378 bp and 458 bp and with allelic frequency of 1.93 %.

The largest allelic frequency in the genome of the grey wolf for the locus FH2361 is 28.85% for the alleles with size of 334 bp, and the smallest allelic frequency of 5.77% showed the alleles with size of 362 bp. The observed heterozygosity was 0.923, while the expected heterozygosity was 0.833. The number of detected alleles with different size is 7.

On the locus FH3924 were detected 6 alleles with different sizes. The observed heterozygosity was 0.538, while the expected heterozygosity was 0.690. Most present was the alleles with size of 354 bp and with allelic frequency of 50.00%, and least present was the alleles with size of 362 bp and 414 bp and with allelic frequency of 3.85 %.

The largest allelic frequency in the genome of the grey wolf for the locus FH3608 is 19.23% for the alleles with size of 400 bp, and the smallest allelic frequency of 5.77% showed the alleles with size of 360 bp and 360 bp. The observed heterozygosity was 0.731, while the expected heterozygosity was 0.873. The number of detected alleles with different size is 8.

The largest allelic frequency in the genome of the grey wolf for the locus FH3489 is 32.69% for the alleles with size of 264 bp, and the smallest allelic frequency of 5.77% showed the alleles with size of 268 bp. The observed heterozygosity was 0.615, while the expected heterozygosity was 0.753. The number of detected alleles with different size is 5.

On the locus FH4027 were detected 15 alleles with different sizes. The observed heterozygosity was 0.577, while the expected heterozygosity was 0.931. Most present was the alleles with size of 392 bp and with allelic frequency of 13.96%, and least present was the alleles with size of 428 bp and 460 bp and with allelic frequency of 1,92 %.

On the locus FH2141 were detected 11 alleles with different sizes. The observed heterozygosity was 0.808, while the expected heterozygosity was 0.912. Most present was the alleles with size of 392 bp and with allelic frequency of 13.96%, and least present was the alleles with size of 428 bp and 460 bp and with allelic frequency of 1,92 %.

CONCLUSIONS

1. The received values, as well as the values for each separate parameter PIC and the number of detected alleles in the genome of the analysed populations indicates that the DNA microsatelite locus which were applied in this study satisfy the criteria which need to meet the microsatelite markers for use in population studies in detection of the allelic frequency and the genetic diversity. The DNA microsatelite locus which were selected for the research of the Grey wolf are highly informative, which is confirmed through the number of detected alleles per locus and the values of the Polymorphic information content (PIC) per locus.
2. The size of the population as well as the number of researched DNA microsatelite markers conditions the number of the alleles which are characteristic for the genome of the analyzed population.
3. Based upon the values of the genetic distance, the genetic identity, the allelic frequency, the expected and observed heterozygosity, as well as the PIC of all researched locus, it can be concluded that the level of divergence of the Grey wolf can be quantified with analysis of DNA level, by using primers which are used in research of other species.
4. According to the received results from this study it can be concluded that there is a relatively low genetic variability in the researched parameters in the population of the Grey wolf.

REFERENCES

1. O'Brien S.J, 1993. Genetic maps. Locus maps of complex genomes. 6th edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory
2. Botstein D., White R., Skolnick M., Davis R. W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics32, 314-331.
3. Sunnucks P, 2000. Efficient genetic markers for population biology. vol. 15 155-203
4. Weber J.L, May P.E, 1989. Abudant class of human DNA polymorphism which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. 44: 388-396
5. Jarne P., Lagoda L., 1996. Microsatellites, from molecules to populations and back. Trend in Ecology and Evolution, 8, 285-288.
6. You-Chun L., Korol A., Fahima. T, Beiles A., Nevo E., 2002. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a Review. Molecular Ecology 11:2453-2465., 2002
7. Neff B. D., Gross M.R., 2001. Microsatellite evolution in vertebrates: Inference from a small number of individuals. Genetics 76: 379-390
8. Levinson G., Gutman G.A. 1987. Slipped-strand mispairing: A major mechanism for DNA sequence evolution. Mol. Biol. Evol. 4: 203-221.
9. Brinkmann B., Junge A., Meyer E., and Wiegand P., 1998. Population genetic
10. diversity in relation to microsatellite heterogeneity. Hum Mutat 11:135–144. 86
11. Epping H., Nadeau J. H., 1995. Comparative maps : the mammalian puzzle. Current Option in Genetics and Developments 5, 709-716.

МИКРОСАТЕЛИТСКА КАРАКТЕРИЗАЦИЈА НА ГЕНОМОТ КАЈ СИВИОТ ВОЛК ВО РЕПУБЛИКА МАКЕДОНИЈА

Есмеров Игор[1], Бранко Атанасов[2], Никола Адамов[3], Катерина Благоевска[1], Стојковски Велимир[1]

[1]Катедра за биохемиј и биологија на клетка, Универзитет „Св. Кирил и Методи- Скопје“
Факултет за Ветеринарна Медицина, Р. Македонија
[2]Катедра за Репродукција, Универзитет „Св. Кирил и Методи- Скопје“
Факултет за Ветеринарна Медицина, Р. Македонија
[3]Катедра за Сточарство, Универзитет „Св. Кирил и Методи- Скопје“
Факултет за Ветеринарна Медицина, Р. Македонија

АПСТРАКТ

DNA микросателитите претставуваа кодоминантен генетски маркер кој наоѓа широка примена во карактеризирањето на биодиверзитетот на одредени популации.

Генетската варијабилност на популацијата на сивиот волк беше одредена преку анализа на 10 DNA микросателитски локуси (FH2361, DGN10, FH3287, FH3924, FH3608, FH3023, FH3489, FH3721, FH4027 и FH2141).

Во студијата беа вклучени 26 примероци на сив волк, кои потекнуваа од планинските региони на западниот дел на Макединија. Информативноста на DNA микросателитските локуси беше одредена според (Полѕморхисм Информатив Цонтент-ПИЦ), како и според бројот на детектирани алели по локус. Сите десет DNA микросателитски локуси беа високо полиморфни.

Во геномот на шсивиот волк највисока вредност за PIC (Polymorphism Informativ Content) беше детерминирана кај локус FH4027 (0.907), а најниска за локусот FH3489 (0.692).

Бројот на детектирани алели по локус кај сивиот волк вариираше од 5 кај локусот FH3489, па до 15 кај локусот FH4027.

Интрапопулациската генетска варијабилност беше утврдена преку бројот на детектирани алели за секој DNA микросателитски локус, просечниот број на алели за сите десет DNA микросателитски маркери и вкупниот број на детектирани алели, карактеристични за даденатa популација.

Кај популацијата на сив волк бројот на заеднички алели беше највисок во локусот FH2361 и изнесуваше 26, од кои 24 хетерозиготи и 2 хомозиготи.

Според добиените резултати од оваа студија може да се заклучи дека постои релативно ниска генетска варијабилност во однос на испитуваните параметри кај популација на сив волк. Ова сознание, согласно сличните студии на поголем број автори имплицира дека сивиот волк е релативно чист вид, кој при размножувањето не е премногу вкрстуван со слични видови на него, како кучето и шакалот.

Клучни зборови: куче, волк, DNA микросателити, генетска варијабилност

UDC: 636.082.22:577.21

POTENTIALS OF MOLECULAR GENETICS FOR IMPROVEMENT OF LIVESTOCK PRODUCTION IN REPUBLIC OF MACEDONIA

Adamov Nikola[1], Pendovski Lazo[2], Esmerov Igor[3], Mickov Ljupco[4], Adamov Mihajlo[1]

[1]Department of Animal Breeding and Genetics, Faculty of Veterinary Medicine-Skopje, Ss. Cyril and Methodius University in Skopje; [2]Department of Functional Morphology, Faculty of Veterinary Medicine-Skopje, Ss. Cyril and Methodius University in Skopje;
[3]Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine-Skopje, Ss. Cyril and Methodius University in Skopje;
[4]Department of Reproduction, Faculty of Veterinary Medicine-Skopje, Ss. Cyril and Methodius University in Skopje
*Corresponding author: adamovn@fvm.ukim.edu.mk

ABSTRACT
Different applications of molecular genetics technologies in livestock improvement programs have been reviewed and their potential for accelerating genetic progress in domesticated animal species has been explained. Several biotechnology methods like sexing of pre-implantation embryos, diagnosis of freemartin heifers, identification of heterozygote disease carriers, parentage verification, QTL mapping and genomic selection have been discussed. The paper also includes the basic features of the genetic markers like microsatellites and single nucleotide polymorphisms (SNPs) and their application in animal genetic and genomic studies.
Key words: molecular genetics, marker assisted selection, QTL mapping, genomic selection.

INTRODUCTION

Molecular genetics is a study of the genotype of individuals at the DNA level. It deals with identification and mapping of the genetic polymorphisms that are present in a certain population and tries to explain their influence on the phenotype of those individuals. With this information available and if applied correctly, it is possible to select improved livestock on the basis of their genetic composition. The use of molecular information in livestock selection schemes has the potential to increase productivity, improve environmental adaptation and maintain genetic diversity. In order to achieve these goals the first step is to understand the genetic control of the trait of interest and its interaction (if any) with the environment.

Conventional livestock breeding programs are based on measuring phenotypic variation and animals with superior performance in the traits of interest are selected as parents of the next generation. Where the trait of interest is sex limited, such as dairy cattle milk production or udder composition, progeny testing allows the genetic value of the bull to be estimated. But there are several drawbacks associated with phenotypic selection: it narrows the genetic diversity of a population; it can only be applied to traits that are easily measured and is relatively expensive. For example in the case of progeny testing for milk production the costs are high because the tested sires have to be raised to certain age and then their daughters themselves raised, bred and calved before the trait can be measured and superior sires selected.

The use of molecular genetics in Marker Assisted Selection (MAS) programs offer a tool to select breeding animals at an early age (even embryos), to select for several traits and to increase reliability in estimating breeding values of the mature phenotypes. Some of the molecular genetics potentials are, but are not limited to:

i) Sexing of pre-implantation embryos,
ii) Identification of freemartin heifers,
iii) Identification of disease carriers,
iv) Parentage determination,
v) Quantitative Trait Loci (QTL) mapping,
vi) Marker assisted selection (MAS) and Genomic selection (MAS on a genome wide scale).

Sexing of pre-implantation embryos

Sexing of pre-implantation embryos can serve as a valuable tool for improving herd for specific production. By transferring only embryos of the desired sex a breeder can significantly reduce unnecessary expenses and time. So far, various methods of embryo sexing have been employed such as (i) non-invasive: immunological assay of HY antigen, quantification of X-linked enzyme and differential growth of male and female embryo and (ii) invasive: cytogenetic analysis, Y specific DNA probes and PCR amplification with Y specific primers.

Ideally the technique to be applied should not have any negative effect on embryo survivability, its implantation success and subsequent development. On the other hand the technique should be simple and easy to carry out, accurate, time-saving and not too expensive for commercial scale. Even though embryo gender can be determined quite accurately by cytogenetic analysis (51), this method is relatively invasive and requires a large piece of an embryo. Sexing of embryos using Y-chromosome specific probes is also accurate method but time consuming and tedious. The use of primers for PCR amplification of SRY specific sequence offers several advantages over other methods: it can be carried out in less than five hours with almost 100% accuracy (39), it is less invasive and requires small quantities of DNA which can be extracted from 2-8 cells biopsied from the embryo (45) and it can be done at an early blastocyst stage (6-8 days) at the 16-32 cells stage (34). Also these cells can be selected from the trophoblast that will make up the placenta, without even touching the inner cell mass. In a recent study (40) three widely used primer sets for bo-

vine embryo sex determination were compared and the results showed that BY/BSP couple primers were able to determine the sex of bovine embryos in all stages with accurate and easy to interpret results.

Identification of freemartin heifers

A freemartin is an infertile female heifer that is born with a male twin which demonstrates incomplete development of the genital tract. This phenotype occurs as a result of XX/XY chimerism which develops in utero through conjoined chorionic blood vessels between the heterosexual twin foetuses. Cytogenetic analysis of cultured lymphocytes has been traditionally used to identify XY cells in suspected freemartins. This test is quite accurate but it has its own drawbacks: if male cells are few in number it requires evaluation of hundreds of cells for a definitive diagnosis (14), it is labor intensive and requires extremely careful collection and handling of samples (15). On the other hand a PCR based technique described by Ennis and Gallagher (1994) detects the polymorphism associated with the bovine amelogenin gene. The AMX/Y allele residing on the Y chromosome contains a 63-bp deletion in the fifth exon when compared to the AMX/Y allele residing on the X chromosome. A single pair of primers is sufficient to amplify a 280-bp fragment from the X chromosome and a 217-bp fragment from the Y chromosome (17). A recent study (15) demonstrated increased sensitivity for the PCR based test with limit of detection between 0.2% to 1% male cells when compared to conventional cytogenetic evaluation which involves evaluation of no less than 100 cells. The use of isolated DNA instead of intact cells (lymphocytes) as a PCR template could improve the sensitivity of this test.

Identification of disease carriers

Many of the most important incurable diseases in cattle occur not as a result of infection with viruses or bacteria but as a result of defects in the genome of the host. The World Holstein Friesian Federation (WHFF) officially recognizes seven genetic disorders in the HF breed of cattle: BLAD, CVM, DUMPS, Syndactyly (mule foot), Factor XI deficiency, Citrullinaemia, and Brachyspina. While the animals affected with syndactyly and factor XI may live to reach puberty, all the other conditions are lethal in homozygous state and lead to embryonic mortality, stillborns or animals die shortly after birth. The heterozygote carriers of these genetic recessives can be easily detected by PCR-RFLP analysis.

Parentage determination and individual identification

Since the breeding value of an animal is estimated using the information from its relatives, the success of genetic gain of a certain population is closely dependent on correct pedigrees among animals. Parentage testing using molecular markers yields much higher exclusion probabilities (>95%) than testing with blood groups (70-90%) or other biochemical markers (40-60%). For example Stevanovic et al. (2010) demonstrated that by using 11 microsatellite markers wrong paternity can be excluded with more than 99% accuracy. The most widely accepted genetic markers were microsatellites mainly because of their high informative value which is provided by the large number of alleles per locus (5). In the past several years SNPs have gained high popularity in parentage testing. Some studies reported SNP marker panels for animal identification and parentage testing in American beef cattle (24) and European dairy breeds (48). But having into consideration that these markers are only bi-allelic, several papers (reviewed by Morin et al. 2004) predict that at least two to six times more SNP markers will be necessary to achieve the same resolution as microsatellites when used for animal identification and parentage assessment (37).

QTL mapping

The main goal of Quantitative Trait Loci (QTL) mapping in livestock is to identify or annotate, using a range of techniques, the genes that underlie complex phenotypes and diseases and to gain a better understanding of their physiology and pathophysiology. The general concept of QTL mapping in domestic animals is to search for correlation between neutral genetic markers (inherited DNA polymorphisms) and a quantitative phenotype or a trait in a structured pedigree (45). Two main approaches have been used to identify genes affecting traits of interest *(i) candidate gene approach* and *(ii) genome scan approach* based on linkage mapping with anonymous DNA markers (1, 21). The difficulty of using the first approach is that there must be an understanding of the biological mechanism controlling the trait before being able to select the potential candidate gene (31). An alternative is the genome scan approach which does not require any prior knowledge of the underlying mechanism controlling the trait. With this approach markers evenly spaced throughout the genome are selected and whole genome linkage mapping is first used in a segregating population to identify a locus with a large effect on the trait of interest. Although these two approaches are often viewed as alternative methods for identifying genes of interest, it is clear that they can be complementary, with a genome scan identifying chromosomal regions that harbor potential QTL, followed by further investigation of the genes known to be located in that region using the candidate gene approach (3). The ultimate goal is to identify the regions (QTLs) and the nucleotide sequences (QTNs) that influence the quantitative trait. The most powerful way to map QTL is to use experimental crossing of inbred strains or lines that are genetically different for the traits of interest (33). But in domestic animals inbred line crossing are practical only in the chicken. In dairy cattle, which have long generation times the use of artificial insemination creates large half-sib families which are useful for QTL mapping experiments. The most popular experimental design has been the granddaughter design (GDD) described by Weller et al. (1990). A single three-generation family of a GDD comprises a grandsire and a random sample of his sons (20 or more) each having 100 or more daughters. Marker genotyping and linkage analysis are performed for the grandsire and his sons and the phenotypic observations are made on the granddaughters (47).

The basic resources needed for QTL mapping are (i) correct pedigrees of the population under study (ii) precise records for the traits of interest and (iii) selection of genetic markers and statistical methods that are able to estimate the QTL's position and effect. Microsatellites, also called STRs (Short Tandem Repeats) or SSRs (Simple Sequence Repeats) have been the predominant marker type in genetic linkage maps until recently (4, 6, 27). They are short motifs of 1-6 nucleotides that are

tandemly repeated mostly in the non-coding regions of the prokaryotic and eukaryotic genomes. They are highly polymorphic with co-dominant inheritance, abundant and distributed throughout the genomes. These properties have made them one of the most widely used markers for gene mapping, parentage testing and population genetics studies. Microsatellites often exhibit tens of alleles per locus that differ from each other in the number of the repeated motifs. In addition, these markers also serve as a useful tool for animal identification, particularly for verification of the semen used for artificial insemination. Although they are widely spread throughout the genome, their evolutionary origin is still not clear and their biological role is unknown. The International Society of Animal Genetics (ISAG) is well advanced in the standardization of panels of microsatellite loci in each of the main livestock species.

In the past decade, a high number of a different kind of genetic markers called single nucleotide polymorphisms (SNPs) have been discovered alongside sequencing of genomes of various species including cattle. SNPs (pronounced as snips) are variations of single nucleotide at a defined position in the genome. A locus is viewed as polymorphic for these markers if it exists in two variants. They are highly abundant and are mostly located in no-coding regions with no direct impact on the phenotype of an individual. However, some introduce mutations in expressed regions and may induce changes in protein structure or regulation. In the past few years SNPs have gained popularity because of their distribution and mode of occurrence, relatively low mutation rate and their ability for low-cost high-throughput automated genotyping. With The Bovine Genome Project completed in 2008 a 7.1 X sequence assembly has been produced with accompanying information on over 2.300.000 SNPs genome wide (26). With the emerging new technologies such as microarray chips, tens or hundreds of thousands SNPs (Single Nucleotide Polymorphisms) can be genotyped for the same price as few hundred microsatellites and therefore such high-density SNP panels are becoming the standard genotyping system in many domestic animal species.

A review of recent publications shows that so far there are many published QTL mapping reports in dairy cattle (29, 50). However, despite the large number of QTL studies in cattle and other species, in only several cases have the casual mutations-QTNs been identified with strong supportive evidence: (i) the DGAT1 on BTA14 (19, 30, 49), GHR on BTA20 (7), ABCG2 (11, 38) and SPP1 gene (10, 42) on BTA6 that affect milk production and health traits in dairy cattle and the IGF2 (28) and GDF-8 (9) regulatory mutations that have been shown to influence muscle mass in pig and sheep, respectively.

Marker assisted selection (MAS) and genomic selection (GS)

The application of molecular genetic information has become an important issue in animal breeding. Traditionally selection of animals for breeding is based on two types of data: pedigrees and phenotypes. A third type of data that can facilitate breeding decisions are DNA markers. Breeding strategies for livestock that utilize molecular genetic information are broadly referred to as marker-assisted selection (MAS, reviewed by Dekkers & Hospital 2002, Dekkers 2004). Until recently, the use of molecular genetics in commercial applications of MAS has focused on the use of individual genes or QTLs linked to markers (12, 13). With the exception of a few genes with relatively large effects on a trait, most candidate genes or QTLs explain only a limited fraction of the total genetic variance (Vg) which means that important part of Vg needs to be selected for in the traditional way.

Depending on the relationship between the markers used and the traits of interest, Dekkers 2004 distinguishes three subcategories of MAS:

1. Gene assisted selection (GAS): utilizes the information of direct markers that are located within the gene of interest. This are the most favorable markers since, by following inheritance of the marker alleles, inheritance of the QTL alleles is followed directly.

2. LD-MAS: the markers used are in linkage disequilibrium (LD) with the QTL throughout the whole population. LD can be deliberately created in livestock population, for example in a F2 QTL mapping experiment LD is created between marker and QTL alleles by crossing two inbred lines (25, 32).

3. LE-MAS: the markers used are in population-wide linkage equilibrium (LE) with the QTL in outbred populations. QTL mapping exploiting LE markers has been performed with success in many livestock species for a great number of traits (2). The difficulty of mapping QTL exploiting LE markers is that the linkage between the markers and QTL is not sufficiently close to ensure that marker-QTL allele relationships persist across the population (as is the case with LD markers), and therefore marker-QTL phase within each family must be established in order to achieve increase in selection response (22). Farnir et al (2002) and Meuwissen et al. (2002) describe for the first time the benefits from the use of combined linkage disequilibrium and linkage (LDL) mapping.

A new form of MAS, called **Genomic Selection (GS)**, bypasses the need of locating QTL region and QTN identification. GS as described by Meuwissen et al. (2001) predicts total breeding values on the basis of a large number of marker haplotypes across the entire genome. This approach is based on the assumption that potentially total genetic variance (Vg) can be captured and explained when high-density SNP markers that are evenly spaced throughout the genome are analyzed because dense SNP genotyping ensures that the markers are in LD with the QTL that they bracket. Hence the marker density must be sufficiently high to ensure that all QTL are in LD with a marker or haplotype of several markers. Advantages of GS over traditional selection methods are: i) identification and validation of QTLs or markers in population LD with causative genes is not necessary; ii) the reliability of estimated breeding values (EBVs) is considerably higher for young test sires and sire dams; and iii) generation intervals may be reduced which would lead to increasing annual genetic gain. GS has become possible very recently with the availability of 10s of thousands of markers and development of high-through output genotyping technology. In the beginning the animals of a large reference population with phenotype information are genotyped for several thousand SNP markers, and their effect inferred from either haplotypes or individual marker alleles are estimated. The genomic estimated breeding value (GEBV) is then calculated by summing up the effects of these dense genetic markers or combinations of their allele haplotypes across

the entire genome. In the subsequent generations, only marker genotypes are needed to calculate GEBV (23). Simulation studies have shown that genomic selection can lead to high correlations between predicted and true breeding value over several generations without repeated phenotyping (20, 35). Calus et al. (2007) used simulated data sets with high and low heritability traits at different marker densities to estimate the accuracy of GEBV and concluded that genomic selection is considerably more accurate than traditional selection, especially for a low-heritability traits. Therefore genomic selection should result in reduced costs and increased genetic progress. In cattle, a microarray chip for simultaneous genotyping of more than 50.000 SNP markers has become commercially available from the beginning of 2008. With this approach genetic evaluation can be performed as soon as DNA is obtained, which allows accurate selection in both genders early in life (44). It is expected that by using genomic selection in dairy cattle breeding, the genetic progress would be doubled while the cost for proving AI bulls could be decreased by up to 90% (41).

POTENTIALS FOR REPUBLIC OF MACEDONIA

So far, in Republic of Macedonia there has been limited investment, either public or private, in animal biotechnology and only modest financial support for livestock molecular genetics research projects. This imposes a serious need for capacity building, at all levels, if these molecular genetic techniques are to be successfully applied to improve the management of livestock species for the benefit of farmers and consumers. There is a serious demand for strengthening competence in the areas of animal molecular genetics and biotechnology, but also for regulatory issues and policy analysis. The national livestock breeding programs should also make use of these technologies as much as possible because if applied correctly they offer a means for faster improvement of genetic gain. In other developing countries, few livestock breeding programs are capable of applying molecular information through marker-assisted and genomic selection as a means for improving farm animal genetic resources. This situation is unlikely to change without significant government support in universities and scientific groups that are able to conduct this kind of research and application. The competent authorities need to consider how to support, in best manner, the private-public sector collaboration in animal biotechnology research and development.

REFERENCES

1. Andersson L. (2001): Genetic dissection of phenotypic diversity in farm animals. Nature Rev. Genet. 2 :130-138.
2. Andersson L., and M. Georges ((2004): Domestic-animal genomics: deciphering the genetics of complex traits. Nat. Rev. Genet. 5 (3): 202-212.
3. Awad Ashraf F.S. (2011): Mapping of quantitative trait loci for milk yield traits on bovine chromosome 5 in the Fleckvieh cattle. Inaugural-disertation for the attainment of doctor degree in veterinary medicine. Faculty of veterinary medicine of the Ludwig-Maximilians-Universitat Munchen. Available at http://edoc.ub.uni-muenchen.de/12692/1/ Awad Ashraf Fathy Said.pdf
4. Barendse W., S.M. Armitage, L.M. Kossarek et al. (1994): A genetic linkage map of the bovine genome. Nat. Genet. 6: 227-235.
5. Bauming R., H. Simianer and I. Hoffmann (2004): Genetic diversity studies in farm animals-a survey. J. Anim. Breed. Genet 121: 361-373.
6. Bishop M.D., S.M. Kappes, J.W. Keele et al. (1994): A genetic linkage map for cattle. Genetics 136: 619-639.
7. Blott S., J.J. Kim, S. Moisio et al. (2003): Molecular dissection of a quantitative trait locus: a phenylalanine-to-tyrosine substitution in the transmembrane domain of the bovine growth hormone receptor is associated with a major effect on milk yeld and composition. Genetics 163: 253-266.
8. Calus M.P.L., T.H.E. Meuwissen, A.P.W. de Roos and R.F. Veerkamp (2007): Accuracy of genomic selection using different methods to define haplotypes. Genetics 178: 553-561.
9. Clop A., F. Marcq, H. Takaeda et al. (2006): A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat. Genet. 38: 813-818.
10. Cohen-Zinder M., E. Seroussi, M.R. Band et al. (2004): SPP1 is a candidate gene for the QTL affecting milk protein concentration on BTA6 in Isreali Holstein. 29th Int. Conf. Ani. Gen. ISAG, F015, Tokyo, Japan.
11. Cohen-Zinder M., E. Seroussi, D.M. Larkin et al. (2005): Identification of a missense mutation in the bovine ABCG2 gene with a major effect on the QTL on chromosome 6 affecting milk yeld and composition in Holstein cattle. Genome Research 15 (7): 936-944.
12. Dekkers J.C. (2004): Commercial application of marker- and gene- assisted selection in livestock: strategies and lessons. J. Anim. Sci. 82 E-Suppl: E313-328.
13. Dekkers J.C., & F. Hospital (2002): The use of molecular genetics in the improvement of agricultural populations. Nat. Rev. Genet. 3: 22-32.
14. Dunn H.O., Johnson R.H. (1981): Sample size for detection of Y-chromosomes in lymphocytes of possible free-martins. Cornell Vet. 71: 297-304.
15. Elizabeth A. McNiel, N.J. Madrill, A.E. Treeful, L.C. Buoen and A.F. Weber (2006): Comparison of cytogenetics and polymerase chain reaction based detection of the amelogenin gene polymorphism for the diagnosis of free-martinism in cattle. J. Vet. Diagn. Invest 18: 469-472.
16. Ennis S., Gallagher T.F. (1994): A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Anim. Genet. 25: 425-427.
17. Ennis S., Vaughan L., Gallagher T.F. (1999): The diagnosis of freemartinism in cattle using sex-specific DNA sequences. Res. Vet. Sci. 67: 110-112.
18. Farnir F., B. GrisartW. Coppieters et al. (2002): Simultaneous mining of linkage and linkage disequilibrium to fine map quantitative trait loci in outbred half-sib pedigrees: Revisiting the location of a quantitative trait locus with major effect on milk production on bovine chromosome 14. Genetics 161: 275-287.
19. Grisart B., W. Coppieters, F. Farnir et al. (2002): Positional candidate cloning of a QTL in dairy cattle: identification of a missense mutation in the bovine DGAT1 gene with major effect on milk yeld and composition. Genome Researh 12 (2): 222-231.
20. Habier D., R.L. Fernando, and J.C. Dekkers (2007): The impact of genetic relationship information on genome-assisted breeding values. Genetics 177: 2389-2397.
21. Haley C. (1999): Advances in quantitative trait locus mapping. From JL Lush to genomics: visions for animal breeding and genetics. Iowa State University Press, 47-59, Ames, IA, USA.
22. Hayes B. (2008): Course notes: QTL mapping, MAS and Genomic Selection.
23. Hayes B.J., P.J. Bowman, A.J. Chamberlain et al. (2009): Invited review: Genomic selection in dairy cattle: progress

and challenges. J. Dairy Sci. 92 (2): 433-443.
24. Heaton M.P., G.P. Harhay, G.L. Benett et al. (2002): Selection and use of SNP markers for animal identification and paternity analysis in US beef cattle. Mamm. Genome 13: 272-281.
25. Hedrick P.W. (2000): Genetics of population. 2nd edn. Joness and Bartlett. pp. 553.
26. http://www. hgsc.bcm.tmc.edu/projects/bovine
27. Ihara N., A. Takasuga, K. Mizoshita et al. (2004): A comprehensive genetic map of the cattle genome based on 3802 microsatellites. Genome Res. 14: 1987-1998.
28. Jungerius B.J., A.S. van Laere, M.F. Te Pas et al. (2004): The IGF2-intron3-G3072A substitution explains a major imprinted QTL effect on backfat thickness in a meishan x European white pig intercross. Genet. Res. 84: 95-101.
29. Khatkar M.S., P.C. Thomson, I. Tammen et al. (2004): Quantitative trait loci mapping in dairy cattle: Review and meta-analysis. Genet. Sel. Evol. 36: 163-190.
30. Kuhn C., G. Thaller, A. Winter et al. (2004): Evidence for multiple alleles at the DGAT1 locus better explains a quantitative trait locus with major effect on milk fat content in cattle. Genetics 167: 1873-1881.
31. Kwon J.M. & A.M. Goate (2000): The candidate gene approach. Alcohol research and health 24: 164-168.
32. Lander E.S. & N.J. Schork (1994): Genetic dissection of complex traits. Science 265: 2037-2048.
33. Lynch M. and B. Walsh (1998): Genetics and Analysis of Quantitative Traits. Sinauer Associates, Sunderland, MA.
34. Machaty Z., A. Paldi, T. Caski, Z. Varga, I. Kiss, Z. Barndi and G. Vajta (1993): J. Reprod. Fertil. 98: 467-470.
35. Meuwissen T.H.E., B.J. Hayes & M.E. Goddard (2001): Prediction of total genetic value using genome-wide dense marker maps. Genetics 157: 1819-1829.
36. Meuwissen T.H.E., A. Karlsen, S. Lien et al. (2002): Fine mapping of a quantitative trait locus for twinning rate using combined linkage and linkage disequilibrium mapping. Genetics 161: 373-379.
37. Morin P.A., G. Luikart, R.K. Wayne and the SNP workshop group (2004): SNPs in ecology, evolution and conservation. Trends Ecol. Evol. 19: 208-216.
38. Olsen H.G., H. Nilsen, B. Hayes et al. (2007): Genetic support for a quantitative trait nucleotide in the ABCG2 gene affecting milk composition of dairy cattle. BMC Genetics 8: 32.
39. Peura T., J.M. Hyttien, M. Turunen and J. Janne (1991): Theriogenology 35: 547-555.
40. Rattanasuk S., R. Parnpai and M. Ketudat-Cairns (2011): Multiplex polymerase chain reaction used for bovine embryo sex determination. J. Reprod. Dev. 5): 539-542.
41. Schaeffer L.R. (2006): Strategy for applying genome-wide selection in dairy cattle. J. Anim. Breed. Genet. 123 (4): 218-223.
42. Schnabel R.D., J.J. Kim, M.S. Ashwell et al. (2005): Fine-mapping milk production quantitative trait loci on on BTA6: analysis of the bovine osteopontin gene. PNAS, USA, 102 (19): 6896-6901.
43. Stevanovic J., Z. Stanimirovic, V. Dimitrijevic, M. Maletic (2010): Evaluation of 11 microsatellite loci for their use in paternity testing in Yugoslav Pied cattle (YU Simmental cattle). Szech J. Anim. Sci. 55 (6): 221-226.
44. Su G., B. Guldbrandtsen, V.R. Gregersen & M.S. Lund (2010): Preliminary investigation on reliability on genomic estimated breeding values in the Danish Holstein population. Journal of Dairy Science 93 (3): 1175-1183.
45. Thibier M., and M. Nibart (1995): Theriogenology 43: 71-80.
46. Viitala S. (2008): Identification of genes controlling milk production in dairy cattle. Doctoral dissertation. MTT Agrifood Research Finland.
47. Weller J.I., Y. Kashi, M. Soller (1990): Power of daughter and granddaughter designs for determining linkage between marker loci and quantitative trait loci in dairy cattle. Journal of Dairy Science 73: 2525-2537.
48. Werner F.A.O., G. Durstewitz, F.A. Hebermann et al. (2004): Detection and characterization of SNP useful for identity control and parentage testing in major European dairy breeds. Anim. Genet. 35: 44-49.
49. Winter A., W. Kramer, F.A. Werner et al. (2002): Association of a lysine-232-alanine polymorphism in a bovine gene encoding acyl-CoA: diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. PNAS, USA 99: 9300-9305.
50. www.animalgenome.org
51. Yadav B.R., W.A. King and K.J. Betteridge (1993): Mol. Reprod. Dev. 36: 434-439.

ПОТЕНЦИЈАЛИ НА МОЛЕКУЛАРНАТА ГЕНЕТИКА ЗА ПОДОБРУВАЊЕ НА ПРОИЗВОДСТВОТО НА ФАРМСКИТЕ ЖИВОТНИ ВО РЕПУБЛИКА МАКЕДОНИЈА

Адамов Никола[1] , Пендовски Лазо[2], Есмеров Игор[3],
Мицков Љупчо[4], Адамов Михајло[1]

*[1]Катедра за сточарство, Факултет за ветеринарна медицина-Скопје,
Универзитет Св. Кирил и Методиј во Скопје;*
*[2]Катедра за функционална морфологија, Факултет за ветеринарна медицина-Скопје,
Универзитет Св. Кирил и Методиј во Скопје;*
*[3]Катедра за биохемија и биологија на клетка, Факултет за ветеринарна медицина-Скопје,
Универзитет Св. Кирил и Методиј во Скопје;*
*[4]Катедра за репродукција, Факултет за ветеринарна медицина-Скопје,
Универзитет Св. Кирил и Методиј во Скопје*
**Автор за кореспонденција: adamovn@fvm.ukim.edu.mk*

АПСТРАКТ
Направен е преглед на неколку различни можности за примена на молекуларно-генетски методи во програмите за одгледување на фармските животни, а објаснет е и нивниот потенцијал за забрзување на генетскиот прогрес кај овие врсти на животни. Дискутирано е за неколку биотехнолошки методи како што се сексирање на пре-имплантациони ембриони, дијагностика на „фримартин“ јуници, идентификација на хетерозиготни носители на наследни заболувања, проверка на родителство, мапирање на локуси за квантитативни својства (QTLs) и геномска селекција. Во трудот се опишани и основните карактеристики на генетските маркери како што се микросателитските и “SNP” полиморфните региони како и нивната примена во студиите за анимална генетика и геномика.
Клучни зборови: молекуларна генетика, селекција со помош на генетски маркери, мапирање на локуси за квантитативни својства, геномска селекција.

UDC: 636.92.09:611.36.088.5

MAGNETIC RESONANCE IMAGING APPLICATION IN RABBIT LIVER ANATOMY. CORRELATION WITH CADAVER CROSS-SECTIONAL CUTS

Stamatova-Yovcheva Kamelia[1], Dimitrov Rosen[1], Chaprazov Zvetan[2], Russenov Anton[3], Yovchev David[1]

[1]*Department of Veterinary Anatomy, Histology and Embryology, Faculty of Vtereinary Medicine, Trakia University, Stara Zagora, Bulgaria*
[2]*Department of Veterinary Surgery, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria*
[3]*Department of Internal Non-infectious Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria*

ABSTRACT

The aim of the present study is to prove correspondence between magnetic resonance imaging anatomical features of the rabbit liver and these of the native cross sectional anatomy of the same organ, in order to apply this imaging technique in modern interpretation of the animal anatomy. We studied ten healthy New Zealand white rabbits, aged 9 months, weighed 2.8 to 3.2 kg. In the magnetic resonance imaging study the animals were positioned in supine recumbency. To obtain best results we used distance between scans's slices. A native topographic anatomical study was performed with five animals. There was proved correlation between magnetic resonance features of the rabbit liver with these of the native anatomical investigation of this organ.

The rabbit liver was homogeneous structure with intermediate intensity, compared to the adjacent soft tissues. There wasn't visible border between liver lobes. The organ was highly contrasted from stomach and its three parts (fundus, body and pyloric part). In the native anatomical cross-sectional frozen study the rabbit liver lobes were found. The organ was in close contact to the same structures, imaged on magnetic resonance. The comparison between the rabbit liver's imaging anatomical and native transversal study could be applied in the interpretation and diagnosis of many rabbit and human liver diseases.

Key words: rabbit liver, magnetic resonance, cadaver

INTRODUCTION

The New Zealand rabbts are suitable individuals to perform many experiments in laboratories conditions. That motivates Brewer (2006) in investigatation the anatomical, histological and physiological features of the organs in this animal species. According to this author the rabbit liver is composed of four lobes , which are subdivised in anterior and posterior lobules. The quadrate lobe is a sublobe of the right hepatic lobe. It is situated medially to the gall bladder and the caudare lobe is too small structure.

The rabbit liver reaches next to the last rib on the right, as caudate processus is situated from 9th to 12th intercostals space, in close contact with the last rib and touches the right kidney. The ventral edge of the right hepatic lobe crosses the costal arch in the ventral end of the 7th rib, at 2-3 cm from xyphoid processus. The left lateral hepatic lobe is extended to the dorsal end of the 10th rib. The gall bladder is in close contact to the median, at the ventral end of the 6th rib. The rabbit liver touches the gastric fund caudally, and caudate processus covers the duodenal cranial part, at the level of right 9th intercostals space (Barone, 1997).

According to Hristov et al. (2006) the rabbit liver is situated perpendicularly to the longitudinal axis of the body, caudally to the diaphragm and cranial dextral to the stomach. 2/3 of it is situated right to the mediamn plane, 1/3-left. The right hepatic lobe is most developed, as it is replaced ahead of the pylor. The quadrate lobe touches the ventral abdominal wall. In the angle between cardia and lesser curvature of stomach is situated papillar process.

The results of Meredith and Raimond (2000) show that the rabbit liver possesses highly developed right and left lobes. The quadrate lobe is poorly developed and it is situated medially to the gall bladder. The caudate lobe is in close contact to the lesser curvature of stomach.

The caudate rabbit liver lobe is with oval to ellipsoid shape, as its place of starting to the whole organ is poorly distinguished, which is connected to torsions and dislocations (Donnelly, 1997).

MR imaging is a safe, accurate technique for evaluating the hepatic anatomy without ionizing radiation. Different hepatic structures can be imagined without used iodinated contrast agents (Catalano et al., 2008).

According to Lee (2001) magnetic resonance imaging is a non invasive qualitative imaging method that determines increased tissue definition, compared to computed tomography and other techniques based on radiation. That is achieved by producing cross-sectional images, allowing best visualization of internal architecture of soft tissues. The soft tissues, determined by magnetic resonance have difference in the magnetic signal intensity.

Many authors (Champetier et al., 1987) suggest that the right lobe of the human liver is more accessible to magnetic resonance imaging study than the left because of its structure and its venous arrangements. It is a suitable techinique for studying the liver anatomy and differing normal from pathologic processings, related to hepatic veins.

With computed tomography and magnetic resonance the liver parenchyma is homogeneous. On T1 weighed magnetic images the liver possesses intermediate intensity, which is higher than that of the spleen. On T2 imag-

es the liver intensity is higher than the close muscles, but lower than that of the pancreas. On T1 weighed images the vessels appeared as low intensity structures, compared to T2 weighed magnetic scans (Lee et al, 2006).

Zotti et al. (2009) perform comparative imaging study of the rabbit internal organs and prove correspondence between the results, imaged on CT scans with these of cadaver frozen cross-sectional cuts.

MATERIALS AND METHODS

The liver of ten sexually mature, clinically healthy New Zealand white rabbits, aged 9 months and weighed 2.8 to 3.5 kg were investigated. The animals were anesthetized with 15 mg/kg Zoletil® 50 (tiletamine hydrochloride 125 mg and zolazepam hydrochloride 125 mg in 5 ml of the solution) Virbac, France. They were positioned in supine recumbency. A magnetic resonance Siemens Magnetom Essenza was used. The device was the following parameters: magnet weight was 3.5 tone, strength of magnetic field 1.5 T, total imaging matrix (TIM) was with 28 coil elements and 5 RF channels. Magnetic cylinder was 70 cm, table mobility, control system length-145 cm, scanning diapason-up 140 cm. The magnetic scanner reconstructed 1131 images per second with matrix 256/256. The device worked with Intel Xeon Host Computer with 2.6 GHz frequency and 4 GB memory. The iPat was 8 PAT. The slices' thickness was from 3 to 4 mm, FOV 244*244, TE-93 and TR 1410. We worked in high resolution. For one series of study the window (W) varied from 745 to 1360. The center was in diapason between 344 and 611.

Four of the studied animals were euthanized with 150 mg Thiopental® (50 mg/kg, I V) (Thiopental sodium 1000 mg) Biochemie, Austria iv (Posner and Burns, 2009). They were frozen frozen at – 18 C. Transverse cross sectional cuts with 5 mm thickness were obtained from cranial abdominal region in order to prove correspondence between magnetic images findings and native topographic characters of the rabbit liver.

The manipulation was in accordance with the requirements of the American Veterinary Association for euthanasia.

RESULTS

By performed magnetic resonance imaging study it was found that rabbit liver was a homogeneous structure with intermediate signal intensity. There wasn't observed visible border between different hepatic lobes. We distinguished only the right and left halves of the organ. The right hepatic lobe was a single structure, while the left was subdivided in left and right part. As orientation for quadrate lobe, the gall bladder was used, because the last covered this part of the liver structure. As bone orientation we used 10th thoracic vertebra was marked dorsally, laterally – the costal arch and ventrally – the abdominal wall (Fig. 1).

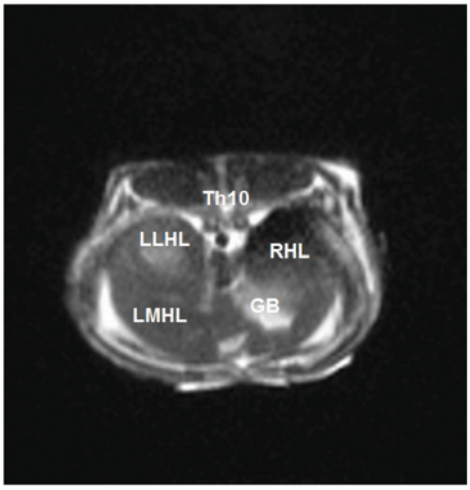


Figure 1. *Transversal magneting resonance image of the rabbit liver: right hepatic lobe (RHL), gall bladder (GB), left medial hepatic lobe (LMHL), left lateral hepatic lobe (LLHL), 10th thoracic vertebra (Th10).*

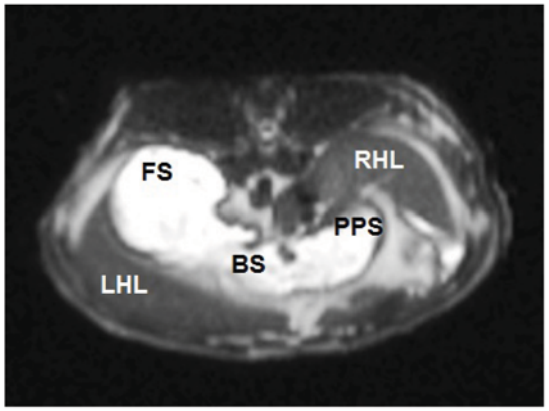


Figure 2. *Transversal magnetic resonance image of rabbit cranial abdominal region: right hepatic lobe (RHL), left hepatic lobe (LHL), stomach fundus (FS), stomach body (BS), pyloric part of the stomach (PPS).*

The left hepatic lobe was distinguishable with intermediate signal intensity toward the stomach fundus and body. The right hepatic lobe was in close contact with pyloric part of the stomach and the beginning of the small intestines (Fig. 2).

On post mortal cross-sectional cuts, the different parts of rabbit liver lobes were observed, because the presence of fine deep clefts between them, distinguished on the native study. As bone marker was used the 10th thoracic vertebra. The gall bladder was distinguished with its different parts and was a marker for quadrate lobe position (Fig. 3).

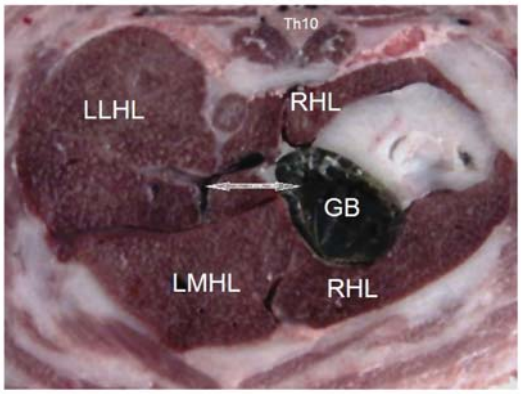


Figure 3. *Transversal cross-sectional frozen cut of the rabbit liver: left lateral hepatic lobe (LLHL), left medial hepatic lobe (LMHL), gall bladder (GB and arrow), right hepatic lobe (RHL), 10th thoracic vertebra (Th10).*

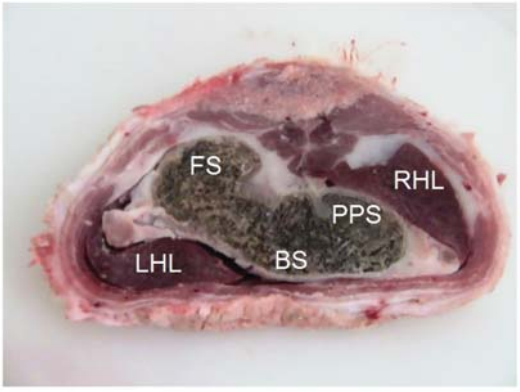


Figure 4. *Transversal cross-sectional frozen cuts of the rabbit cranial abdominal region: left hepatic lobe (LHL), right hepatic lobe (RHL), fundus of stomach (FS), stomach body (BS), pyloric part of the stomach (PPS).*

On transversal cross-sectional frozen cuts of the cranial abdominal region the was close contact between rabbit liver and different parts of the stomach was found. The right hepatic lobe was close to the stomach pylor part and the left one – close to the stomach fundus and body (Fig. 4).

CONCLUSION

The correspondence of findings, concerned the magnetic resonance imaging anatomy of the rabbit liver with topogaphrical parameters of this organ on native frozen cross-sectional cuts, proved that the magneting imaging resonance is suitable technique in the interpretation of small animals anatomy. The data could be used as base for diagnosis many rabbit and other small animals and human liver diseases.

REFERENCES

1. Breweer, N., Historical special topic overview on rabbit comparative biology. *Journal of the American Association for Laboratory Animal Science*, **45**, 8-24, 2006.
2. Barone, R., Chapitre VII - Foie. In: Anatomie comparée des mammifères domestiques. Splanchnologie I. Tome troasième, Troisième edition, Editions Vigot, Paris, pp. 507-560, 1997.
3. Hristov, H., Kostov, D., Vladova, D., Topographical anatomy of some abdominal organs in rabbits. *Trakia Journal of Sciences*, **4**, 7–10, 2006.
4. Meredith, A., Rayment, L., Liver Disease in Rabbits. *Seminars in Avian and Exotic Pet Medicine*, **9**, 146-152. 2000.
5. Donnelly, T., Basic Anatomy, Physiology, and Husbandry. In: E. Hillyer and K. Quesenberry, editors. Ferrets, Rabbits, and Rodents. Philadelphia: WB Saunders; pp 147-159. 1997.
6. Catalano, O. A., Singh, A.H., Uppot, R. N. Hahn, P.F., Ferrone, C. R., Sahani, D.V, Vascular and biliary variants in the liver: implications for liver surgery. *Radiographics*, **28**, 359-378, 2008.
7. Lee, R., Introduction. In: Manual of Small Animal Diagnostic Imaging. Second edition, British Small Animal Veterinary Association, England, pp. 15-30, 2001.
8. Champetier, J., Le Bas, J. F., Yver, R., Létoublon, C., Hou, K. Y, Magnetic resonance imaging of the liver by frontal (coronal) sections. *Surg Radiol Anat*,. 9, 107-121, 1987.
9. Lee, J., Sagel, S., Stanley, R., Heiken, J., Chapter 13- The Liver. In: Computed Body Tomography with MRI correlation. First Volume, Fourth Edition, Lippincott Williams & Wilkins, Philadelphia, USA, pp. 934-967, 2006.
10. Zotti, A., Banzato, T., Cozzi, B., Cross-sectional anatomy of rabbit neck and trunk: Comparison of computed tomography and cadaver anatomy. *Research in Veterinary Science*, 87: 171-176, 2009.

ПРИМЕНА НА МАГНЕТНАТА РЕЗОНАНЦА ЗА ПРИКАЗ НА АНАТОМИЈАТА НА ЦРН ДРОБ ОД ЗАЈАК. КОРЕЛАЦИЈА СО ТРАНСВЕРЗАЛНИ РЕЗОВИ ОД КАДАВЕР

Стаматова Јовчева Камелија[1], Димитров Росен[1], Чапразов Цветан[2], Русенов Антон[3], Јовчев Давид[1]

[1]Катедра за Ветеринарна Натомија, Хистологија и Ембриологија, Факултет за Ветеринарна Медицина, Тракиски Универзитет Стара Загора, Бугарија
[2]Катедра за Ветеринарна Хирургија, Факултет за Ветеринарна Медицина, Тракиски Универзитет Стара Загора, Бугарија
[3]Катедра за Внатрешни Неинфективни Болести, Факултет за Ветеринарна Медицина, Тракиски Универзитет Стара Загора, Бугарија

АПСТРАКТ
Целта на оваа студија е да се докаже врската на анатомските карактеристики на зајачки црн дроб при користење на магнетна резонанца и нативниот анатомски пресек на истиот орган, за примена во модерната интерпретација во анималната анатомија. Проучувавме десет здрави Ново Зеланндски бели зајаци, на возрасто од 9 месеци, со тежина од 2,8-3,5 кг. Живптните на магнетна резонанца беа во легната положба. За да добиеме најдобри резултати оставивме простор помеѓу слајдовите. Нативната топографско анатомска студија беше направена врз пет животни. Беше докажана корелацијата помеѓу резултатите од магнетната резонанца на зајачкиот црн дроб и нативното истражување на истиот орган.
Зајачкиот црн дроб беше со хомогена структура и среден интензитет во споредба со околните меки ткива. Немаше видливи граници помеѓу црнодробните лобуси. Органот имаше повисок контраст во неговите три дела (фундусен, тело и пилоричен дел). При нативната анатомска секција на смрзнат зајачки црн дроб лобусите може да се видат. Органот беше во контакт со истите структури како и на магнетната резонанца. Компарација на овие две методи може да се искористи при дијагноза на многу болести на црниот дроб кај луѓето и зајаците.
Клучни зборови: зајачки црн дроб, магнетна резонанца, мртов

UDC: 636.4.09:611.366.018.26

DISTRIBUTION OF MAST CELLS IN THE PORCINE GALL BLADDER

Stefanov Ivaylo[1], Vodenicharov Angel[1]

[1]*Department of Veterinary Anatomy, Histology and Embryology, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria.*

ABSTRACT

Mast cells are concentrated within the mucosa of several organs such as the respiratory and gastrointestinal tract. By virtue of the release of the large number of preformed and newly formed biologic active substances, mast cells have a great potential to influence mucosal functions such as ion transport of mucosal epithelium. It is also well known the role of these cells in the regulation of gastric mucosal blood flow. The data concerning the examination of mast cells in gall bladder of animals are scarce. There is no data in the literature about the distribution of mast cells in normal pig` s gall bladder, which motivated us to undertake the present study in order to determine the localization and density of mast cells in the wall of porcine gall bladder fundus, body and neck, and in the *ductus cysticus*, as well.

The material was obtained from the wall of gall bladder of 6 castrated male and 6 female pigs, aged 6 months, slaughtered for meat consumption in a slaughterhouse. The samples were fixed in Carnoy's liquid at room temperature for 4 h and further they were processed for serial paraffin sections that were stained with 0.1% solution of toluidine blue in McIlvane's buffer, pH 3 for assessment of metachromasia of the mast cells.

The mast cells distribution was established in the propria, muscular and serosal (or adventitial) layers of gall bladder, and its excretory duct. They were localized predominantly in the vicinity of blood vessels. Some of them were observed near the epithelium of gall bladder and around the glands of gall bladder neck.

The distribution of mast cells in normal porcine gall bladder has been described for the first time. Based on our results, we could assume that mast cells are involved not only in the maintenance of local homeostasis but in the regulation of the gall bladder function, as whole.

Key words: mast cells, gall bladder, swine

INTRODUCTION

Mast cells (MCs) originate from haematopoietic stem cells in the bone marrow and are released to the circulation as immature cells. The MCs mature in different tissues under the influence of local growth factors and are, unlike basophils, usually not found in the circulation after maturation. MCs are found throughout the body, primarily in tissues that come in contact with the outside of the body for example mucus membranes in the digestive system and the respiratory tract. MCs are important cells in various inflammatory conditions by releasing the contents of their secretory granules which promotes inflammation and in combating infections caused by extracellular parasites (Hallgren *et al* 2006). Mast cells contain a variety of biologically active compounds and mediators like proteoglycans, arachidonic acid derivates and different neutral proteases like carboxypeptidase, cathepsin G, tryptase and chymase (Schwartz, 1994). The latter may be specific for mast cells, since tryptase is found only in basophils, in very small amounts, and chymase is exclusively produced by mast cells (Hallgren *et al* 2006). Furthermore, mast cells are able to synthesize a multitude of cytokines and growth factors, suggesting the involvement of these cells in various biological processes (Paul et al., 1993).

The distribution of mast cells mainly in guinea-pig and human gall bladder was established by several authors (Jennings et al., 1995; Friesen et al., 2011).

There is no data in the literature about the distribution of mast cells in normal porcine gall bladder. This motivated the present study that aimed to determine the localization and density of mast cells in the wall of porcine gall bladder and in the *ductus cysticus*, as well.

MATERIALS AND METHODS

1. *Animals*
   The material was obtained from the wall of gall bladder of 6 castrated male and 6 female pigs (Landras X Bulgarian White), aged 6 months, slaughtered for meat consumption in a slaughterhouse.
2. *Histochemical detection of metachromasia in mast cells*
   The samples were fixed in Carnoy's liquid at room temperature for 4 h and further they were processed for serial paraffin sections (5–6 μm) that were stained with 0.1% solution of toluidine blue in McIlvane's buffer, pH 3 for assessment of metachromasia of the mast cells (Pearce, 1960).
3. *Statistics*
   Using cross sections of the wall of the gallbladder fundus, body and neck, and of the wall of *ductus cysticus,* as well the number of mast cells per mm^2 was estimated.

Data for number are given as mean ± SD. For that purpose it was used a light microscope (ZEISS Primo Star, Germany), camera (Progres, Capture 2.6 - JENOPTIK) and software analysis programme (Soft Imaging Sistem GmbH). Statistical data processing was done using Data Analysis tool and Student`s t-test by means of the StatMost for Windows software. A p value < 0.05 was considered significant.

RESULTS AND DISCUSSION

In this study the mast cells localization and density in the propria, muscular and serosal (or adventitial) layers of porcine gall bladder, and its excretory duct were established for the first time. Similar investigation was

performed by Jennings et al. (1995) who established the distribution of histamine-containing mast cells in the mucosa and muscularis/serosa layers of guinea pig gall bladder. In our study it was established that mast cells were localized predominantly in the vicinity of blood vessels: arteries, veins, arterioles, venules and capillaries. Most mast cells were observed in the adventitial layer of blood vessels but few of them were situated in tunica media. Our findings about the localization of mast cells near the blood vessels confirm the data reported by May et al. (2000) and give us a reason to suggest that observed mast cells take part in regulation of vascular tonus in studied organ.

In the propria, mast cells also were detected near the epithelium of gall bladder. Intraepithelial localization of single mast cells and their location in the lumen of porcine gall bladder, as well was observed for the first time. These results correspond with the findings of Toledo et al. (1981) who established the existence of mast cells in an epithelial location in the gallbladders of both cattle and sheep. The authors performed optical- and electron microscopic observations which demonstrated that, in both species, mast cells from the connective tissue of the gallbladder diapedese across the basal lamina and migrate through the epithelium all the way to the luminal surface, and that a degranulation process takes place during this migration. The probable function of these cells in transporting and liberating glycosaminoglycans into the bile is discussed.

Because of presence of glands in gall bladder neck mast cells were localized around them (Fig. 1).

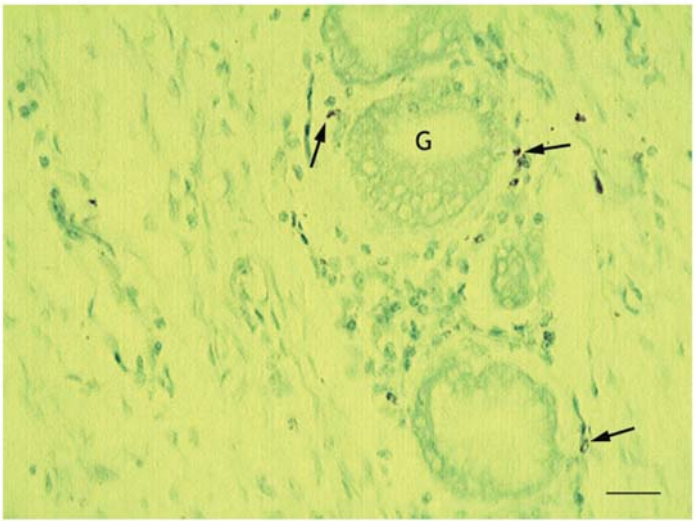


Figure 1. *Mast cells (arrows) near the glands (G) of gall bladder neck. Bar=20μm.*

We assume that the localization of mast cells betwen the glands may be related to the role of these cells for normal functioning of the glands on one side and for pathological changes in them on the other side. To confirm this hypothesis further investigation need to be performed.

In the muscle layer of gall bladder mast cells were located between and into the smooth muscle cells bundles (Fig. 2).

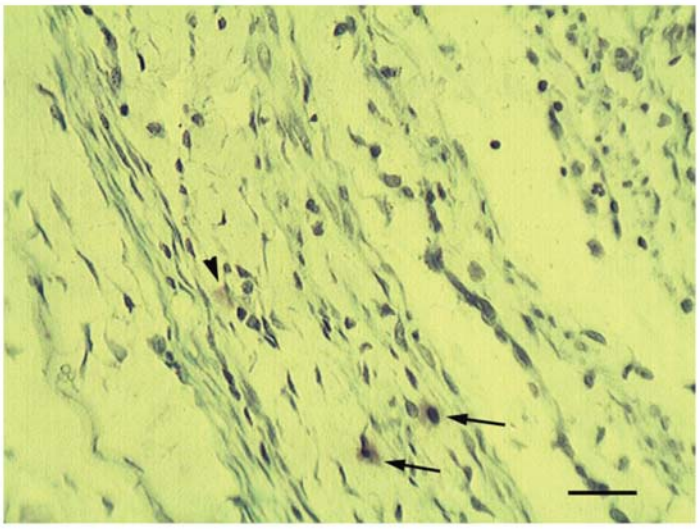


Figure 2. *Some of the mast cells were located next to the smooth muscle cells (arrows) and other ones (arroheads) near the blood vessels in the muscle layer of gall bladder. Bar=20μm.*

These findings may be explained with the results of some authors who established that histemine elicits its effects on guinea pig gallbladder motility through direct actions on gallbladder smooth muscle (Hemming et al., 2000). According to these authors histamine released from mast cells produces a depolarization of gallbladder smooth muscle, indicating that mast cell degranulation could contribute to pathophysiological changes in gallbladder tone that are associated with cholecystitis.

The results about the density of toluidine blue stained mast cells in different layers of the gall bladder fundus, body, neck and its excretory duct in both genders are presented in Table 1.

Comparing density of mast cells in both genders, it was found out that there was no sexual dimorphism (Table 1). The highest mast cell density per 1 mm^2 in both genders was observed in the propria. In this layer of gall bladder, MC were significantly more abundant compared to MC in the muscle layer in both males and females (P<0.001, Student`s t – test). The results of this investigation showed that the distribution of mast cells in porcine gall bladder wall is similar to that in the intestinal wall (Xu et al., 1993). In the gall bladder body and neck, and in the *ductus cysticys*, as well the number of mast cells was higher in the muscle layer than in the subserosal layer. However, in the gall bladder fundus there was no statistically significant difference between mast cells number in the muscle layer and that in the subserosal layer (Table 1).

The mean values of mast cells density in the entire wall of different gall bladder parts was higher in its body (32.43±10.56) and neck (33.9±12.17) than in the fundus (28.66±10.42) and ductus cysticus (30.03±9.71) with statistical significance p< 0.01.

Table 1. *Mast cells in different layers of the gall bladder fundus, body, neck and its excretory duct (ductus cysticus) in both genders.*

MCs localization	MCs number/mm^2 male pigs	MCs number/mm^2 female pigs
Gall bladder fundus	*42.20±5.84*	*42.21±5.89*
-Lamina propria mucosae		
Gall bladder fundus	*21.8±2.33****	*21.9±2.42****
- Tunica muscularis		
Gall bladder fundus	*22.0 ±3.25*	*22.1±3.38*
-Tela subserosa		
Gall bladder body	*43.20±4.8*	*43.25±5.01*
- Lamina propria mucosae		
Gall bladder body	*31.80±9.17****	*32.10±9.00****
- Tunica muscularis		
Gall bladder body	*22.30±2.95****	*22.45±2.93****
- Tela subserosa		
Gall bladder neck	*47.0±3.43*	*46.75±3.55*
- Lamina propria mucosae		
Gall bladder neck	*32.70±10.54****	*32.5±110.57****
- Tunica muscularis		
Gall bladder neck	*22.0±2.51****	*22.03±2.57****
- Tela subserosa		
Ductus cysticus	*39.8±6.32*	*39.75±6.34*
- Lamina propria mucosae		
Ductus cysticus	*29.2±6.62****	*30.01±6.69****
- Tunica muscularis		
Ductus cysticus	*21.10±4.86****	*21.21±4.89****
- Tela subserosa		

*** *p< 0.001 – statistically significant difference vs mast cells in previous layer (Student`s t- test).*

REFERENCES
1. Schwartz, L.: Mast cells: function and contents. Curr. Opin. Immunol., 1994, 6, 91-7.
2. Hallgren, J., Pejler, G.: Biology of mast cell tryptase, an inflammatory mediator. Review article. FEBS Journal,2006, 273, 1871-1895.
3. Paul, W., Seder, R., Plaut, M.: Lymphokine and cytokine production by FcsRI+ cells. Adv. Immunol., 1993, 53, l-29.
4. Jennings, L., Salido, G., Pozo, M., Davison, J., Sharkey, K., Lea, R., Singh, J.: The source and action of histamine in the isolated guinea-pig gallbladder. Inflamm. Res., 1995, 44, 447–453.
5. Friesen, G, Neilan, N, Daniel, J., Radford, K, Schurman, J., Li, D., Andre, L., Peter, S., Holcomb, G.: Mast cell activation and clinical outcome inpediatric cholelithiasis and biliary dyskinesia. BMC Research Notes, 2011, 4, 322-329.
6. Pearce, A.: Histochemistry Theoretical and Appllied, 2nd ed., J. & A. Churchill, Ltd., London, 1960; 472-475, 692, 888- 889.
7. May, A., Fusbs, M., Scheib, M., Lutjen-Drecoll, E.: Characterization of nitrergic neurons in the porcine and human ciliary nerves. *Inv. Ophthalmol. Vis. Sci.*, 2002, **43**, 581-86.
8. Toledo, O., Morales, C., Pereyra, L., Jordão T., Montes G.: Migrating mast cells in the gallbladder epithelium of cattle and sheep. Histochem. Cell. Biol., 1981, 72(3), 433-442.
9. Hemming, J., Guarraci, F., Fith, T., Jennings, L., Nelson, M., Mawe. G.: Actions of histamine on muscle and ganglia of the guinea pig gallbladder. *Am. J. Physiol. Gastrointest. Liver Physiol.,* 2000, **279**, 622–630.
10. Xu, L., Car, M., Bland, A., Hall, G.: Histochemistry and morphology of porcine mast cells. *Histochem. J.*, 1993, **25**, 516–522.

ДИСТРИБУЦИЈА НА МАСТОЦИТИ ВО ЖОЛЧНО ЌЕСЕ КАЈ СВИЊИ

Стефанов Ивајло[1] , Воденичаров Ангел[1]

[1]Катедра за ветеринарна анатомија, хистологија и ембриологија,
Факултет за ветеринарна медицина, Тракиски Универзитет, Стара Загора, Бугарија

АПСТРАКТ
Мастоцитите се сместени во лигавицата на некои органи, како што се органите од респираторниот и гастроинтестиналниот систем. Врз основа на ослободувањето на голем број претходно формирани и новоформирани биолошки активни супстанции, мастоцитите имаат голема моќ да влијаат врз функциите на лигавицата, како што е јонскиот транспорт низ мукозниот епител. Исто така, добро е позната и улогата на овие клетки во регулирање на крвниот проток низ желудечната лигавица. Податоците во врска со испитувањето на мастоцитите во жолчното ќесе кај животните се доста оскудни. Во литературата не постојат податоци за дистрибуцијата на мастоцитите во жолчното ќесе кај свињите, што беше наш мотив за ова испитување, а со цел да се утврди местоположбата и густината на мастоцитите во ѕидот на фундусот и вратот на жолчното ќесе кај свињите, како и во неговиот канал.
Материјалот е земен од кланица и тоа од ѕидот на жолчното ќесе од 6 кастрирани машки и 6 женски свињи, со старост од 6 месеци. Примероците беа фиксирани во Carnoy-ев фиксатив на собна температура, во времетраење од 4 часа, а потоа беа изготвени сериски парафински пресеци и обоени со 0,1% раствор на толуидинско сино во McIlvane-ски пуфер со pH 3, за оценување на метахромазијата на мастоцитите.
Дистрибуцијата на мастоцитите беше евидентирана во пропријата, мускулните и серозните (или адвентициски) слоеви на жолчното ќесе и неговиот изводен канал. Мастоцитите беа лоцирани претежно во близина на крвните садови. Некои од нив беа забележани во близина на епителот и околу жлездите во вратот на жолчното ќесе.
За прв пат е опишана дистрибуцијата на мастоцитите во жолчното ќесе кај свињи. Врз основа на нашите резултати, би можеле да претпоставиме дека мастоцитите се вклучени не само во одржување на локалнатата хомеостазата, туку и во регулирање на функцијата на жолчното ќесе.
Клучни зборови: мастоцити, жолчно ќесе, свиња

UDC: 636.4.09:611.146.2

RENAL VEIN BRANCH PATTERNS IN PIG KIDNEYS

Pendovski Lazo[1], Ilieski Vlatko[1], Petkov Vladimir[1],
Popovska-Percinic Florina[1], Tososka - Lazarova Dobrila[2]

[1]*Department of Functional Morphology, Faculty of Veterinary Medicine*
University Ss. Cyril and Methodius in Skopje,
[2]*Institute for Anatomy, Medical Faculty University of Ss. Cyril and Methodius in Skopje*

ABSTRACT

The aim of this paper is to present the renal vein branching patterns in pig kidneys and to investigate if there are differences in venous branching patterns between kidneys obtained from two different pig breeds.

The material was encompasses 69 kidneys taken from two hybrid pig breeds (*31 kidneys from landars /yorkshire and 38 kidneys from dalland*). The anatomy of renal venous vessels was studied on three-dimensional silicone S-10 corrosion casts prepared together with the arteries and kidney collecting system.

In all investigated kidneys we found one renal vein. In 80.65% in a landras/yorkshire breed and in 78.94% in a dalland breed the renal vein was formed by two primary branches ($p>0.05$), while in the rest of 19,35% (landras /yorkshire) and 21.6% (dalland) of cases an additional intermedial branch was found ($p>0.05$).

According the results, the pig kidneys don't have a segmental venous structure which is necessary condition for segmental resection of kidneys during vascular partial nefroctomy.

Key words: pig kidneys, renal veins, S-10 corrosion casts

INTRODUCTION

The intrarenal anatomy of the blood vessels and its relationship with the collecting system in pig kidneys is the key for practicing the vascular reconstruction in urinary medicine, especially for the clinical xenotransplantation(1-4)

The segmental arterial structure and collecting system on the pig kidneys is well studied (6-9). In the past years there are also some investigations for the venous blood vessels in pig kidneys (5, 10) but the knowledge for their ramification is still scars due to the lack of anatomical details for the distribution of primary renal vein branches.

Therefore, the aim of this paper is to present the renal vein branching pattern in pig kidneys based on the renal vein primary branches and to investigate if there are differences in venous branching patterns between kidneys taken from two different pig breeds.

MATERIAL AND METHODS

The material was encompasses non-fixed 69 kidneys taken from two hybrid pig breeds (*31 kidneys from landars/yorkshire and 38 kidneys from dalland*) with an average weight of 95 kg (mean) slaughtered in arbitrary at age of 5.5 months. The kidneys were dissected together with the large blood vessels (aorta and caudal vena cava) in order to preserve the urinary–vessel loop intact. The intrarenal anatomy of the venal branches was analyzed on the 3-dimensional corrosive casts prepared together with branches of renal artery and kidney collecting system according the method described previously(8, 9).

Briefly, the corrosive casts were prepared by injecting a mixture of silicon S10/S3 (100:1) with additional S6-hardener in ratio 0.5% of the injected mass. Before the silicone mixture injection, the collecting system and blood vessels were prewashed with 50ml saline solution added with heparin (250 i.e / NaCl) to prevent clotting of residual blood in blood vessels (9) and washed out with running tap cold water for 2 hours. Thereafter in the main trunks of renal artery and renal vein, by using a syringe and continuous gentle pressure, we applied 5-8ml silicone mass added with a red pigment (BIODUR Paste Red AC50) for arteries and blue pigment (BIODUR Paste Blue AC52) for the veins. The kidney collecting system was injected through the ureter with 15ml colorless silicon. For better visualization of pelvicaliceal system, a silicon mixture with yellow pigment (BIODUR Paste AC53) was injected in several kidneys.

The corrosion was made with commercial concentrated HCl until complete decomposition of organic matter was archived leaving the three-dimensional casts of the internal systems that were injected.

RESULTS

In all examined kidney corrosion casts we found one renal vein (v.renalis) per kidney that was a lateral branch of caudal vena cava. The renal venous blood vessels were distributed along arterial segmental branches, generally following their ramification.

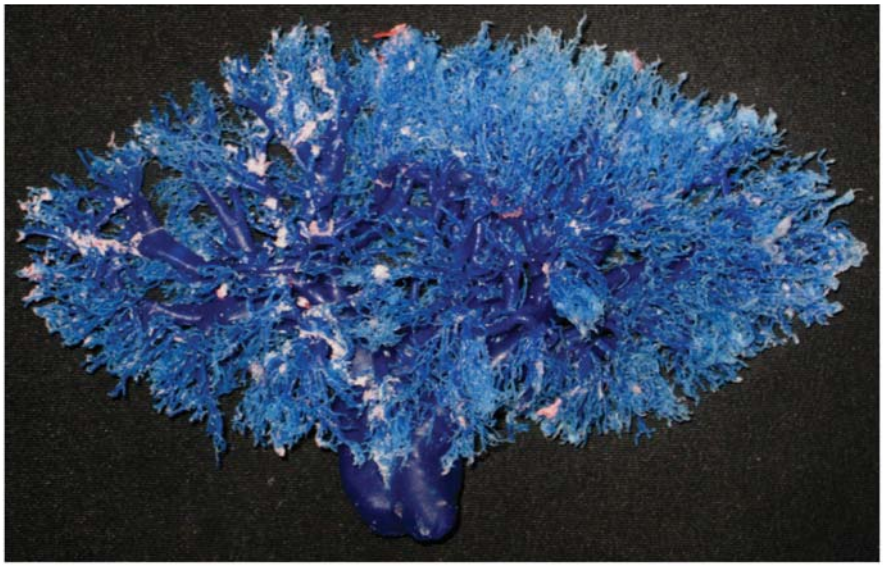


Figure 1. *Corrosive cast from the venous capillary system*

The renal vein was emerging from the renal hilus of kidneys by amalgamation of several venous trunks. According to the number of venous branches we found two different types of venous systems, classified as type I and type II (Table 1).

Table 1. *The results for the proportional distribution of different types of renal venous systems for the both pig breeds.*

Types of venous systems	**landras/yorkshire** (%) number/total number	**dalland** (%) number/total number	**p-level**
type I	80.65% (25/31)	78.94% (30/38)	0.8401
type II	19.35% (6/31)	21.06% (8/38)	0.8373

In the renal venous pattern ***type I***, the renal vein was formed by two branches, one cranial branch (ramus cranialis) and one caudally branch (ramus caudalis) from the cranial and caudal pole of kidney respectively. On the uretero-pevinel junction the both branches were united forming the renal vein. (Figure 2)

This venous pattern in our research was founded in 80.65% in a landras / yorkshire breed and in 78.94% of dalland breed.

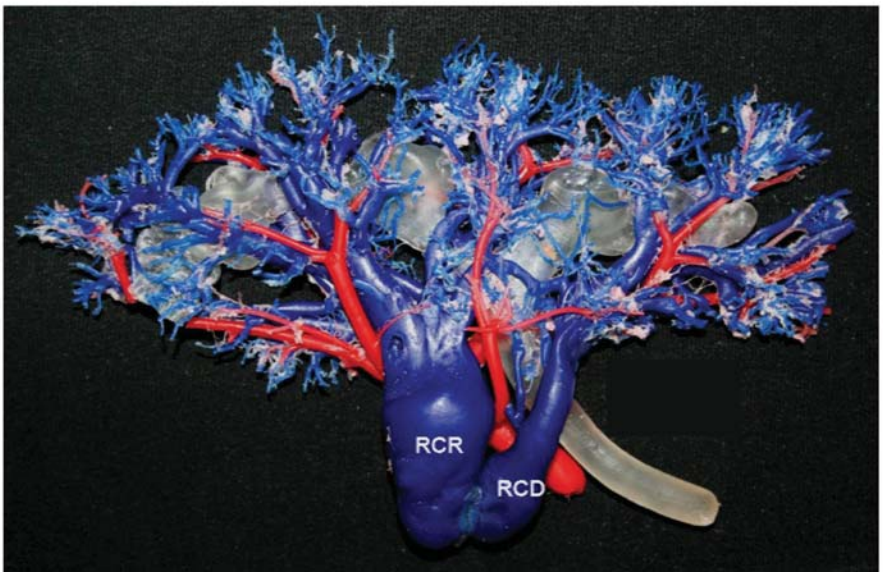


Figure 2. *The ventral surface of the left pig kidney collecting system together with veins (blue) and arteries (red).* ***The venous pattern I****. The renal vein has two branches, one cranial (RCR) and one caudal (RCD) branch.*

In the renal venous system ***type II***, beside the cranial and the caudal branches, we found an intermedial third branch (ramus intremedius) that enters into renal vein where the cranial and the caudal branch were amalgamated and form the renal vein. (Figure 3)

This venous type was represented in 19,35% in a landras /yorkshire breed and in 21.6% in a dalland breed.

However there were no differences in the renal vein branch patterns between the kidneys in the investigated pig breeds.($p>0.05$)

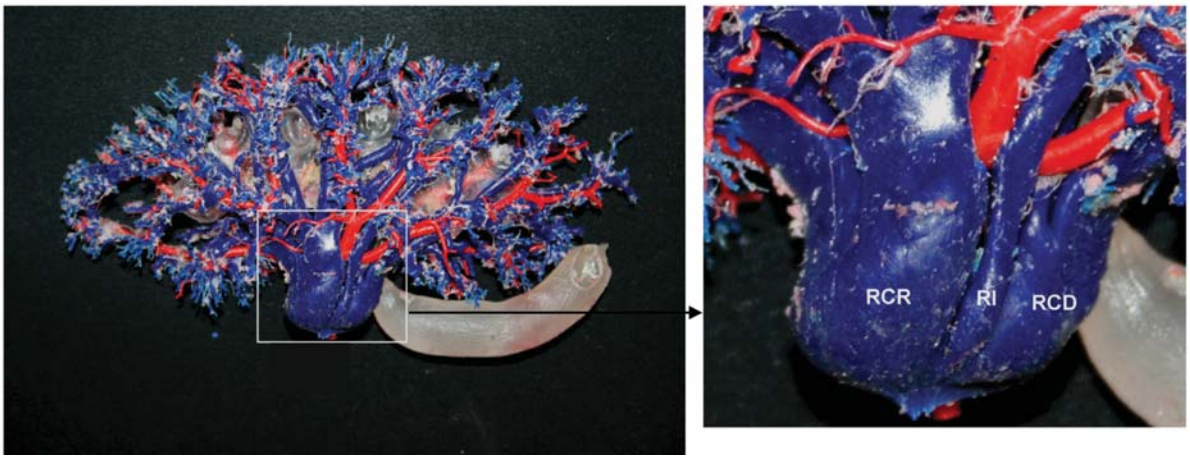


Figure 3. *The ventral surface of the left pig kidney collecting system together with veins (blue) and arteries (red).* ***The venous pattern II.*** *The renal vein has three branches, one cranial (RCR), one caudal (RCD) and one intermedial (RI) branch.*

Future more, in all investigated kidneys there were well developed anastomoses between the venous blood vessels arranged in three longitudinal levels. (Figure 4)

The first level was near the pelvio-uretral junction where mainly two adjacent perilobal veins were connected. In the middle part of the renal pelvis (from both sides) was the second longitudinal system of anastomosis connecting a two neighbouring prelobar veins. Peripherally was the third longitudinal anastomosis system between the veins along lateral border of the renal pelvis. This system formed anastomoses in the form of arcs.

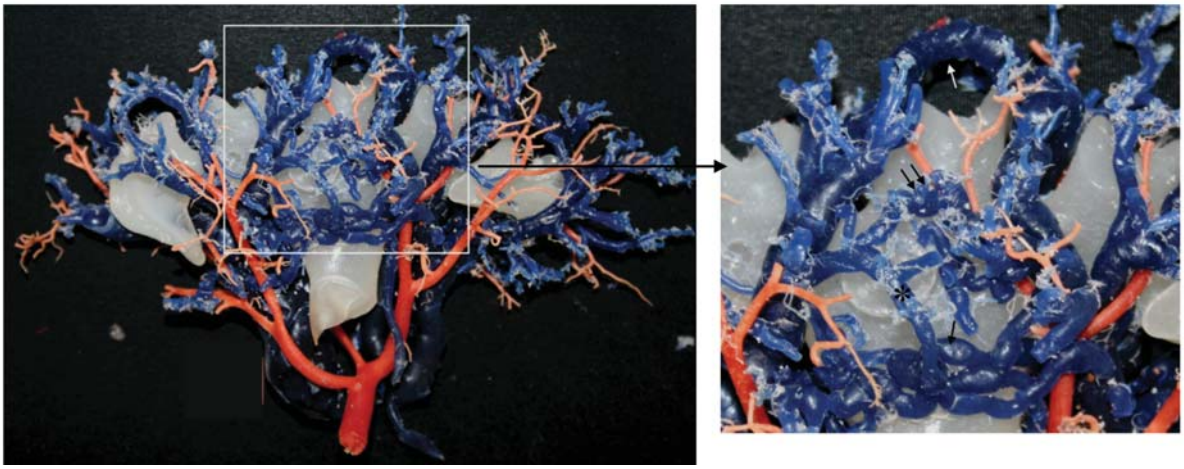


Figure 4. *The dorsal surface of the left pig kidney collecting system together with veins (blue) and arteries (red). Between the veins there are well developed anastomoses arranged in three longitudinal levels. The first level is near the pelvio-uretral junction(black arrow). The second anastomoses system is on the middle of the renal pelvis from the both sides (double black arrows). The peripherally is the third longitudinal anastomosis system along lateral border of the renal pelvis (white arrow). There is a transversal anatstomose connecting the perilobal veins from dorsal and ventral kidney surfaces(✻).*

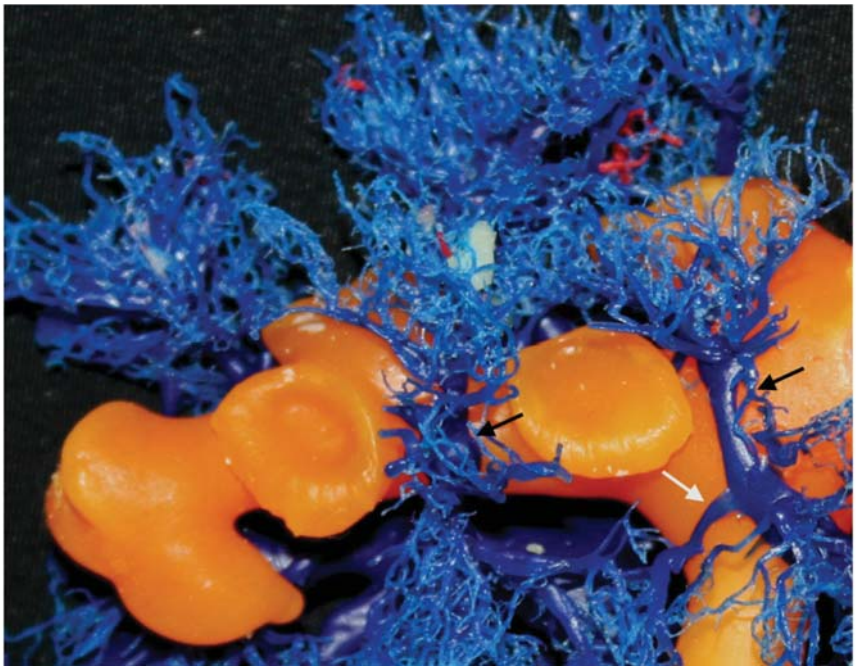


Figure 5. *The ventral surface of the right pig kidney collecting system (yellow) together with veins (blue). Multiple transversely anatstomoses with significantly smaller diameter connecting the perilobal veins from the dorsal and ventral kidney surfaces (white arrow). The venous connections around the base of small cups are circular (collar) anastomoses (black arrows).*

Besides the longitudinal anastomoses, in the investigated pig kidneys we found a transversely anatstomoses that usually connect the perilobal veins from dorsal and ventral kidney surfaces. There were around the base of small cups forming the circular (collar) anastomoses. (Figure 5)

CONCLUSION

According the results, the venous system in pig kidneys could be predicable. Different form the arterial system, the venous renal system doesn't have a segmental structure due to the well developed anastomoses between the perilobal renal veins which is necessary condition for practicing a kidney segmental resection in vascular partial nefroctomy as well for their use in clinical experimental medicine.

REFERENCES

1. Bucher P.; Morel P.; Buhler L. Xenotransplantation: an update on recent progress and future perspectives. Transparent international 2005 18:894-901
2. Cozzi E., White DJ. The generation of transgenetic pigs as potential organ donors for humans. Nat med 1995; 1:965
3. Deschamps JY., Roux FA., Gouin E. history of xenotransplantation Xenotramsplantation 2005; 12:91-109
4. Graves F.T., The itrarenal veins and their evolution. Anatomical studies for renal and intrarenal surgery. Bristol Wright, chapt.8 1986; 62-71
5. Helio J. S. Bagetti, Filho, Marco A. Pereira-Sampaio, Luciano A. Favorito and Francisco J. B. Sampaio. Pig Kidney: Anatomical Relationships between the Renal Venous Arrangement and the Kidney Collecting System. J Urol., 2008; 179: 1627-1630
6. Sampaio FJB., Aragao AHM., Anatomical relationship between the renal venous arrangement and the collecting system. Journal of Urology 1990 144: 1089-1093
7. Sampaio FJB., Pereira-Sampaio MA., Favorito LA. The pig kidney as an endourologic model: Anatomic contribution., Journal of Endourology 1998 12: 45-50
8. Pendovski L., Ilieski V., Petkov V., Popovska-Percinic F., Anatomical classification at the segmental arterial branches on the renal artery in pig kidneys. Macedonian Veterinary Review. Vol. 32, No.1: 55-69; 2009
9. Pendovski L**.** Morphologic study for blood vessels and pelvio-caliceal system in pig kidneys Macedonian Journal of Medical Sciences. 2008 Dec 15; 1(2):64-74.
10. Vodenicarov A., Golubova M. Renal venous architectonic in domestic swine. Anatomy Histology, Embryology 1995 24:149-153

ТИПОВИ НА РАЗГРАНУВАЊЕ НА БУБРЕЖНАТА ВЕНА *(A.RENALIS)* ВО СВИНСКИ БУБРЕЗИ

Пендовски Лазо[1], Илиески Влатко[1], Петков Владимир[1], Поповска-Перчиниќ Флорина[1], Тососка- Лазарова Добрила [2]

[1] Катедра за функционална морфологија, Факултет за ветеринарна медицина, Универзитет "Св. Кирил и Методиј" во Скопје
[2]Институт за анатомија, Медицински факултет Универзитет "Св. Кирил и Методиј" во Скопје

АПСТРАКТ

Целта на овој труд е да се прикажат различните типови на разгранување на бубрежната вена во свински бубрези односно да се утврди дали постои разлика во разгранувањето на бубрежната вена во бурезите земени од различни раси на свињи.

За материјал се користени 69 бубрега земени од две хибридни раси на свињи (*31 бубрег од расата ландрас/јоркшир и 38 бубрега од расата даланд*). Анатомијата на бурежните венозни крвни садови е анализирана на тридимензионални корозивни одливки од силикон С-10 подготвени заедно со артериите и собирачкиот систем на бубрезите.

Кај сите испитани бубрези е утврдена една бубрежна вена. Во 80,65% кај расата ландрас/јоркшир и во 78,94% кај расата даланд утврдно е дека бубрежната вена е изградуваат две примарни гранки ($p>0.05$), додека во останатите 19,35% кај расата ландрас/јоркшир односно 21,6% кај расата даланд е пронајдена додатна интремедијална венозна гранка ($p>0.05$).

Според резултатите може да се заклучи дека свинските бубрези немаат сегментална венска структура која е неопходна при изведувањето на сегменталната ресекција на бубрези при парцијалната нефроктомија.

Клучни зборови: свински бубрези, бубрежни вени, С-10 корозивни одливки

ANIMAL REPRODUCTION

Plenary Lecture

UDC: 636.3.09:618.39-02(495)

CAUSES OF ABORTION IN SMALL RUMINANTS

Nektarios D. Giadinis[1]

[1]*Clinic of Farm Animals, School of Veterinary Medicine, A.U.Th., Thessaloniki, Greece*
**Correspondence to: Nektarios D. Giadinis, Thessaloniki, Greece*
(ngiadini@vet.auth.gr)

ABSTRACT

Abortions consist a severe problem in small ruminant flocks in Greece and worldwide. Abortions can be sporadic or massive. In this review article are discussed the causes of massive abortions in small ruminant flocks. The causes of massive abortions in small ruminant flocks are infectious (mainly bacterial and parasitic) and non-infectious.

From the bacterial causes of abortions in small ruminants the most important in the Greek flocks are chlamydiosis, brucellosis, contagious agalactia, listeriosis, anaplasmosis and colibacillosis. Other bacterial causes of abortion can be Q-fever, salmonellosis and campylobacteriosis (vibriosis). The most common parasitic (protozoic) causes of abortions are toxoplasmosis (very common) and neosporosis (rare). As infectious causes of abortion in small ruminants is considered also Bluetongue and Border disease, two viral diseases with a great impact in the small ruminant industry of the affected country, as well as of the neighboring countries. Very rarely, abortions of fungal aetiology can be observed.

Also abortions in small ruminant flocks can be the result of non-infectious agents, as selenium deficiency, vitamin A deficiency, iodine deficiency, as well as consumption of toxic plants.

For diagnosis of abortions is necessary a careful history, a full necropsy of one or more fetuse(s) and certainly laboratory examinations, including bacterial cultures, serology, cytology and histopathology.

Although abortions in small ruminant flocks are a very important problem, in the literature the existing data for systematic investigation of abortion outbreaks are scarce.

Key words: small ruminants, abortions, causes, diagnosis

INTRODUCTION

Abortions consist a severe problem of small ruminant flocks that has impact upon the economics of the farms. The economical consequences are the result of lamb or goat kid losses and also of the reduced or minimal milk production at the subsequent milking season (Giadinis et al 2011). Also, they are very important for the public health, as most of the causative agents that cause abortions in small ruminants are zoonotic (Pugh 2002).

Although abortions consist a very severe problem of sheep and goat industry, in the existing veterinary literature the available data for systemic investigations of abortions in small ruminant flocks are scarce (Giadinis et al 2012).

Abortions in small ruminants can be sporadic or massive. Taking into account the severe economic impact of the massive abortions, this article will emphasize upon these ones. Massive abortions in small ruminant flocks can be the result of infectious agents or of metabolic problems (deficiencies or toxicities).

Enzootic abortion-Chlamydiosis (*Chalmydia abortus*, former *Chlamydia psittaci*) is the most common cause of abortions in small ruminant flocks worldwide (Pugh 2002, Aitken 2008) and also has been diagnosed many times in Greece. It causes abortion at the last month of gestation in sheep and at any stage of gestation in goats. The affected animals have normal appetite and sensorial condition, while sometimes retained placenta in the same flock can be observed. Animals that abort once do not re-abort from the same cause. Also stillbirths in the same flock can be observed. Diagnosis is confirmed by finding the presence of *C. abortus* mainly in smears from placenta and also in smears from fetal stomach. Also, paired sera can lead to diagnosis. After the establishment of diagnosis the condition can be treated by one oxytetracycline LA injection (20 mg/kg bodyweight) to the rest pregnant animals and abortions stop after 2-3 days. This disease can be prevented by vaccines live and attenuated (Giadinis 1997, Pugh 2002, Aitken 2007, Menzies 2011).

Brucellosis is another important cause of abortion in small ruminant flocks, that it has also a severe zoonotic impact. The most common cause of small ruminant brucellosis in para-mediterranean countries is *Br. melitensis*. In case that non-pregnant sheep or goats are infected, they usually remain asymptomatic and become immune (Samadi et al 2010a). When non-immune sheep or goats are infected during pregnancy, then abortions can occur at 3rd to 5th months of gestation or stillbirths are observed in the affected flocks. The affected female sheep or goats usually don't exhibit other signs except for abortions or retained placentas; either high mortality rate due to brucellosis has been reported to date. The fertility of ewes or goats that aborted due to *Br. melitensis* is not affected. The excretion of the organism in milk is not accompanied by clinical mastitis in sheep, but cases of mastitis have been reported in goats with changes of the milk and nodules in the udders (Papadopoulos 1987, Samadi et al. 2010a, Samadi et al 2010b, Minas 2011). Male animals infected by *Br. melitensis* are usually asymptomatic, but more rarely can exhibit orcheo-epididimytis, frequently uni-lateral, that can result to infertility. Both sexes can present osteoarthritis, synovitis or nervous signs, but these clinical findings seem to be very rare (Papadopoulos 1987, Samadi et al 2010a, Minas 2011). From the other *Brucella* species, *B. ovis* usually causes orchitis and sterility and rams, while *B. abortus* causes an asymptomatic infection in

small ruminants (Minas 2011). The condition can be diagnosed by *Brucella* isolation in fetal membranes or fetal stomach content, while diagnosis can be also confirmed serologically by paired sera. In case that abortions are observed in a flock due to brucellosis, the best results in our Clinic have been observed by administration of injectable oxytetracycline LA (20 mg/kg) twice 7 days apart the one from the other injection (Giadinis-unpublished data). The best way of prevention is vaccination with the live vaccine Rev-1, that is administered in the eye or by injection. Also, periodical serological examination of the rams or bucks is necessary (Samadi 2010a, 2010b, Minas 2011).

Contagious agalactia syndrome caused mainly by *Mycoplasma agalactiae* and also other *Mycoplasma* species can cause abortions in small ruminant flocks except for other signs, as keratoconjunctivitis, arthritis, mastitis and hypogalactia (Rodriguez et al 1996, Giaidnis et al 2008). It is known, that mycopalasmatic infections can occur also *in utero* (Filioussis et al 2011). Diagnosis is based upon *Mycoplasma* spp. isolation in placenta or fetal stomach content. Usually these abortions are sporadic and treatment seems not necessary, but prevention with vaccinations is useful (Pugh 2002).

Listeriosis mainly caused by *Listeria monocytogenes* can cause sporadic abortions in small ruminant flocks that are characterized by necrotic lesions in fetal liver. This condition can be controlled by avoiding feeding spoiled silages (Pugh 2002).

Anaplasma phagocytophilum and other *Anaplasma* species that are mainly transmitted by ticks can cause abortions in small ruminant flocks. Also these abortions seem to be sporadic and any treatment seems necessary, but tick control is useful in reducing the abortion cases (Chianini et al 2004).

Some strains of *Escherichia coli* can cause abortions in sheep flocks, as have been shown by studies in UK. During the last years, cases of abortions attributed to *E. coli* have been diagnosed in sheep and goat flocks, also in Greece. It was characteristic, that most of the aborted fetuses had hemorrhages in muscles. These abortions usually respond very well to antibiotics like oxytetracycline LA (Giadinis et al 2012).

Although *Coxiella burnetii* infection is usually asymptomatic, many times can cause abortions in small ruminant flocks; goats are more susceptible to this infection compared to sheep. The agent is zoonotic and causes tick-borne fever in humans. In initial outbreaks the abortion rate can be 5-35 %. PCR or paired sera are helpful for diagnosis. Treatment with oxytetracycline LA seems to be effective. Prevention is based upon biosecurity measures and vaccinations (Menzies 2011).

Campylobacteriosis is a severe cause of abortions in many countries, but it is not common in Greece. *Campylobacter jejuni* and *C. fetus* are implicated in abortions of small ruminants. Usually *C. jejuni* cause sporadical abortions and the ewes can present diarrhea. The aborted lambs or kids have swollen liver with necrotic focuses. *C. fetus* causes a cyclic disease, with abortion storms to be repeated every 4-6 years. Fetal liver or fetal stomach content are considered the best materials for the isolation of the microorganism. Both cases can be treated by antibiotics, but the clinicians should take into account the possible antimicrobial resistance. Commercial vaccines are available in USA and other countries (Menzies 2011, Rodi-Burriel 2011).

Various *Salmonella* species can cause abortions in small rumiants flocks. Fever, diarrhea or mortality of pregnant animals can also occur. The microorganisms can be isolated from fetal membranes, fetal internal organs and vulvar exudates. Treatment with antibiotics is helpful, while the condition can be prevented by vaccinations (Rodi-Burriel 2011).

From protozoal agents, mainly toxoplasmosis and also neosporosis cause abortions in small ruminant flocks. Toxoplasmosis is transmitted mainly by cat feces infected by *Toxoplasma gondii*. It can cause abortions between the 3rd to 5th month of gestation. The aborted fetuses are usually mummified, while also malformations or stillbirths can be observed. Diagnosis is based upon the isolation of *Toxoplasma* cysts in fetal brain by direct smears, PCR or histopathology. Serology of the fetal blood can also be diagnostic. Treatment is based upon administration of various substances during pregnancy (sulfadimidine, decoquinate, monensin), while vaccinations with attenuated vaccines help in the disease prevention, as well as biosecurity measures (Aitken 2007, Giadinis et al 2011).

Neosporosis is considered a common cause of abortion in cattle, but in small ruminants it is considered a rare cause of abortion. Also congenital malformations or stillbirths can be observed (Varaschin et al 2012). The condition can be diagnosed by the parasite isolation and by serology. An effective treatment has not been found to date, while prevention is based upon biosecurity measures (Papadopoulos 2011).

Other not very common causes of infectious abortion can be Bluetongue and Border virus, as well as fungal infections (Papadopoulos 1987, Winter 1991, Billinis and Spyrou 2011).

Selenium deficiency has been implicated in abortions of small ruminant flocks. These conditions can be diagnosed and confirmed by necropsy and selenium determination, while can be treated by selenium administration to the pregnant animals (Giadinis et al 2011). Also, other deficiencies, like vitamin A, iodine, as well as consumption of toxic plants can cause abortions in small ruminant flocks.

REFERENCES

1. Aitken I.D. (2007) Disease of Sheep, Blackwell Science, UK.
2. Billinis H., Spyrou V. Border disease. In: Reproduction of Small Ruminants, G.C. Fthenakis (editor), Tziolas Publications, Thessaloniki, Greece.
3. Chianini F., Adams C., Buxton D. (2004) Neuropathological changes in ovine fetuses caused by tick-borne fever. *Veterinary Record* 155, 805-806.
4. Giadinis N.D. (1997) The effect of selenium and/or vitamin E administration upon chlamydiosis (*Chlamydia psittaci*) antibody production in sheep. Doctoral Thesis, A.U.Th, Thessaloniki, Greece.
5. Giadinis N.D., Petridou E.J., Filioussis G., Siarkou V., Terpsidis K., Papadopoulos E. (2012) Investigation upon the abortion aetiology in Greek small ruminant herds. In: Proceedings of the 12th Greek Veterinary Congress, April 2012, Athens, Greece.
6. Giadinis N.D., Petridou E.J., Sofianidis G., Filioussis G., Psychas V., Hatzopoulou E., Karatzias H. (2008) Adult goat mortality attributed to *Mycoplasma capricolum subsp. capricolum*. *Veterinary Record* 163, 278-279.
7. Giadinis N.D., Terpsidis K., Diakou A., Siarkou V., Loukopoulos P., Ridvan O., Karatzias H., Papazahariadou

M. (2011) Massive toxoplasma abortions in a dairy sheep flock and treatment with different doses of sulfadimidine. *Turkish Journal of Veterinary and Animal Sciences* 35, 207-211.
8. Menzies P.I. (2011) Control of important causes of infectious abortion in sheep and goats. *Veterinary Clinics of north America-Food Animal Practice* 27, 81-93.
9. Minas A. (2011) Brucellosis. In: Reproduction of Small Ruminants, G.C. Fthenakis (editor), Tziolas Publications, Thessaloniki, Greece.
10. Papadopoulos E. (2011) Neosporosis. In: Reproduction of Small Ruminants, G.C. Fthenakis (editor), Tziolas Publications, Thessaloniki, Greece.
11. Papadopoulos O. (1987) Infectious diseases of domestic animals, A.U.Th. Publication Service, Thessaloniki, Greece.
12. Pugh D.G. (2002) Sheep and Goat Medicine, Saunders, USA.
13. Rodi-Burriel A. (2011) Salmonelosis, leptospirosis, Q fever, campylobacteriosis. In: Reproduction of Small Ruminants, G.C. Fthenakis (editor), Tziolas Publications, Thessaloniki, Greece.
14. Rodriguez J.L., DaMassa Al.J., Brooks D.L. (1996) Caprine abortion following exposure to *Mycoplasma capricolum* subsp. *capricolum*. *Journal of Veterinary Diagnostic Investigation* 8, 492-494.
15. Samadi A., Ababneh M.MK., Giadinis N.D., Lafi, S.Q. (2010a) Ovine and caprine brucellosis (*Brucella melitensis*). *CAB Reviews: Prespectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 5, No 42.
16. Samadi A., Ababneh M.MK., Giadinis N.D., Lafi, S.Q. (2010b) Ovine and caprine brucellosis (*Brucella melitensis*) in aborted animals in Jordanian sheep and goat flocks. *Veterinary Medicine International* 2010, 458695.
17. Varaschin M.S., Hirsch C., Wouters F., Nakagaki K.Y., Guimarães A.M., Santos D.S., Bezerra P.S., Costa R.C., Peconick A.P., Langohr I.M. (2012) Congenital neosporosis in goats from the State of Minas Gerais, Brazil. Korean Journal of Parasitology 50, 63-67.
18. Winter A. (1991) Outline of clinical diagnosis in sheep, UK.

ПРИЧИНИ ЗА АБОРТУС КАЈ МАЛИ ПРЕЖИВАРИ

Нектариос Д. Гианидис[1]

[1]Клиника за фармски животни, Школа за ветеринарна медицина,
Аристотелис Универзитет Солун, Солун, Грција
**Коресподенција: Нектариос Д. Гианидис, Солун, Грција*
(ngiadini@vet.auth.gr)

АПСТРАКТ
Абортусите претставуваат сериозен проблем кај малите преживари во Грција и во целиот свет. Абортусите може да бидат спорадични или масовни. Во овој прегледен труд се дискутира за причините за масовен абортус во стада од мали преживари. Причинители за масовниот абортус во стадата на мали преживари можат да се инфективни (главно бактериски и паразитски) и неинфективни.
Меѓу најважните бактериски причини за абортуси кај малите преживари во стадата во Грција спаѓат хламидиозата, бруцелозата, заразната агалакција, листериозата, анаплазмозата и колибацилозата. Други бактериски причинители на абортусот може да се и Q-треската, салмонелозата и кампилобактериозата (вибриоза). Најчестите паразитски (протозои) причинители на абортуси се токсоплазмозата (многу честа појава) и неоспорозата (ретка). Како инфективни причинители за абортус кај малите преживари се сметаат, исто така, и болеста Син јазик и Бордерова болест, кои претставуваат две вирусни заболувања кои имаат големо влијание врз индустријата на самата држава, како и на соседните земји. Многу ретко се забележуваат абортуси од габична етиологијата.
Исто така, абортусите може да бидат резултат и на неинфективни агенси, како што се недостаток од селен, витамин А или јод, како и консумирање токсични растенија.
За дијагностицирање на абортуси потребно е да се знае историјатот на болеста, да се направи целосна некропсија на еден или повеќе фетус(и) и, секако, да се направат лабораториски испитувања, вклучувајќи бактериски култури, серологија, цитологија и хистопатологија.
Иако абортусите кај малите преживари претставуваат многу важен проблем, во литературата ретко се среќаваат податоци за систематско проучување на масовниот абортус.
Клучни зборови: мали преживари, абортус, причинители, дијагноза

Plenary Lecture

UDC: 636.1.083.453.5

MANAGEMENT OF EQUINE REPRODUCTION DURING THE BREEDING SEASON (FRESH, COOLED AND FROZEN SEMEN: WHAT, WHEN AND HOW)

Lacalandra GM, Nicassio M,

Department of animal production, University of bari aldo moro, Italy

ABSTRACT

The management of equine reproduction during the breeding season of mares that will be submitted to artificial Inseminantion (AI), was described. Horses are long-day breeders. Seasonal reproductive activity is stimulated by photoperiod together with exogenous factors. During the breeding season, cycle length is about 22 days with 5-7 days of oestrus. The anovulatory season can be differentiated into an autumn transitional phase, a mid-anovulatory period and a spring transitional phase bringing the mare back into cyclic activity. During the mid-anovulatory period, follicular development is minimal. The beginning of the spring transitional period is characterized by the development of 1-3 anovulatory follicular waves before ovulation occurs and the most important factor for the re-initiation of ovulatory activity is the occurrence of repeated pronounced increases in circulating LH. Equine artificial insemination (AI) has been by now widely established in the warm blood horse industry. Mares can be artificially inseminated with fresh, chilled or frozen semen to increase the revenue from their offspring. In the last decade, there has been a significant increase in the quality and commercial use of frozen equine semen. Rectal palpation and ultrasonography are described as a combined method used for monitoring follicular development in the mare allowing objective observation and measurement of follicular growth until the identification of the more convenient moment for AI. The emergence of new reproductive technologies, coupled with the high prices for an insemination dose from some stallions, the increasing costs of import and export and the marketing policies of stallion agents or owners in the sport horse industry has stimulated the fractionation of doses for insemination. Consequently, the sperm number and the volume of an insemination dose are significantly reduced. Lower doses of semen can be delivered hysteroscopically or by rectally guiding a flexible pipette to the tip of the desired uterine horn.

Key words: artificial insemination , management, follicular growth, equine reproduction

INTRODUCTION

The practice of artificial insemination (AI) in domestic animals is presumed to have begun in the equine species. The literature reports that in 1332 an Arabian chief fertilized his mare by using semen "stolen" from a stallion owned by an enemy.

Artificial insemination is a technique by which an adequate number of live spermatozoa with progressive motility is deposited in the uterus of a mare in estrus. Although this procedure seems to be simple, only the precise coordination of procedures and a proper manual ability can provide an optimal pregnancy rate.

The spread of AI has partially solved the problem of over-demand for a specific stallion's semen and, in the course of time, has gradually reduced the need to send mares to stallion operation centers. Now doses of semen "travel." Furthermore, the development of AI has enabled stallions of high genetic value to be used to breed large numbers of mares, through the use of refrigerated and frozen semen. In many equine breeds, AI has almost completely superseded natural mount, with all its accompanying technical and health problems, in which males might be handled incorrectly and exposed to the risks of venereal diseases and trauma.

REPRODUCTIVE ACTIVITY

Equine reproductive activity is concentrated within a limited period of the year. In fact, the mare cyclic activity is defined as *seasonally polyestrous with positive photoperiodism*. The mare has repeated cycles in the period starting in February-March through to August-September, so in the months characterized by a longer day length. The mare goes into seasonal anestrous during winter (November-January), when the ovaries gradually become smaller and hard, due to the absence of follicles and corpora lutea. The transition from anestrous to the breeding season occurs during February-March in conjunction with the increase in day length, which gradually stimulates ovarian activity. In this period, follicular development and heat can be irregular, with follicles that develop and regress without ovulating, until a normal ovulatory cycle is established.

While photoperiod is the most important factor in determining seasonality, other variables may influence the course of the reproductive cycle, including environmental factors, farm conditions, nutritional level, the use of mares for work or competitive activity, use of drugs – especially those with hormonal action -- or individual metabolism. In sporting breeds, such as Trotters, the onset of the breeding season is bound by certain regulations that officially identify this period from 15 February to 15 July.

During the breeding season, there are repeated estrous cycles lasting 19-23 days. When the mare is shown to be receptive to the stallion, and it is possible to detect one or more growing follicles (2.5-3.5 to 7 cm diameter) on transrectal examination, estrus (follicular phase), lasting 4-6 days, starts. Ovulation occurs consistently 24-48

h before the end of estrus. After this, diestrus (the luteal phase) starts, lasting 16-17 days, during which the female is not receptive to the stallion. A corpus luteum forms on the ovaries within 3-4 days after ovulation.

The persistence of the corpus luteum in the ovary is linked with the presence or absence of pregnancy. In the case that the oocyte is not fertilized, the corpus luteum regresses (cyclic corpus luteum) whereas in pregnant females, it persists (gravid corpus luteum). The cyclic corpus luteum, which produces progesterone, starts to regress around the twelfth day after ovulation, due to the action of uterine prostaglandins. The gravid corpus luteum persists much longer, because it is indispensable for the maintenance of pregnancy, representing the main source of progesterone for at least the first 50 days of gestation. The primary corpus luteum is assisted later by the accessory corpora lutea resulting from ovulation or luteinization of a second generation of follicles.

The length of estrus decreases as the breeding season progresses into its peak (May-June) probably due to an acceleration of folliculogenesis before ovulation with the increase of favorable photoperiod. The climate and cyclical components that regulate these reproductive rhythms depend on the interplay of several hormones, whose release is triggered by numerous exteroceptive stimuli. In a specialized part of the brain, the hypothalamus, there are certain factors that control hormone release. Among these factors, the most important for reproductive activity is GnRH (Gonadotropin Releasing Hormone). GnRH travels to the pituitary gland through the hypothalamic-hypophyseal portal system, and stimulates the secretion of specific hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH). The mode and frequency of GnRH secretion (pulsatility) is important; during anestrus this frequency is very low due to the depression induced by melatonin. Melatonin is secreted by the pineal gland. Its production considerably increases during the hours of darkness and it depresses, through channels that are not yet clearly defined, the release of GnRH. Secretion of GnRH is also reduced during cyclic diestrus due to negative feedback from progesterone. FSH stimulates the early growth of follicles in the ovaries during diestrus. Blood levels of FSH are elevated during estrus and mid-diestrus.

In the mare, a small number of follicles develops during the final phases of diestrus. One of this cohort of follicles continues to grow, matures during estrus and ovulates. Meanwhile, the remaining follicles may continue to grow; and one or more can ovulate during the first days of the subsequent luteal phase even in the face of a persistent high level of LH, while the remaining follicles fall into atresia. All this implies, in the equine species, a high incidence of multiple ovulations. During the development of the preovulatory follicle the concentration of 17-β-Estradiol increases. The peak of secretion of this hormone occurs in the second half of estrus and about 24 hours before the peak of LH. In the mare, estrogens coming from the ovary and the granulosa cells serve several functions.

These estrogens:

- stimulate the behavioral centers of the brain and induce sexual receptivity;
- feed back positively on the hypothalamus and hypophysis, resulting in the secretion of FSH and LH;
- increase the uterine, cervical and vaginal secretions that facilitate mating and spermatozoa transport through the genital tract.

During maturation, ovarian follicles secrete progressively greater amounts of a protein hormone, inhibin, which feeds back to inhibit the release of FSH, presumably by altering, at the pituitary level, the sensitivity of FSH-producing cells to GnRH.

During the greater part of diestrus, serum LH concentrations are very low. The secretion of LH increases near the beginning of estrus and reaches a peak during estrus. After ovulation, the decline of LH is relatively slow compared to other species, and baseline values are not reached again until at least 5 or 6 days after ovulation. The low LH levels during diestrus are due to negative feedback by progesterone secreted by luteal cells of corpus luteum. Progesterone is also secreted, to a small extent, by the granulosa cells of the maturing follicle before ovulation. The LH increase during estrus is responsible for final maturation of the preovulatory follicle, for ovulation induction and for corpus luteum formation. A deficiency of LH, as occurs during the transition period, usually prolongs the follicular phase and does not allow ovulation. Under normal conditions, ovulation usually occurs 1 or 2 days before the end of estrus and can be detected by examination of the ovaries via transrectal palpation or ultrasonography. Immediately after ovulation, the follicular cavity fills with blood, giving rise to the corpus haemorrhagicum. Granulosa cells organize themselves in the follicular cavity to form the corpus luteum. Progesterone secretion by the corpus luteum results in an increase in blood progesterone levels to over 1 ng/ml within 2 or 3 days. The blood progesterone concentration continues to increase until it reaches 6-8 ng/ml after about 6 days.

The corpus luteum continues to produce progesterone until about 14-15 days after ovulation, when prostaglandin F2α (PgF2α) produced by uterus, causes luteolysis. This prostaglandin probably reaches the ovaries through a peripheral circuit. The resulting decrease in progesterone plasma concentrations allows the increase of LH secretion, the acceleration of follicular growth, the increased secretion of estrogen and the return to estrus. Estrogen and progesterone affect uterine and cervical tone and uterine, cervical and vaginal secretions.

MARE MONITORING

Mares properly restrained in stocks are subjected to palpation and ultrasound examination per rectum. On palpation, the ovaries, uterus and cervix are evaluated. The ovary is evaluated for its shape, size and consistency. Then, size and tone of the uterus and finally the degree of closure of the cervix are always evaluated.

Data for each mare must be recorded on an appropriate sheet or kept stored on computers with special software in single reports in which the mare's name, its reproductive condition, the assigned stallion, the date of the mounts or inseminations to which it may have already been submitted, and the result of previous examinations or treatments are recorded.

Ultrasound examination follows a careful clinical examination per rectum. Ultrasound examination without a good transrectal clinical examination cannot be considered a sufficient diagnostic tool. With ultrasonography, uterus, ovaries and cervix are again evaluated. Transrectal ultrasound allows the operator to perform accurate measurements of uterine wall thickness and edema of the uterine folds, typical estrus-phase invaginations of the

uterine wall to which we give subjective values: Folds 0.5-1-1.5 or Edema 1-2-3. On ultrasonography, edematous uterine folds give an unmistakable "wagon wheel" image. It is also possible to detect the presence of certain conditions such as single or multiple uterine cysts which may cause infertility (can make it difficult for embryonic vesicles to communicate properly with the endometrium if they are located near the cysts). In addition, uterine cysts may lead to diagnostic errors, as they are similar to an embryonic vesicle in size and shape. It is also possible to detect the presence of fluid in the uterus.

In the ovaries it is possible measure follicular dimensions, or to detect the presence of a corpus haemorrhagicum, luteal tissue, or the presence of formed fully functional corpus luteum (at 5 days after ovulation) on ultrasonography per rectum. In the presence of a corpus luteum, we can decide to accelerate the return of the mare in estrus by inducing luteolysis with prostaglandin. In addition to its luteolytic effect, prostaglandin induces the release of FSH and LH, which have follicle-stimulating activity. If multiple follicles of unimportant size (2-2.5 cm) are present, the mare not in estrus can be in the proestrus phase, and on the board we will write the data "small multiple follicles" (SMF). In these situations, in most cases we will find follicles of 20-22 mm in both left and right ovaries before one or two become dominant and become potentially preovulatory follicles. There are cases in which, because of endocrine alterations, small follicles that do not exceed 5-7 mm in diameter are found in both ovaries and these do not reach ovulation for several cycles. In cases in which two follicles reach considerable size (around 40 mm in diameter) and are both considered preovulatory, it is important to follow both because often it is more successful to fertilize the second ovulated follicle instead of the first (to avoid twins). It also could happen that one of the two does not ovulate and instead regresses. Another useful parameter evaluated on ultrasound is follicular wall thickness, or signs of a tenuous shading in the follicular fluid, characteristic of an anovulatory follicle. We can obtain some information related to the cervix with ultrasonography, although this area is better explored by direct investigation through the vagina. After ultrasound exam all the clinical and ultrasound findings are added to the mare's clinical board, establishing whether or not to proceed with the mating and fixing the time for the next examination. In mares in which a follicle is found that, due to its size and characteristics via palpation and ultrasonography, is considered near to ovulation, it is possible to induce ovulation by intravenously administering hCG (Human Chorionic Gonadotropin). Ovulation typically occurs between 30 and 48 hours after hCG administration, within the time that fresh semen can keep its viability within the reproductive tract of the mare.

AI WITH FRESH, COOLED AND FROZEN-THAWED SEMEN

The management of the mare to be inseminated differs according to type of semen to be used (fresh, cooled or frozen-thawed semen). Fresh semen is used when the stallion is on the farm but, for obvious reasons, is not used in natural mating. This situation gives us the ability to have semen at any time, but it is still preferred to inseminate the mare only once, after administering hCG to induce ovulation. After collecting the semen and preparing the insemination doses, AI is performed as soon as possible. One at time, the mares which must be inseminated are led into the stocks. Before proceeding with cleaning of the perineal region, it is always important to clean the rectum of feces that may impede the next phase of insemination. After lifting the tail and tying it or otherwise holding it away from the perineum, the veterinarian cleans the perineum several times with clean water or, if necessary, with Betadine. After donning an examination glove, the veterinarian inserts the hand and arm into the vagina, carrying the catheter connected with the syringe containing the semen. After locating the external cervical os with the finger he slides the catheter through cervical canal as deeply as possible, while being careful not to apply undue pressure, and deposits the semen into the uterus. After removing the catheter it is recommended to grasp the cervix and lift it from the vaginal floor in order to prevent spillage of semen. At the same time, this cervical massage increases uterine contraction, favoring the ascent of spermatozoa. After 24 or 36 hours, the mare is again examined to be sure that ovulation occurred. If the examination is made at 24 hours and you have semen collected the day before, you can safely use it again. Alternatively, insemination can be repeated after 48 hours using semen collected on the same day. It is important to ensure that ovulation occurs no later than 48 hours after insemination. For the use of cooled and transported semen, the main difficulty is synchronizing ovulation with the arrival of the semen. Stallion Centers traditionally collect stallions and prepare semen doses on odd-numbered days and a request for semen must be done no later than ten o'clock on the day of collection. For this reason, it is important to examine all mares in estrus that are intended for insemination with cooled semen in the early morning hours of odd-numbered days, and administer hCG to those with preovulatory follicles, after confirming the availability of the desired semen. The following morning, these mares are examined to ensure the continued presence of the preovulatory follicle, and then are inseminated as described above. In this way, we typically perform insemination 10-12 hours before ovulation. The day after insemination the mare is examined to determine if ovulation occurred.

If using frozen-thawed semen it is important to monitor the mare precisely, to ensure that insemination is performed immediately before impending ovulation (maximum 6 hours before) or immediately following ovulation (maximum 6 hours afterward).

In recent years, especially in the saddle horse world, there has been a progressive reduction in the number of straws of frozen semen contained in an insemination dose for commercial purposes. This has led to the spread of new AI techniques in order to deposit the semen at or near the uterotubal junction of the uterine horn ipsilateral to the dominant follicle, because it was demonstrated that this increases the number of spermatozoa found in the oviduct, compared with that found after AI in the uterine body. Such "deep AI" uses hysteroscopic techniques or special catheters. The hysteroscopic technique has been gradually abandoned because it is impractical, thus the use of catheters for deep AI is preferred. We prefer to use the traditional technique, performing only one insemination before ovulation or, when there is only a single dose, immediately after ovulation.

In conclusion all the above-mentioned strategic solutions require high professional skill by the veterinarians dealing with frozen semen but they can improve

in some instances the pregnancy rate at the end of the season. On the other hand, this peculiarities show clearly how complex is the fertilisation process and how many mechanisms, still not well understood, are involved in the biology of reproduction. Further field studies are needed to demonstrate positive results in the use of artificial insemination with frozen semen in equine breed industry compared to fresh-chilled semen and finally to let this technological tool become extensively diffused among breeders.

REFERENCES

1. Aurich C., Reproductive cycles of horses. Anim Reprod Sci. 2011 Apr;124(3-4):220-8. Review.
2. Hughes JP, Stabenfeldt GH, Evans JW., The oestrous cycle of the mare., J Reprod Fertil Suppl. 1975 Oct;(23):161-6.
3. Buratini J, Rosa e Silva AA, Barros CM, Papa FO, Caldas MC, Meira C., Follicular dynamics in Mangalarga mares. Equine Vet J Suppl. 1997 Dec;(25):7-11.
4. Davies Morel MC, O'Sullivan JA., Ovulation rate and distribution in the thoroughbred mare, as determined by ultrasonic scanning: the effect of age. Anim Reprod Sci. 2001 Apr 30;66(1-2):59-70.
5. Miro J., Ovarian ultrasonography in the mare., Reprod Domest Anim. 2012 Jun;47 Suppl 3:30-3. doi: 10.1111/j.1439-0531.2012.02043.x.
6. Hemberg E, Lundeheim N, Einarsson S., Successful timing of ovulation using deslorelin (Ovuplant) is labour-saving in mares aimed for single ai with frozen semen. Reprod Domest Anim. 2006 Dec;41(6):535-7.
7. Batellier F, Vidament M, Fauquant J, Duchamp G, Arnaud G, Yvon JM, Magistrini M., Advances in cooled semen technology. Anim Reprod Sci. 2001 Dec 3;68(3-4):181-90.
8. Carbonara D., Buono R., Nicassio M., Lacalandra G.M., Impiego del seme congelato in un centro di fecondazione equina. Risultati in un periodo di 4 anni (1998-2001). 2003, IPPOLOGIA, vol. 14, p. 31-34, ISSN: 1120-5776.
9. Crowe CA, Ravenhill PJ, Hepburn RJ, Shepherd CH. A retrospective study of artificial insemination of 251 mares using chilled and fixed time frozen-thawed semen. Equine Vet J. 2008 Sep;40(6):572-6.
10. Degl'Innocenti S., Agresti P., Rubei M., *Esperienze di inseminazione artificiale con seme congelato nella cavalla da trotto*, 1996, Atti L Convegno S.I.S.Vet., 50: 43-44.
11. Hayden SS, Blanchard TL, Brinsko SP, Varner DD, Hinrichs K, Love CC., Pregnancy rates in mares inseminated with 0.5 or 1 million sperm using hysteroscopic or transrectally guided deep-horn insemination techniques. Theriogenology. 2012 Jun 13.
12. Miller CD., Optimizing the use of frozen-thawed equine semen. Theriogenology. 2008 Aug;70(3):463-8.
13. Sieme H, Martinsson G, Rauterberg H, Walter K, Aurich C, Petzoldt R, Klug E., Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. Reprod Domest Anim. 2003 Apr;38(2):134-40.

МЕНАЏМЕНТ НА РЕПРОДУКЦИЈА КАЈ ЕКВИДИ ВО СЕЗОНА НА РАЗМНОЖУВАЊЕ (НАТИВНА, ОЛАДЕНА И ЗАМРЗНАТА СПЕРМА: ШТО, КАКО И КОГА)

Лакаландра Џ М., Никасио М.,

Оддел за сточарство
Универзитет во Бари, Алдо Моро, Италија

АПСТРАКТ

Во трудот е опишан менаџментот на репродукцијата кај еквидите, за време на репродуктивната сезона кај кобилите кај кои ќе биде изведено вештачко осеменување. Еквидите се размножуваат во текот на сезоната со долги денови. Сезонската репродуктивна активност е стимулирана од фотопериодизмот, заедно до други надворешни фактори. За време на сезоната на размножување, траењето на естралниот циклус е околу 22 дена, од кои на еструсот отпаѓаат 5-7 дена. Ановулаторната сезона може да се подели на есенска транзициска фаза, среден ановулаторен период и пролетна транзициска фаза, која ги враќа кобилите во повторна циклична активност. Во текот на централниот ановулаторерн период, развојот на фоликулите е минимален. Почетокот на пролетниот транзициски период се карактеризира со развој на 1-3 ановулаторни фоликуларни бранови, пред да се случи овулацијата и многу важни фактори за враќање на овулаторната активност претставува појавување на повторено нагласено зголемување на циркулаторен LH. Вештачкото осеменување кај еквидите (ВО) моментално е широко распространето кај топлокрвните раси коњи. Кобилите може да се осеменат со нативно, оладено и замрзнто семе, за да се зголеми генетската вредност на нивното потомство. Во последната деценија, забележан е значителен пораст на квалитетот и комерцијалната употреба на замрзната сперма од еквиди. Ректалната палпација и ултрасонографијата се опишани како комбинирана метода која се употребува за следење на фоликуларниот раст кај кобили, овозможувајќи објективна опсервација и мерење на фоликуларниот раст се до идентификацијата на најпогодниот момент за осеменување. Појавата на нови репродуктивни технологии, поврзано со високата цена на една доза за осеменување од пастуви, ја зголемува цената на увозот и извозот и пазарната легислатива на застапниците и сопствениците, кои се употребуваат за спортски манифестации, што доведува до потреба за фракционирање на дозите за осеменување. Последователно, бројот на сперматозоиди и волуменот на дозата за осменување, значително се намалени. Намалените дози сперма можат да се администрираат хистероскопски или по пат на ректално водење на флексибилен катетер се до врвот на соодветниот рог на матката.

Клучни зборови: вештачко осеменување, менаџмент, раст на фоликули, репродукција на коњи

UDC: 636.234.09:612.616.2.086.13

ACTIVITY OF NADH-TETRAZOLIUM REDUCTASE IN BULL SEMEN AFTER CRYOPRESERVATION IN RELATION TO THEIR MOTILITY

Stefanov Rossen, Chervenov Mihail, Georgiev Boyko, Kacheva Dimitrina, Taushanova Paulina, Kistanova Elena, Sabev Milko

Institute of Biology and Immunology of Reproduction, BAS, Sofia, Bulgaria

ABSTRACT

Enzyme system NADH-Tetrazolium Reductase can be used as a marker for the functional status of mitochondria. Its activity gives us information about the intracellular oxidation, and its quantitative characteristics are expressed by mean cytochemical coefficient (MCC).

The aims of this study were to establish the relationship between motility in bull semen and the MCC of NADH- Tetrazolium Reductase in spermatozoa.

In this study 42 ejaculates from 10 Holstein bulls, were used. After obtained by using of artificial vagina, the semen was freezed according to Cassou paillette technology . Computer sperm class analyzer (CASA) was used for evaluation of semen motility, both after thawing and after incubation at 39ºC for 5 hours. NADH- Tetrazolium Reductase activity in spermatozoa was determined.

The results demonstrate that along with increased motility there is increase of MCC. The data from this survey, suggests that there is a relationship between motility and activity of the enzyme system NADH- Tetrazolium Reductase.

INTRODUCTION

The metabolism of sperm is dependent on the need for energy generation, which is transformed, mainly into mechanical to maintain their motility. Published data show that the catabolic dominates over anabolic processes (Mita et al., 1994). An important factor in controlling the transformation of the energy in sperms is the ratio between NAD and NADH. This ratio is influenced by group of enzymes which catalyze the oxidation - reduction processes. (Kleniewska et al., 2012). Correlations have been found between sperm motility and tetrazoll-redox activity in the midpeace of the spermatozoa, which can be used as criteria for assessing the biological properties of these cells (Цветкова, 2000).

Enzyme system NADH-Tetrazolium Reductase can be used as a marker for the functional status of mitochondria. Its activity gives us information about the intracellular oxidation, and its quantitative characteristics are expressed by mean cytochemical coefficient (MCC).

Aim: The aims of this study were to establish the relationship between motility in bull semen and the MCC of NADH- Tetrazolium Reductase in spermatozoa.

MATERIAL AND METHODS

In this study 42 ejaculates from 10 Holstein bulls, were used. The animals were 3 to 5 years old, clinically healthy and with good reproductive performance. Ejaculates were obtained by using of artificial vagina and then their volume, concentration and motility were determined. After assesment, the semen was diluted with Triladil extender, equilibrated at 4 °C for 4 hours and freezed according to Cassou paillette technology (1964).

Computer sperm class analyzer (CASA) was used for evaluation of semen motility, both after thawing and after incubation at 39ºC for 5 hours. NADH- Tetrazolium Reductase activity in spermatozoa was performed by the Kiernan method (Kiernan, 1981), modified by Subev and Yonkov (Subev and Yonkov, 1992)

Intensity of the reaction is expressed by the amount of accumulated diformazan in mitochondria (Fig.1). From those data, using the method of Astaldi et Verga (1957) is calculated the mean cytochemical coefficient (MCC).

Two experiments were conducted with three different media for semen thawing. Medium №1 was 2.8% sodium citrate solution, and media № 2 and № 3 consisted of different combinations of carbohydrates. In experiment 1 were used ejaculates with greater motility than those which were used in experiment 2.

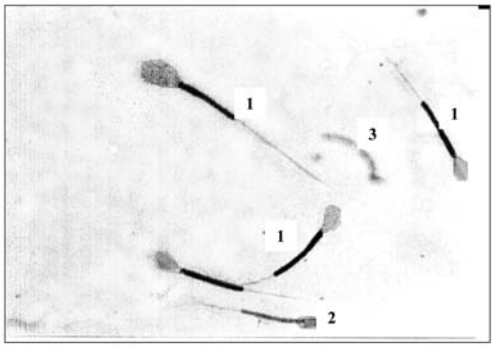


Figure 1. *Different intensity of NADH- Tetrazolium Reductase in spermatozoa of bull: 1 strong reaction, 2-moderate reaction, 3-weak reaction (Magnification: 40x)*

RESULTS AND DISCUSSION

In Table 1, 2 and 3 are presented data on motility and MCC of NADH- Tetrazolium Reductase from bull sperm after thawing. In media 2 and 3, containing different combinations of carbohydrates was observed higher MCC in sperm, compared with medium 1.

Table 1. *Motility after thawing and after incubation and MCC after incubation of bull spermatozoa treated with medium 1*

Experiments	Medium 1		
	Motility %		MCC
	After thawing	After incubation	
1	32.46+0.3	17.38+ 0.61	2.98+0.2
2	18.32+0.5	7.37+1.02	2.28+0.4

Table 2. *Motility after thawing and after incubation and MCC after incubation of bull spermatozoa treated with medium 2*

Experiments	Medium 2		
	Motility %		MCC
	After thawing	After incubation	
1	37.52+0.71	17.42+ 0.71	3.06+0.4
2	23.68+0.53	10.34+0.60	2.74+0.3

Table 3*. Motility after thawing and after incubation and MCC after incubation of bull spermatozoa treated with medium 3*

Experiments	Medium 3		
	Motility %		MCC
	After thawing	After incubation	
1	40.26+0.58	17.44+ 0.58	3.09+0.3
2	22.78+0.68	7.20+0.64	2.86+0.5

The results demonstrate that along with increased motility there is increase of NADH- Tetrazolium Reductase activity in spermatozoa.

The intensity of enzyme cytochemistry, reaction depends on the possibility of mitochondria to oxidize exogenous NADH, which is a source of electrons and protons. At the same time in mitochondria reduction of exogenous tetrazolium salt, an artificial acceptor of hydrogen atoms, takes place(Piasecka et al., 2001). Di-formazan deposits occur in the mitochondria, which are indicative for highly active organelles (Dariusz et al., 2003). Higher MCC, is exponential for the functional activity of cells and is a prerequisite for better flow dynamics of cellular metabolic processes. As a result

stimulating of the viability of male gametes is observed.

Conclusion: The data from this survey, suggests that there is a relationship between motility and activity of the enzyme system NADH- Tetrazolium Reductase expressed by MCC

REFERENCES:

1. Mita M., S. Oguchi., S. Kikuyama, R. de Santis and M. Nakamura. 1994, Endogenous substrates for energy metabolism in spermatozoa of the Sea Urchins Arbacia Luxula and Paracentrotus lividus. Biol. Bul. 186: 285-290.
2. Kleniewska P., A.Piechota, B. Skibska, A. Goraca, 2012,The NADPH Oxidase Family and its Inhibitors, Arch. Immunol. Ther. Exp. http://www.springerlink.com
3. Цветкова П. 2000. Постпаротиден орхид и инфертилитет. НФ Андрология. С, 65.
4. Cassou R.V., 1964. La methode des pailletes en plastique adaptee a la generalisation de la congelation. V Congr. Inter. Reprod. Anim. Fecond. Artif., Trento, v. 4, 540-546.
5. Kiernan J.A .,1981. Histological and Histochemical Methods: Theory and Practice. Oxford: Pergamon Press pp 116-153.
6. Subev M, Yonkov Y.,1992. Cytochemical study of NADH and NADPH-tetrazolium reductases in boar spermatozoa. In: Proc. 12th Congress International Pig Veterinary
7. Astaldi G., L. Verga. ,1957. The glycogen content of the cells of lymphatic leukemia. Acta Haematol. 17,129-135.
8. Piasecka M., L. Wenda-Rozewicka , T. Ogonski, 2001. Computerized analysis of cytochemical reactions for dehydrogenases and oxygraphic studies as methods to evaluate the function of the mitochondrial sheath in rat spermatozoa. Andrologia, 33: 1-12.
9. Dariusz G., M. Piasecka, J. Udala, B. Blaszczyk, M. Laszczynska, A. Kram, 2003. Oxidoreductive capability of boar sperm mitochondria in fresh semen and during their preservation in BTS extender. Reproductive Biology, 3 (2):161-172

АКТИВНОСТ НА NADH-ТЕТРАЗОЛ РЕДУКТАЗА ВО СПЕРМА ОД БИКОВИ ПО КРИОПРЕЗЕРВАЦИЈА ВО ПОГЛЕД НА НИВНАТА ПОДВИЖНОСТ

Стефанов Росен, Червенов Михаил, Георгиев Бојко, Качева Димитрина, Таушанова Паулина, Кистанова Елена, Сабев Милко

Институт за биологија и имунологија на репродукцијата, БАС, Софија, Бугарија

АПСТРАКТ

NADH-тетразол редуктазниот ензимски систем може да се користи како маркер за функционалниот статус на митохондриите. Неговата активност ни дава информација за интрацелуларната оксидација, а неговите квантитативни карактеристики се изразени преку средниот цитохемиски коефициент (МСС).

Целите на ова истражување беа да се воспостави врска меѓу подвижноста на биковската сперма и средниот цитохемиски коефициент на NADH-тетразол редуктазата во сперматозоидите.

Во ова истражување беа користени 42 ејакулати од 10 Холштајн бикови. По добивањето на семето со помош на вештачка вагина, тоа беше замрзнато според Cassou paillette технологија. За евалуација на подвижноста на спермата по одмрзнување и по инкубација на 39º С за време од 5 часа, беше користен компјутерски асистиран анализатор на сперма (CASA). На тој начин беше одредена NADH-тетразол редуктазната активност во сперматозоидите.

Резултатите покажуваат дека заедно со зголемената подвижност постои зголемување на МСС. Податоците од ова истражување укажуваат на тоа дека постои врска меѓу подвижноста и активноста на ензимскиот систем NADH-тетразол редуктаза.

UDC: 636.4.09:612.616.2.086.13

DISTRIBUTION OF THE SPERMATOZOA SUBPOPULATIONS IN CRYOPRESERVED BOAR SEMEN

Mickov Ljupco[1], Dovenski Toni[1], Petkov Vladimir[1], Atanasov Branko[1], Adamov Nikola[1], Nikolovski Martin[1], Blagoevska Katerina[1], Tomeska-Mickova Svetlana[2]

[1] *Institute of Reproduction and Biomedicine, Faculty of Veterinary Medicine – Skopje;*
[2]*Food and Veterinary Agency of R. Macedonia*

ABSTRACT

Ejaculates obtained by 3 different breeds of boars, were cryopreserved using 2 different cryopreservation protocols. The samples were assessed by CASA before freezing, and after thawing. The objective of the study was to estimate the difference in the distribution of subpopulation of spermatozoa in liquid semen, and in semen cryopreserved by two different cryo-protocols. The samples taken by glowed hand technique, were extended to ratio 1:1 and taken to laboratory for CASA analysis. The first group of ejaculates was criopreserved according the procedure described by Westendorf et al. modified by Thurnston et al. In the second group, only P1 of SRF was cryopreserved according the procedure by Rodriguez-Martinez and Wallgren. The analysis was performed by CASA, using the standard set-up for boar semen. The proportions of Rapid subpopulation of spermatozoa ranged from 77.45 ± 0.47 %, 25.02 ± 0.63 %, and 28.90 ± 1.17 %, in liquid semen, TCP semen, and SCP semen, respectively. The proportion of Medium subpopulation of spermatozoa was on levels of 3.45 ± 0.10 %; 4.20 ± 0.18 % and 4.14 ± 0.41 % respectively in the mentioned groups. The Slow subpopulation of spermatozoa was represented by 3.45 ± 0.10 %; 4.20 ± 0.18 % and 4.14 ± 0.41 %, respectively, and the Static subpopulation was in proportions of 12.90 ± 0.36; 61.37 ± 0.70 and 55.68 ± 1.52 % respectively (LS, TCPS, XCPS). The results showed significant difference in all assessed parameters ($p < 0.05 – p < 0.00005$).

Key words: boars semen, CASA analysis, cryopreservation

INTRODUCTION

Despite the widespread of use of cryopreserved semen in cattle, its utilization in swine is limited, mostly as a result of the poor cryo-survival of boar spermatozoa. In order to overcome the mentioned problem, most of the work so far was focused on designing a suitable cryo-protocol for boar semen.

The evidences derived from a number of studies strongly suggests that different subpopulations of spermatozoa coexist within any typical mammalian ejaculate (Chang et al., 1975; Bedford et al., 1983). These, as was suggested is a result to variations in the assembly of individual spermatozoa during spermatogenesis as well as to differential maturational status and age of the sperm cells during the process of mixing or the different spermatozoa generations in the epididymis.

In theory, computer-assisted semen analysis (CASA) systems permit the study of motion characteristics in sperm subpopulations to an unprecedented degree of sophistication. As the trajectories of individual spermatozoa are determined by their flagellar function, characteristics such as sperm velocity, flagellar beat frequency, and amplitude of the lateral head sidplacement should, with a high percent of certainty reflect the physiological status of individual cells. Large sets of data derived from hundreds or thousands of individual sperm measurements are routinely provided by CASA systems, so the problem of dissecting them should be amenable to established methods of multivariate analysis. This valuable potential of CASA has, however, been rather neglected (Abaigar et al., 1999).

According the WHO guideline, for CASA evaluation of the human semen, spermatozoa in the ejaculates were classified in 4 subpopulations according the velocity of the average path (VAP): Rapid, Medium, Slow and Static, and most of the CASA systems are equipped with an option of differentiation of the mentioned subpopulations in different animal species.

The objective of this study was to determine the differences of the subpopulations of the spermatozoa, classified according the VAP in liquid boar semen, and cryopreserserved boar semen, by two different cryo-protocols.

MATERIALS AND METHODES

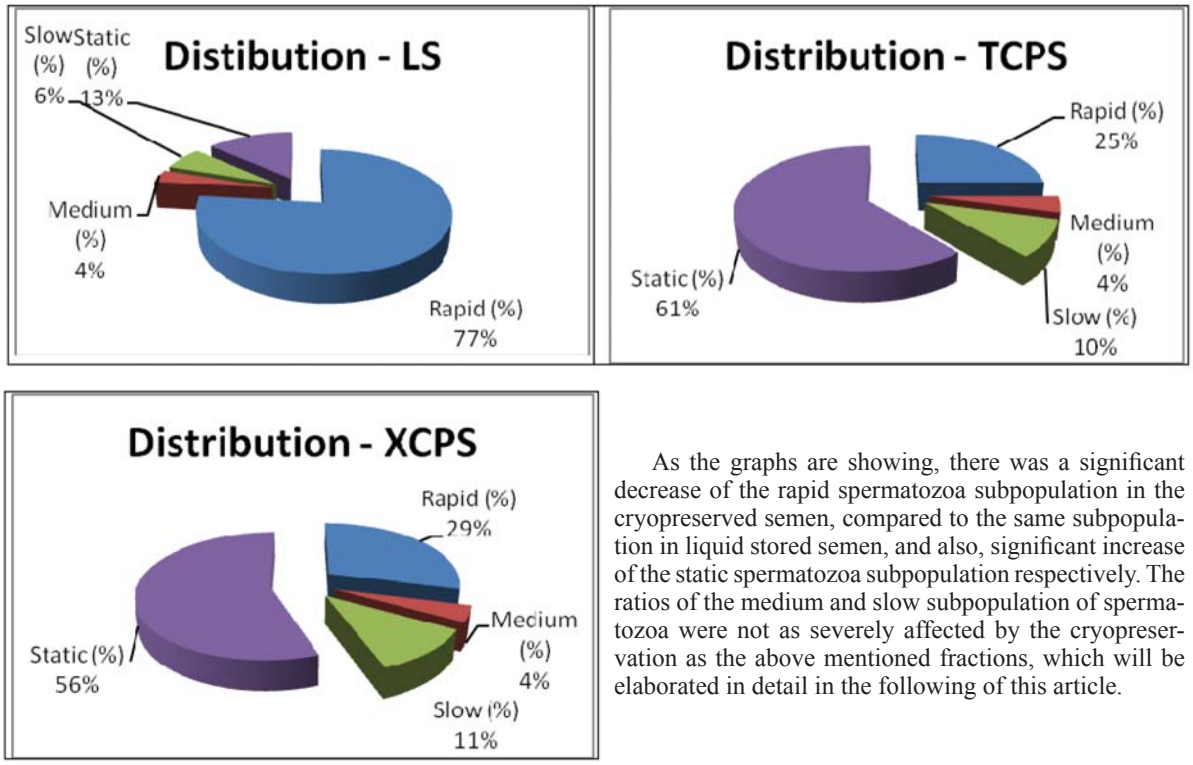
Total of 713 ejaculates, obtained from 19 boars of 3 different breeds (Landrace, Yorkshire and Durock) were used in our investigation. The ejaculates were obtained traditionally, by 'glowed hand' technique, in field conditions. The first, contaminated, fraction of the ejaculate was separated, while the rest of the P1 or full SRF of the ejaculate was collected in a plastic bag, which is filtering the gelatin fraction from the spermatozoa rich fraction. After the routine examination of the quantitative parameters of the obtained ejaculates, by phase-contrast microscopy, the semen was diluted (Androhep®, Minitub, Tiefenbach, Germany) in ratio 1:1 and transferred in glass containers. Chilled on 17°C were transported to the laboratory, where were assessed by CASA (HTM-IVOS V.12.0, Hamilton Thorne Research, MN, USA), and cryopreserved.

The first group of ejaculates (389) consisted by the complete SRF, were cryopreserved according the protocol described by Westendorf et al. (1975) modified by Thurnston et al.(2002) and the second group (171), consisted of P1 of SRF, was frozen according to the protocol described by Rodriguez-Martinez and Wallgren (2011) while the group of 713 evaluated ejaculates of liquid semen, were considered as a control group. The thawing was performed in a water-bath preheated on

39°C, and all the samples were submitted for CASA evaluation of the subpopulations of spermatozoain liquid as well as in cryopreserved semen. Statistical analysis was carried out in Statistica 7.0 (StatSoft Inc. Tulsa, OK, USA).

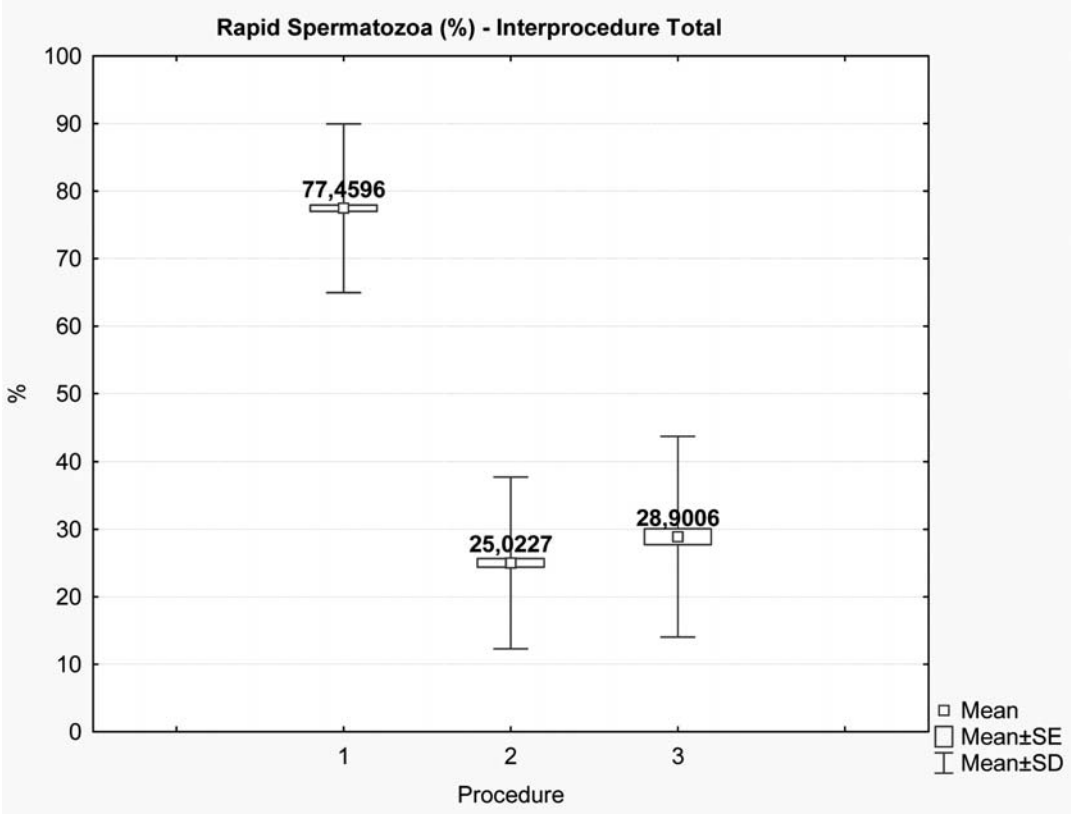
RESULTS AND DISSCUSION

The preview of the obtained results regarding the subpopulations of spermatozoa contained in liquid and cryopreserved semen, are given in following figure.



As the graphs are showing, there was a significant decrease of the rapid spermatozoa subpopulation in the cryopreserved semen, compared to the same subpopulation in liquid stored semen, and also, significant increase of the static spermatozoa subpopulation respectively. The ratios of the medium and slow subpopulation of spermatozoa were not as severely affected by the cryopreservation as the above mentioned fractions, which will be elaborated in detail in the following of this article.

The values of the Rapid subpopulation of spermatozoa are showed on the following graph:



Breakdown Table of Descriptive Statistics (Spreadsheet143)
Smallest N for any variable: 1262

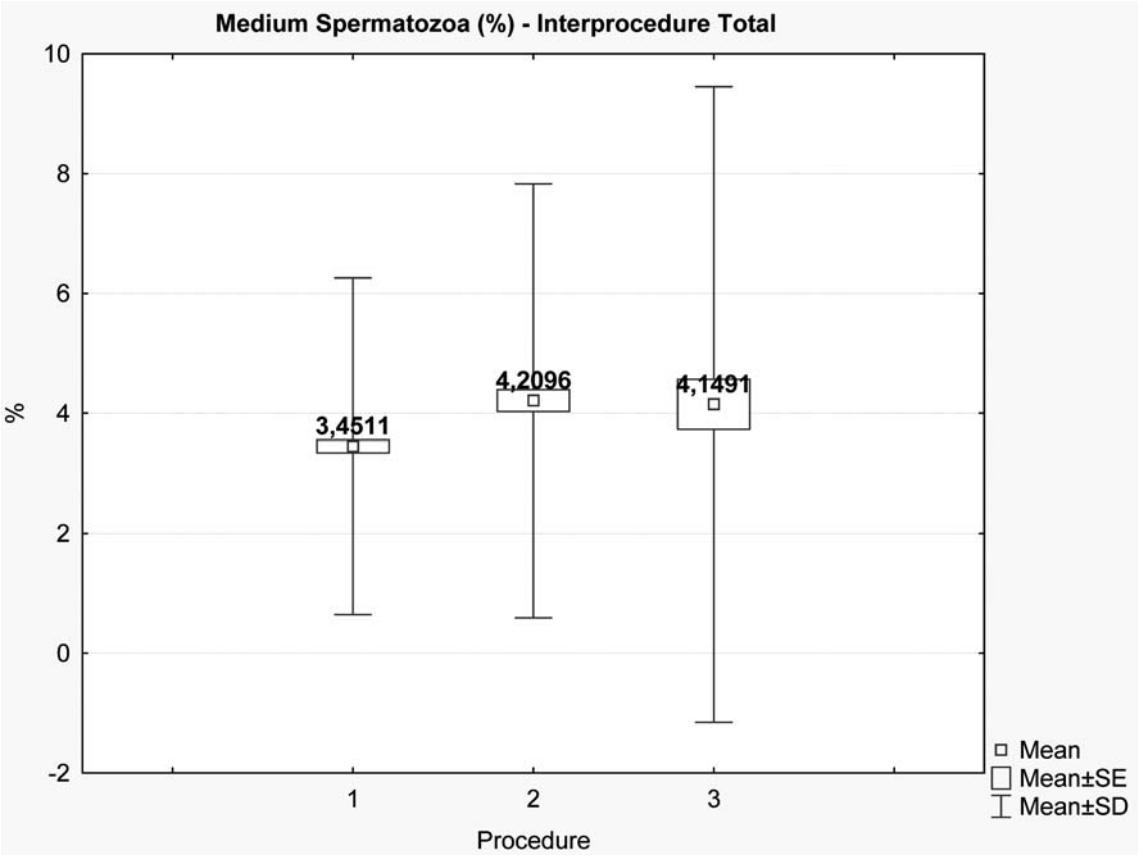
	Means	N	Std.Dev.	Variance	Std.Err.	Minimum	Maximum
LS	77,45957	705	12,49324	156,0811	0,470523	14,00000	98,00000
TCPS	25,02273	396	12,71646	161,7083	0,639026	1,00000	77,00000
XCPS	28,90062	161	14,86826	221,0651	1,171783	1,00000	62,00000
All Grps	54,81062	1262	28,58319	816,9990	0,804602	1,00000	98,00000

Analysis of Variance (Spreadsheet143)
Marked effects are significant at p < ,05000

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Var5	821109,4	2	410554,7	209126,3	1259	166,1051	2471,657	0,00

The means of the rapid sperm subpopulation were 77.45 ± 0.47 % in LS, 25.05 ± 0.63 % in TCPS and 28.90 ± 1.17 % in XCPS. There was high significance in the difference of the levels of this subpopulation of spermatozoa between the LS and the both groups of cryopreserved semen ($p < 0.00005$), and the difference of the proportion of this subpopulation of spermatozoa was also significant between both cryopreservation protocols ($p < 0.05$). The difference between the LS and the cryopreserved semen was expected, according the postulated loss of motility during the cryopreservation due to irreparable damages occurring during the both freezing and thawing process (Morris 2006; Morris *et al* 2007). The analysis of variance was significant as well.

The values of the Medium subpopulation of spermatozoa are showed on the following graph:

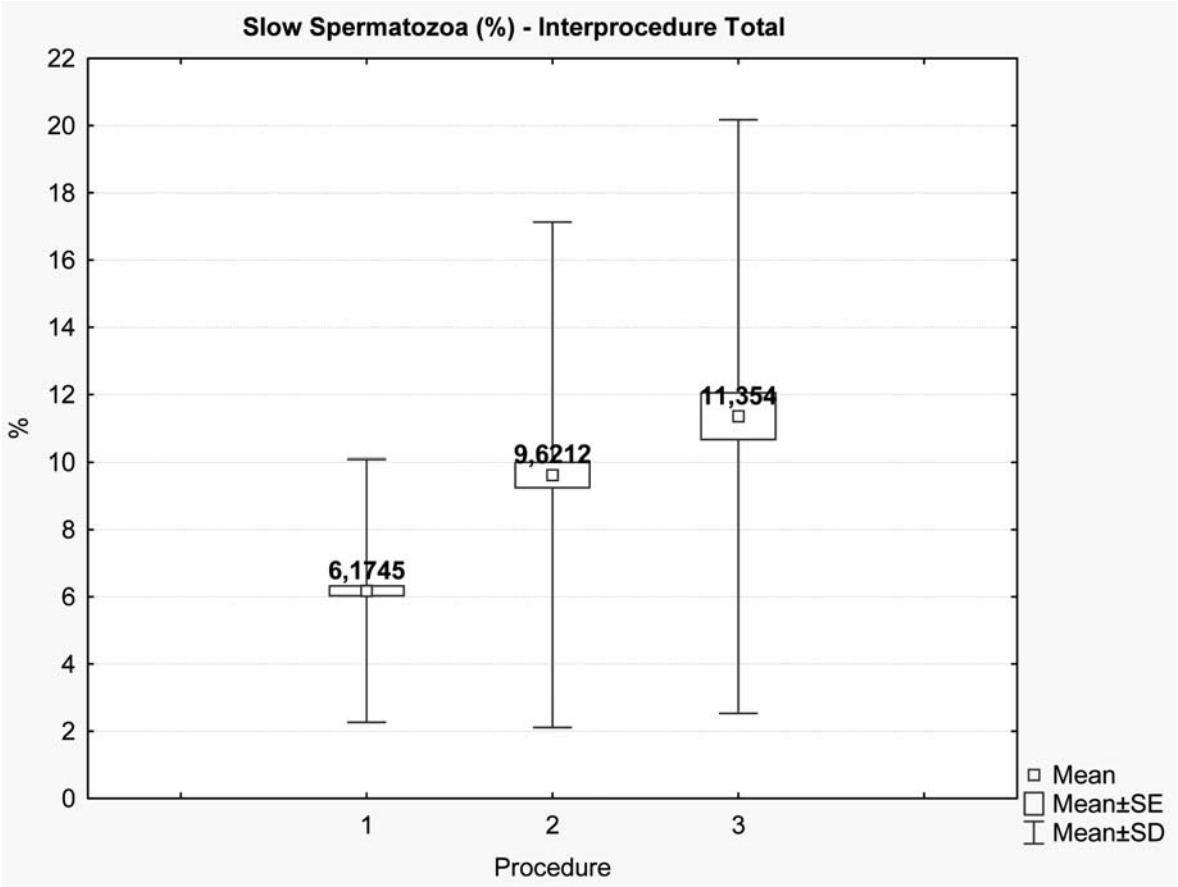


Var4	2-Way Tables of Descriptive Statistics (Spreadsheet147) Smallest N for any variable: 1262						
	Means	N	Std.Dev.	Variance	Std.Err.	Minimum	Maximum
LS	3,451064	705	2,805879	7,87296	0,105676	0,00	27,00000
TCPS	4,209596	396	3,619778	13,10279	0,181901	0,00	31,00000
XCPS	4,149068	161	5,301192	28,10264	0,417792	0,00	31,00000
All Grps	3,778130	1262	3,493040	12,20133	0,098327	0,00	31,00000

Variable	Analysis of Variance (Spreadsheet147) Marked effects are significant at p < ,05000							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Var5	171,2888	2	85,64439	15214,59	1259	12,08466	7,087034	0,000870

The average values of the Medium spermatozoa subpopulation were 3.45 ± 0.10 % in LS, 4.20 ± 0.18 % in TCPS and 4.14 ± 0.41 % in XCPS. There was significance in the difference of the levels of this subpopulation of spermatozoa between the LS and the TCPS ($p < 0.01$). The analysis of the variance was significant as well ($p < 0.001$). According the recent studies, it is the subpopulation consisted mostly by spermatozoa demonstrating hyperacctivated pattern of kinetic activity (Taitzoglou et al. 2001), and supports the recent hypothesis that cryopreservation has a capatitation-like and hyperactivation-like effect on boar spermatozoa (Schmidt and Kamp, 2004).

The values of the Slow subpopulation of spermatozoa are showed on the following graph:



Breakdown Table of Descriptive Statistics (Spreadsheet151)
Smallest N for any variable: 1262

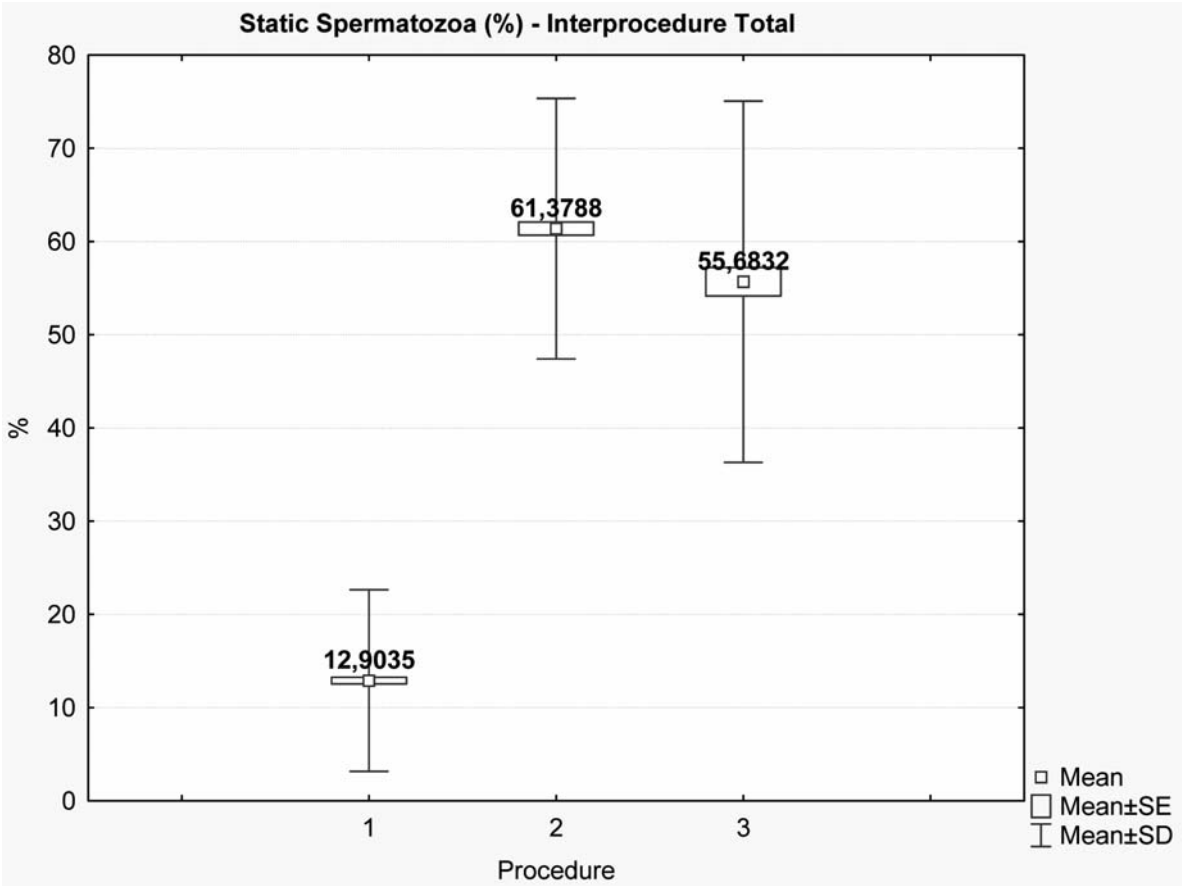
	Means	N	Std.Dev.	Variance	Std.Err.	Minimum	Maximum
LS	6,17447	705	3,907587	15,26923	0,147168	1,000000	39,00000
TCPS	9,62121	396	7,509855	56,39793	0,377384	1,000000	56,00000
XCPS	11,35404	161	8,820013	77,79262	0,695114	0,000000	55,00000
All Grps	7,91680	1262	6,338725	40,17943	0,178432	0,000000	56,00000

Analysis of Variance (Spreadsheet151)
Marked effects are significant at p < ,05000

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Var5	5192,722	2	2596,361	45473,54	1259	36,11878	71,88396	0,000000

The average values of the Slow spermatozoa subpopulation were 6.17 ± 0.14 % in LS, 9.62 ± 0.37 % in TCPS and 11.35 ± 0.69 % in XCPS. There was high significance in the difference of the levels of this subpopulation of spermatozoa between the LS and the both groups of cryopreserved semen ($p < 0.00005$), and the difference of the proportion of this subpopulation of spermatozoa was also significant between both cryopreservation protocols ($p < 0.05$).. The analysis of the variance was significant as well.

The values of the Medium subpopulation of spermatozoa are showed on the following graph:



Breakdown Table of Descriptive Statistics (Spreadsheet155)
Smallest N for any variable: 1262

	Means	N	Std.Dev.	Variance	Std.Err.	Minimum	Maximum
LS	12,90355	705	9,74457	94,9566	0,367002	0,000000	61,00000
TCPS	61,37879	396	13,97314	195,2486	0,702177	7,000000	88,00000
XCPS	55,68323	161	19,38151	375,6428	1,527477	7,000000	98,00000
All Grps	33,57211	1262	26,56891	705,9072	0,747901	0,000000	98,00000

Analysis of Variance (Spreadsheet155)
Marked effects are significant at p < ,05000

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Var5	686073,5	2	343036,7	204075,5	1259	162,0933	2116,292	0,00

The average values of the Slow spermatozoa subpopulation were 12.90 ± 0.36 % in LS, 61.37 ± 0.70 % in TCPS and 55.68 ± 1.52 % in XCPS. There was high significance in the difference of the levels of this subpopulation of spermatozoa between the LS and the both groups of cryopreserved semen ($p < 0.00005$), and the difference of the proportion of this subpopulation of spermatozoa was also high significant between both cryopreservation protocols ($p < 0.0005$). The analysis of the variance was significant as well.

CONCLUSIONS

1. The distribution of Rapid subpopulation of spermatozoa was significantly lower in cryopreserved semen, compared to liquid semen ($p < 0.00005$), and the differences in the distribution of the mentioned subpopulation, between the two cryo-protocols were also significant ($p < 0.05$).
2. The distribution of the Medium subpopulation of spermatozoa was significantly increased in TCPS compared to the one in the LS ($p < 0.01$)
3. The increase of the distribution of the Slow subpopulation of spermatozoa was significant between LS and cryopreserved semen ($p < 0.00005$), and the difference of the proportion of this subpopulation of spermatozoa was also significant between both cryopreservation protocols ($p < 0.05$).
4. The increase of the Static subpopulation of spermatozoa was significant in cryopreserved semen, compared to LS ($p < 0.00005$), and the difference of the distribution of this subpopulation of spermatozoa was also significant between the studied cryo-protocols.

REFERENCES

1. Westendorf et al. Deep freezing of boar sperm. Laboratory and insemination results using the Hulsenberger paillete method. Deutsche Tierärztliche Wochenschrift 82:261-267 (1975);
2. Thurston et al. Semen cryopreservation: a genetic explanation for species and individual variation? Cryo Letters 23: 255-262(2002);
3. Rodriguez-Martinez, Wallgren, Advances in Boar Semen Cryopreservation. SAGE-Hindawi Veterinary Medicine International, Vol. 2011, Art.ID 396181. (2011).
4. Chang MC, Hunter RHF. Capacitation of mammalian sperm: biological and experimental aspects. In: Hamilton DW, Greep RO (eds.), Handbook of Physiology. Endocrinology V. Washington: American Physiological Society; 1975: 339–351.
5. Bedford JM. Significance of the need for sperm capacitation before fertilization. Biol Reprod 1983; 28:108–120.
6. Abaigar, T., Holt, W.V., Harrison, R.A., & Del Barrio, G. 1999. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biology of Reproduction 60*: 32–41.
7. Morris, G.J. 2006. Rapidly cooled human sperm: no evidence of intracellular ice formation. *Human Reproduction 21*: 2075–2083.
8. Morris, G.J., Faszer, K., Green, J.E., Raper, D., Grout, B.W.W., & Fonseca, F. 2007. Rapidly cooled horse spermatozoa: loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. *Theriogenology 68*:804–812.
9. Schmidt H., G. Kamp - Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis, 2004, Reproduction, 128,171-179.

ДИСТРИБУЦИЈА НА СУБПОПУЛАЦИИТЕ НА СПЕРМАТОЗОИДИ ВО КРИОПРЕЗЕРВИРАНА СПЕРМА ОД НЕРЕЗИ

Мицков Љупчо[1], Довенски Тони[1], Петков Владимир[1], Атанасов Бранко[1], Адамов Никола[1], Николовски Мартин[1], Благоевска Катерина[1], Томеска-Мицкова Светлана[2]

[1] Институт за репродукција и биомедицина Факултет за ветеринарна медицина – Скопје;
[2]Агенција за храна и ветеринарство

АПСТРАКТ

Ејакултатите добиени од 3 различни раси нерези, беа криопрезервирани со користење на 2 различни протоколи за криопрезервација. Примероците без проценети со помош на CASA пред замрзнувањето и по одмрзнувањето. Целта на истражувањето беше да се проценат разликите во дистрибуцијата на субпопулациите на сперматозоиди во течна сперма и во сперма криопрезервирана по пат на два различни крио-протоколи. Примероците земени по пат на мануелна фиксација на главата на пенисот без екстендирани во размер 1:1 по што беа пренесени во лабораторија каде беше изведена CASA анализа. Првата група ејакулати беше криопрезервирана според процедурата опишана од Westendorf et al. (1975) модифицирана од страна на Thurnston et al. Кај втората група, само P1 одбеше криопрезервирана според процедурата на Rodriguez-Martinez and Wallgren. Анализата беше изведена со помош на CASA, со користење на стандардниот сетап за сперма од нерези. Процентите на Брзата суппопулација на сперматозоиди се движеа од 77.45 ± 0.47 %, 25.02 ± 0.63 %, и 28.90 ± 1.17 %, во течна сперма, TCP сперма, и XCP сперма, респективно. Процфентите на субпопулацијата Средно брзи сперматозоиди без на ниво од 3.45 ± 0.10 %; 4.20 ± 0.18 % и 4.14 ± 0.41 % респективно кај споменатите групи. Субпопулацијата Бавни сперматозоиди беше претставена со 3.45 ± 0.10 %; 4.20 ± 0.18 % и 4.14 ± 0.41 %, респективно, а субпопулацијата Статични сперматозоиди беше во рамките од 12.90 ± 0.36; 61.37 ± 0.70 и 55.68 ± 1.52 % респективно (LS, TCPS, XCPS). Резултатите покажаа значајна разлика кај сите испитани параметри ($p < 0.05 – p < 0.00005$).

Клучни зборови: сперма од нерези, CASA анализа, криопрезервација.

UDC: 636.2.09:618.14-072.1

UTEROSCOPY EXAMINATIONS OF UTERINE DISORDERS IN DAIRY COWS

Pavlović Miloš[1], Pavlović Vojislav[1], Vakanjac Slobodanka[1],
Pavlović Ivan[2], Jakić-Dimić Dobrila[2]

[1]Department for obstetrics, fertility and a.i., Faculty of veterinary medicine, Belgrade University, Serbia
[2]Scientific veterinary institute „Serbia", Belgrade, Serbia[2]

ABSTRACT
Endoscopy of uterus (uteroscopy) is one of many methods in diagnosis of the female genital diseases. In bovine practice uteroscopy as diagnostic and therapeutic technic is not very common in use. It describes limiting reproductive performance in a precise way. Thirty Holstein-Friesian cows were exposed to uteroscopy, all of them 150 and more days after parturition. Various types of endometritis are causing decreased fertility and large economic losses. High producing dairy cows are subfertile during lactation. Ovarian inactivity is associated with enhanced uterine involution. Reduced reproductive performance in dairy cattle is often caused by uterine disorders. Beside acute metritis and chronic endometritis, the objective of present study is to show negative impact of subclinical endometritis. In earlier postparturient period the local inflammation of uterus by many authors is called metritis. Endoscopy examination of the uterus (uteroscopy) in cows allows to receive the real intrauterine picture and recognise nonpalpatoral changes . Endoscopy has the advantage of recognizing endometrial changes and damages. It needs special, expensive equipment, conditions and competence not so easy for stable use. In bovine practice histeroscopy is occasionally used. The common method of detection of pathological features concerning gynecological examinations with vaginal or rectal exploration – palpation provides little information about inflammation of the endometrium itself. Endoscopic view of uterus in cows may be important especially when general or untypical symptoms occur. It gives veterinarians wide intrauterine inspection and imagination like an „open window view" about the health problem what is more than palpable impressions. Endometrium of cows without uterine discharge is a common result of dystocia or retained placenta and concerns deeper layers of uterine wall and various damage of itself. Different forms of endometritis or tissue damages could be diagnosed and treated, even to a small target of endometrial tissue through endoscopic examination of uterus .
Key words: cows, uteroscopy, uterus

INTRODUCTION

Fertility of cattle is the most important element in the economics of keeping dairy cows. Pregnancy rates after the expected percentage of the service period, the cows, is often known to be reduced from once visible and understandable reasons, but there are also those that are not available in routine diagnostic methods. Changes that may occur in the reproductive tract of calved cows are causing infertility, which dramatically reduces the economic impact. It is reflected in the extended service period, reducing the secretion of milk and the lack of several semen doses to be used or for renewal of the herd or for fattening and resale. Farmers who do not adequately monitor the state of fertility felt long after all the losses incurred.

Factory Farming cows with emphasis on high milk yield has resulted in cows becoming subfertile in the lactation period. The reasons for this statement are numerous and are not unique. Numerous studies indicate that factors such as reproductive diseases (retention of the placenta, endometritis and ovarian cysts) or greater importance of seasonal calving have a huge impact on the reproductive performance of cows. Yet it must be clear that the physiological process of lactation is more closely associated with reduced fertility of high yielding cattle than it is the case with beef cattle throats.

Application of uteroscopy, as a method of choice in diagnosing and treating clinical and subclinical endometritis cows becomes more and more present. Enlarging the number of dairy cows on individual farms in order to increase the quantity and improve the safety of milk, results in consequent changes in the reproductive tract after calving. Increasing the intensity of milk production has as a consequence of the post- calving period appearance of pathological changes in the endometrium. The change, which is caused by the extension of service period, increases the consumption of seed dose and increasing housing costs.

The most common indication for flexible endoscopes for gynecological treatment of cows is an inspection of the interior of the uterus, and can be followed by taking samples for biopsy, and treatment of the working channel of the instrument visual control. This technique is applied to fertility disorders, which are early clinical methods such as rectal palpation, ultrasonography, vaginoscopy, or bacteriological tests could not be resolved.

Endoscopic inspection of the uterus includes an overview of all the associated parts of the uterus, diagnoses changes in the mucosa and detection of defects. This method helps in the diagnosis of endometritis, sampling and uterine secretions, detection of changes in macroscopic appearance of the endometrium. Uteroscopy is used to determine the amount, nature and localization of secretion. Under visual control of the browser, the uterine lavage or administer targeted therapies is to performed. This method visualizes and the *sterilitas sine materia*.

Uteroscopy should be considered a complementary diagnostic method for medical checkup of cows. The introduction of the endoscope in the uterus is principally a burden for cow uterus and should be a good indication of

the measure. It is wrong to consider uteroscopy a routine diagnostic method.

Endoscopy is constantly being improved, in particular their flexibility and mobility. There were designed biopsy forceps for grasping, brushes, vacuum devices, surgical electrical coagulants, a separate working channel that runs through these instruments and handled under the control of the eye.

Construction endoscopes are classified as non-crimp, which can be flexible and rigid. Mode of operation is the same for both forms. Fixed endoscopes are most commonly used in endoscopic surgery through the working channel.

Endoscopic apparatus with fiber optic fibers, used in uteroscopy of cows, belong to the flexible group. At complex surgery to remove a lesion, surgical treatment of salpingitis or the transplantation of embryos used rigid endoscopes, laparoscopy. The diameter itself, is flexible endoscope for uteroscopy of cows must not exceed the diameter of 1 cm.

Flexible endoscopes are fundamentally the same construction, but according to the manufacturers differ in details. The cable for the implementation of light is a combination of light source to the endoscope. It consists of an incoherent fiber glass. Light is carried to the head of the endoscope into the device through one or two, also, for the implementation of incoherent beam of light, reaches its peak, which rises and illuminates the object to be examined.

The light reflected by the object accepts an optical device (lens system) on top of an endoscope, a coherent beam of light to conduct him forward, and an optical device on the head of the endoscope it reflects. Ocular increases the picture that transports the beam from eight to twelve times to carry out light. With most flexible and a rigid endoscope eyepiece part can be adjusted according to the sphere of examiners.

Mechanical devices for flexible endoscopes with glass fibers with larger instruments consist of wire pulling the tip of the endoscope, which rotates in all directions. Instruments with a small diameter often have only a device to run in two directions, while very small uroscopies, as a rule, do not tow wire to activate the motion. For larger models, it is done using the command buttons, while others prefer manufactured structure with handles.

If the endoscope must be made in sampling, biopsying or smaller operation, it should be noted that the instrument has one or two working channels. Through them may be set for taking a biopsy forceps and pincers to capture the foreign objects, brush cytology, suction tubes or diathermic loop. Diameter of the channels on the instrument, as a rule, is two to three millimeters. Channel hopper, whose distal end is turned so that the lens of the eye can reach the water and air, is used for insufflations of air and bringing the wash liquid.

Preparing for an ideal uteroscopy includes epidural anesthetion. The animal is placed in the box for inspection. Before the introduction of an endoscope, it is brewed in the area of the caudal vertebrae, in terms of hair cutting and shaving the hair and skin iodine to be applied by shallow epidural anesthesia . Cleaning and disinfection of the rectum, vulva and labia perineum follows. Epidural anesthetic effect is achieved by temporarily stopping peristalsis and relaxation of the cervix to facilitate the passage of endoscope .The examination is possible without such preparation with the endoscope diameter about 5 mm (or up to 1cm for wider endoscopes) if there is atony of the uterus with an open cervical canal.

Implementation of uteroscopy of cows is relatively short. For the viewer, it is important to have an instrument which is narrower than the diameter of the cervical canal, to avoid injury and hemorrhagic lesions and the occurrence of defects that would have further consequences on the already existing sterility. The review begins in the vagina, the finger guiding hand of the browser to access *Porcia vaginalis* and gently pushing the instrument into the cervix under close visual control.

The very use of endoscopy in terms of views of barned cows is satisfactory in modern conditions. The existence of separate milking parlor stalls to the introduction of the endoscope apparatus which is comprised of cold light source includes a supporting base for the apparatus, equipment to be protected from the animal's unforeseen reactions which may lead to damage. It is also necessary to provide the most hygienic conditions of the apparatus before and after the inspection or disinfection of endoscopes and working channels that can reach different contents of the uterus and the infective material would be transferred from one animal to another.

Uteroscopy is the method that a browser will choose for application and it is justified in those cases of valuable dairy cows, and in whom previous standard methods of examination and diagnosis weren't giving results . This method is commonly used with cows that have a long service period with unknown causes of infertility. These cases entail the lack of quality offspring , which increases the economic loss.

Double layered muscular structure of the cervix and the inability of manual dilatation of the cervical canal as is the case in mares is certainly one of the preconditions why this method has not been previously introduced in the cow. The openness of the cervix in puerperium and estralne stage uterine atony have created the conditions for the application of uteroscopy as a diagnostic method in cows.

Uteroscopy has a major role in controlling the status of postpartum metritis induced by retained placenta and a difficult delivery. Caused inflammatory process can be visible and monitored through the instrument itself. In this way, the method receives a full justification for the diagnosis and treatment of cows as a preparation for conception. By using this advanced diagnostic technology greater opportunities arise to resolve a significant number of causes of infertility, increased conception, as well as a reduction of service period to a lower economic and biological cost. Endoscope inspection allows visualization of the cervical canal and the status of cervical rings, access and review of the mucous membrane of the uterus, accompanying horn, base of the uterus. This method allows you to discover the presence of any abnormalities that explain the causes of sterility.

In cows, postpartum changes often occur as a result of the endometrium or lack of retention of placenta separated parts in the area placentoma. In case of a late diagnosis and lack of adequate therapy approaches, such inflammatory conditions may advance to the chronic, lasting sterility. Such changes are a direct cause of a prolonged calving period, unsuccessful insemination and sterility, as well as a highly threatening source of infection with possible fatal outcome for the embryo if conception occurs.

Application of uteroscopy is equally important in the

diagnosis of manifested and non-manifested endometritis. Manifested clinical endometritis is clearly visible, tangible and practitioners resort to traditional methods in diagnosis and therapy, such as rectal examination and use of hormone therapy with the use of antibiotics and abrasives to perform a mass effect on endometrial lining of the uterus separated parts of the present changes. At therapy and repeated unsuccessful insemination, uteroscopy becomes the method of choice because of the possibility of monitoring the effects caused by making excessive application of lavage of the uterus that can cause negative effects such as:

- The application of an inadequate concentration of solution for lavage of the uterus
- Damage to the endometrial surface layer due to excessive number of lavage.

Decisions on the application of this method have a special significance in endometritis. This subclinical form of pathological changes cannot be diagnosed by rectal palpation, and if the browser uses ultrasound, it is also impossible to detect fine and very small changes in the so-called dry or endometritis placentitis.

Postpartum uterine diseases are known to be the leading cases of reproductive inefficiency in dairy cows in veterinary literature. Endometritis is defined as inflammatory processes in the endometrium and to a minimum 21 days and more after calving, and are not associated with systemic diseases. The fallacy of a diagnosed endometritis in cows is in the absence of a universally accepted definition of the disease and a failure to identify a simple, effective diagnostic technique. Major part of the problem is that in the postpartum period, all cows have some degree of inflammatory changes of the endometrium in combination with normal involution of the uterus. The vet, however, is to establish and identify the disease and adequate access curing . Recently, endometritis has, according to literature, been divided into subclinical and clinical categories. Clinical endometritis is defined as the presence of purulent uterine contents or mucopurulentar after 21 or more days of birth. Subclinical endometritis is defined by the presence of> 18% polymorphonuclear (PMN) cells in cytological samples of uterine data collected 21-33 days after delivery, or> 10% PMN in the sample taken between 34-37 days.

Uteroscopy can be considered a valid method for researching a particular interpretation of the state of the uterus in the postpartum period. Uteroscopy with vaginoscopy is a direct method of evaluation of the endometrium with clinical endometritis (CE) in cows.

Subclinical endometritis is much easier, less expensive equipment, the presence of neutrophils (PMNs) in endometrium is proven and present content without uterus. Subclinical endometritis can be used and cytological analysis of endometrial cells that are collected by low-flow technique liquide.This method is called "cyto brush ", though the technique can be performed through the working channel of the endoscope.

Improvement of gynecological diagnosis in cows resulted in an introduction of cytological examination of endometrium uterusa. "Cyto brush" technique with the use of low-volume uterine lavage is used to collect endometrial cells. Proportion of polimorfonuclearneutrofil between the number of cells (PMN) in relation to the total number of endometrial cells in the sample are used for the detection of subclinical endometritis. Variation of results ranges from 5-18%. This technique is limited in terms of clinical diagnosis of endometritis. Uteroscopy through its working channel allows the visualization of changed sites, and lavage conducting fluid (0.9% sodium chloride sol.), as well as passing a sterile cotton swab, which is fixed to the working alligator clamps to the target spot area of 3 cm, and all in aseptic and sterile conditions.

Cows with subclinical endometritis have uterine discharge, although there are problems that affect the reproductive performance. Uteroscopy, as a poorly invasive method to determine the degree of inflammation of the endometrium, includes uterine lavage and cytology content of collected liquid. The examination of intrauterine fluid with bacteriological findings with respect to the involution of the uterus shows the nature of the inflammatory process. Polymorphonuclear cells as the predominant finding in intrauterine fluid and their relationship to the findings may indicate the reproductive performance of cows in the postpartum period.

Uteroscopy is a quick and simple technique which gives a positive diagnosis of the presence of cervical exudate to confirm the pathology of the uterus. Vaginoscopy is a sensitive method for improvement.

Justification of uteroscopy and vaginoscopy with rigid and flexible endoscopes

Uteroscopy as a diagnostic tool for dairy cows allows the vet to notice changes such as:

-Lesions of the cervical mucus, which lead to closing during pregnancy and causes early embryonic death.
- Partial endometritis and changes in caruncules
-Localized-metrorrhagia
-The presence of pathological-fermented liquid
-The remains of the fetal membranes caruncula with necrotic and hemorrhagic content

Uteroscopy is a quick and simple technique that allows a positive diagnosis based on the presence of exudates visually located.

Visual examination of uterine cavity was surgically relieved that allows the browser to work with a perfect diagnostic tool . Except visual inspection of uterine cavity, it is possible to biopsy suspicious tissue sections under direct visualization. The introduction of the endoscope inside the uterine cavity, which can be smaller in diameter (4-5 mm), and without using speculum, makes for comfortable review of maximum generation patient .New uteroscope, with an oval profile, allows an easy passage through the cervical canal. Instrument with a smaller diameter allows the normal review and possible biopsy, as well as the possibility of treatment of pathological changes, without the use of anesthesia. This feature is called "look and treat" procedure. Visually - the operational methods lie in a single instrument which gives uteroscopy a great role, followed by the expected improvement of the apparatus in the last 20 years.

Indications for uteroscopy

Uteroscopy is one of the diagnostic methods for diseases of the genital tract of cows, which is not a routine procedure.This method requires special, expensive equipment, environmental conditions for its implementation as well as the competence of the browser. It is used by a smaller number of practitioners, and mostly with horses and female dogs. With cows, it is used individually and is focused on specific cases. The method used most commonly to detect pathological conditions of the

uterus with a vaginal and rectal exploration is palpation. This method is usually sufficient for adequate diagnosis and therapy. The early postpartum period with the local inflammatory process in the uterus, called metritis, is often considered a consequence of dystociae or retained placenta and imply that occurs consequent thickening of the uterus. After this, there is a disorder of postpartum involution, which leads to greater reproductive disorders and infertility. It is recommended that in the early postpartum stage, different methods of gynecological examination should be performed in order to prevent fatal consequences . Influence of manual exploration of the vagina, which would have the effect of bacterial contamination of the uterus during this period is not significant in terms of normal hygiene. Manual exploration, whether vaginal or rectal, in the clinical control uterus in the postpartum period after physiological or pathological labor is always good therapy to allow the evacuation of remaining parts of the placenta, massage.

Infertility is the most common indication for performing uteroscopy. It enables visibility of endometrium. Lysis of adhesions, removal of localized changes in the endometrium, residual parts of the placenta, the fermented content, diagnosis and treatment of endometritis can be performed by uteroscopy. Uteroscopic review should be applied when abnormal uterus cannot be detected by rectal palpation. Uteroscopy is a "window" into the reproductive tract. Other abnormalities in the uterus can be detected by ultrasound or combining these techniques. The reason for the frequency of the use of endoscopes in pathology of reproduction is the absolutely accurate determination of the presence of various fluids in the uterus, connective tissue distension and other changes in the appearance of the mucosal. This method, which was previously used with a lot of reservations because of the sensitivity of instruments, gains primacy in diagnosis in the recent years due to its reliability.

The most common diagnostic methods of clinical endometritis were rectal palpation and vaginal inspections. However, they provide very little information about the inflammatory condition of the endometrium. Finding false positive results such as vaginitis or a false negative result could not be found when endometritis can lead to wrong diagnosis. Uteroscopy is applied for various reasons:

-Diagnosing endometritis
-With less biopsy sample (biopsy), uterine secretions, and lavage
-Establishing the amount, characteristics and localization of secretion in the uterus
-Controlled drainage
-Sterilitas sine materia
-Cervical defects
-Evacuation of abnormal contents
-Early embryo mortality and embryo absorption
-Habitual abortion
-Cannulated tubules

Uteroscopy implementation and application of drugs

Uteroscopy can be done in estrus or diestrus, although diestrus is recommended. Endoscope is carefully introduced with a sterile glove and the entire procedure is performed aseptic. Uteroscopy is best done with an optical fiber flexible endoscope that is known to be used for bronchoscopy or child gastroduodenoscopy. Forefinger endoscope is introduced into the cervix and then uterus. At the same time, the penetration of the top lens of the endoscope is visually monitored. When ostum internum uteri cervix and reach the entrance to the uterus, further advancing should be performed under visual control .If uterine lumen is reduced, air is insufflated through the working channel of the endoscope, causing the endometrium to withdraw and reveal the uterine lumen. Dilatation with insufflated air should continue until a good view of the interior of the uterus begins to expand cranially. For the inspection of the entire body of the uterus, the endoscope should be moved in a circular fashion. Cranial movement of the tip of the endoscope allows examination of the partition wall that separates the horns of uterus. Uterotubal link is found with light movement of the endoscope. Papila can be oval, round or pointed, and can vary in size.

MATERIALS AND METHODS

Cattle Farm "Radosavljevic," Surcin municipalities in Belgrade has 150 cows Holstein Friesian breed beef. It is privately owned. Cattle were originally imported from Germany. The average annual production of milk on this farm was 6600 lit. Renewal of the herd per year is 18.2%. This farm has a system of keeping open all year with the so-called "League of boxes." The property also owns milking cow stalls with separate 2 x 6 stands, and special boxes for examination and insemination of cows. Cows are milked with the machine and this takes place twice a day, morning and evening, three times a day with freshly calved cows. Maternity care is separated and cows are introduced to it two weeks before partus. Calved cows are kept for 10 days after the calving. Calves are kept retained in a nearby stall for about three weeks after birth. Mechanical cleaning is conducted once a week, and hygienic measures are taken every day in the maternity care

For this experiment, 30 cows were used that had an extended service period after calving, which amounted to 150 - 270 days. They were repeatedly treated with hormones and uterine lavage was performed with the use of vitamin preparations and in some animals was carried out over 5 insemination, even with double doses of semen. It is also important to note that animals that were used in the application of diagnostic uteroscopy had already experienced pregnancy. Their milk production was lower at calving, but the first 100 days of lactation have reached a satisfactory level of production. The average production of milk was above an average of 20 liters per day, which is the reason why more energetic measures were not taken.

Methods:

Endoscopic mucosal determining the status of the vagina and portio vaginalis, cervix and uterus

Endoscopic inspection was performed on all cows, the introduction of the endoscope being manual and visually controlled, in maximum aseptic conditions. The endoscope brand "Olympus" in diameter 5.0 mm is a universal human bronchoscope, flexible with a cold light source, located on a separate base and connected to the mains voltage of 220 V.

In endoscopy of vaginal area, and the vestibular fundus in order to see the portio vaginalis in the presence of discharge did not require the presence of the hand during the examination but combined the mobility of the lens outer navigation.

Endoscopic inspection of the cervical canal

The endoscope was manually introduced through portio vaginalis with visual control. This section reviews the most delicate and is kept under close visual control in order to prevent harming the mucous membranes of the endometrium during the examination and so that this finding cannot be attributed to previously implemented trauma cervix. This opportunity to inspect the lining of the Rings orefitium internum uteri.

Uteroscopy of body of the uterus

Is done manually, by pushing the flexible endoscope through the cervical canal and manual navigation with two operating systems that run round the top of the endoscope and give it the possibility of rotation in a 360 degrees radius

When viewing, sterile water is always used for washing and cleaning the lens as a medium that allows the effect of hydrometers for displaying uterine cavity

Uteroscopy review of the mucous membrane of the uterus horns

The lining of uterine horns often hides the identity of the present pathological changes, especially in terms of subclinical endometritis and state carancula. It is known that placentitis is a common reason for abortion or embryonic death. Uteroscopy is a privileged method, which can be directly visual to give us information about the state of the mucous membrane in the immediate vicinity of the carancula.

RESULTS

Changes in the vagina

Table 1. *The frequency of changes in the vagina of tested cows*

Change	Number	%	% from total
1. Secretion of portio vaginalis	7	63,64	23,33
2. Atypical tissue	5	45,46	16,17
TOTAL	**12**	**100,00**	**39,50**

Statistical significance is shown in capital letters. The same letters A, B, C show the significance ($p \leq 0.05$), and the same letters X, Y, Z, showing statistical significance ($p \leq 0.01$)

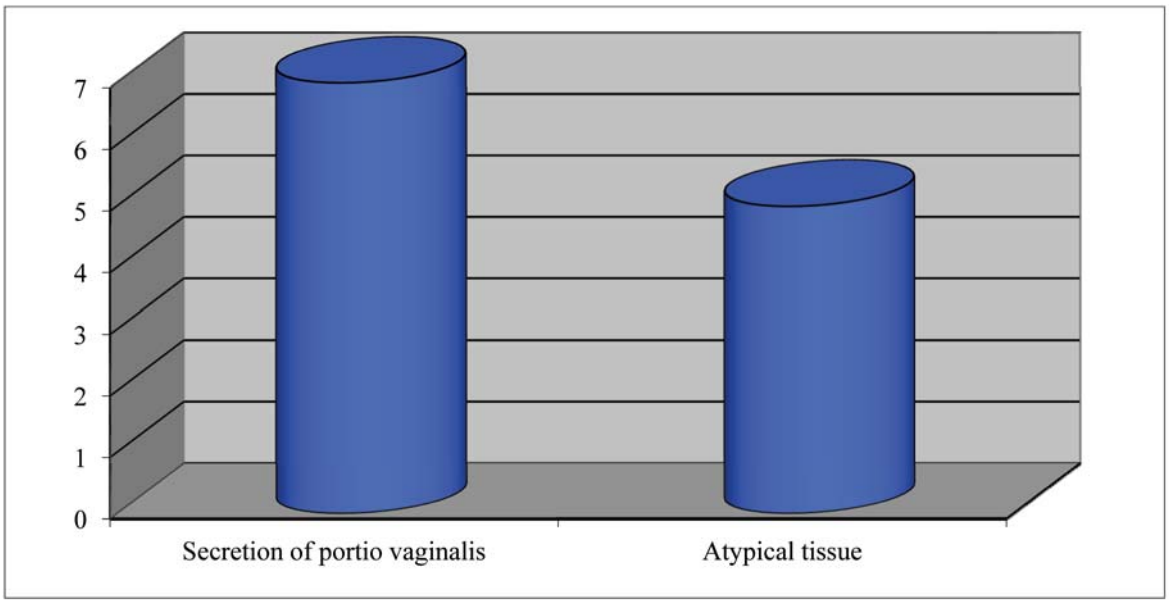


Figure 1. *Display pathological changes in the vagina*

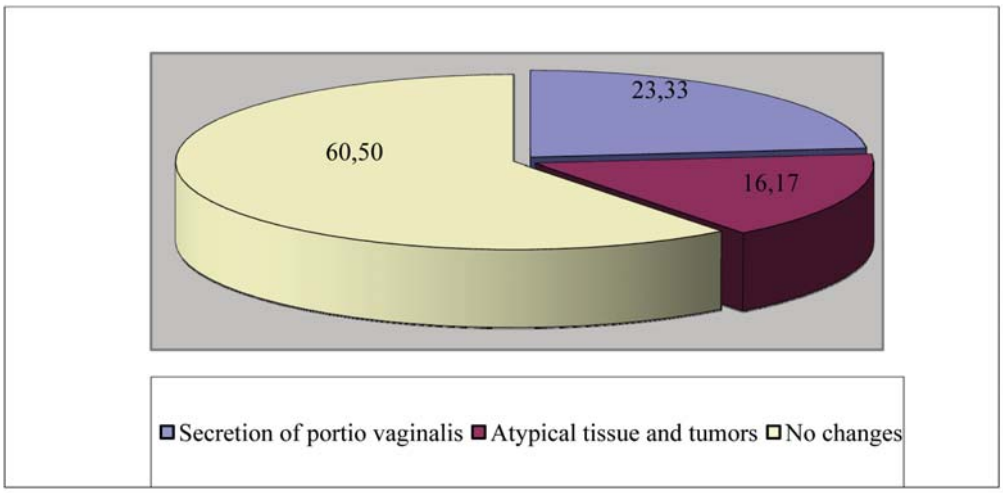


Figure 2. *The structure of pathological changes in the vagina*

The changes in the cervix

Table 2. *The frequency of changes in the cervix of the tested cows*

Change	Number	%	% from total
1. Lesions of the cervix	12^{X}	75,00	40,00
2. Atypical tissue and tumors	4^{X}	25,00	13,33
TOTAL	**16**	**100,00**	**53,33**

Statistical significance is shown in capital letters. The same letters A, B, C show the significance ($p \leq 0.05$), and the same letters X, Y, Z, showing statistical significance ($p \leq 0.01$)

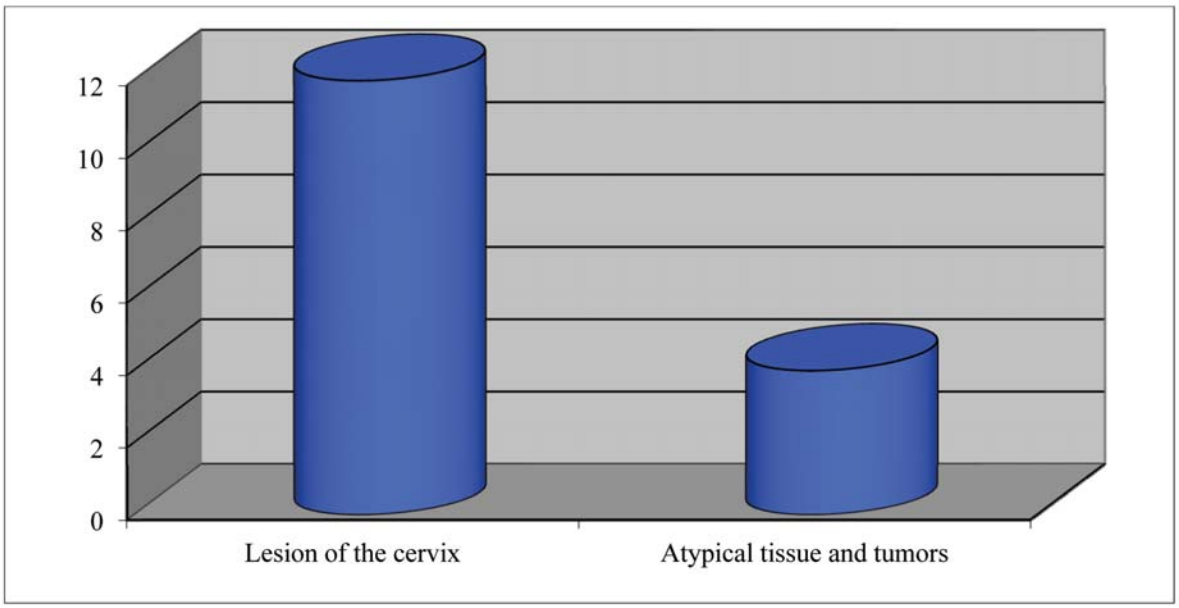


Figure 3. *Display pathological changes in the cervix*

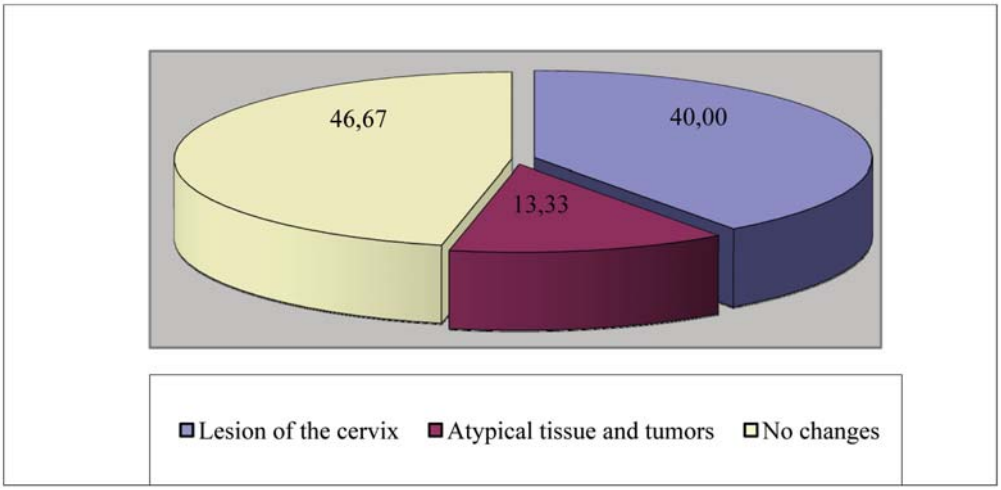


Figure 4. Structure of the pathological changes in the cervix

Changes in the uterus

Table 3. *The frequency of the uterus of cows tested*

Change	Number	%	% of total number
1. Clinical endometritis	8^{AB}	29,63	26,67
2. Subclinical endometritis	17^{XA}	62,96	56,67
3. Atypical tissue and tumors	2^{XB}	7,41	6.67
TOTAL	**27**	**100,00**	**90,01**

Statistical significance is shown in capital letters. The same letters A, B, C show the significance (p≤0,05), the same letters X, Y, Z, show the significance (p≤0,01)

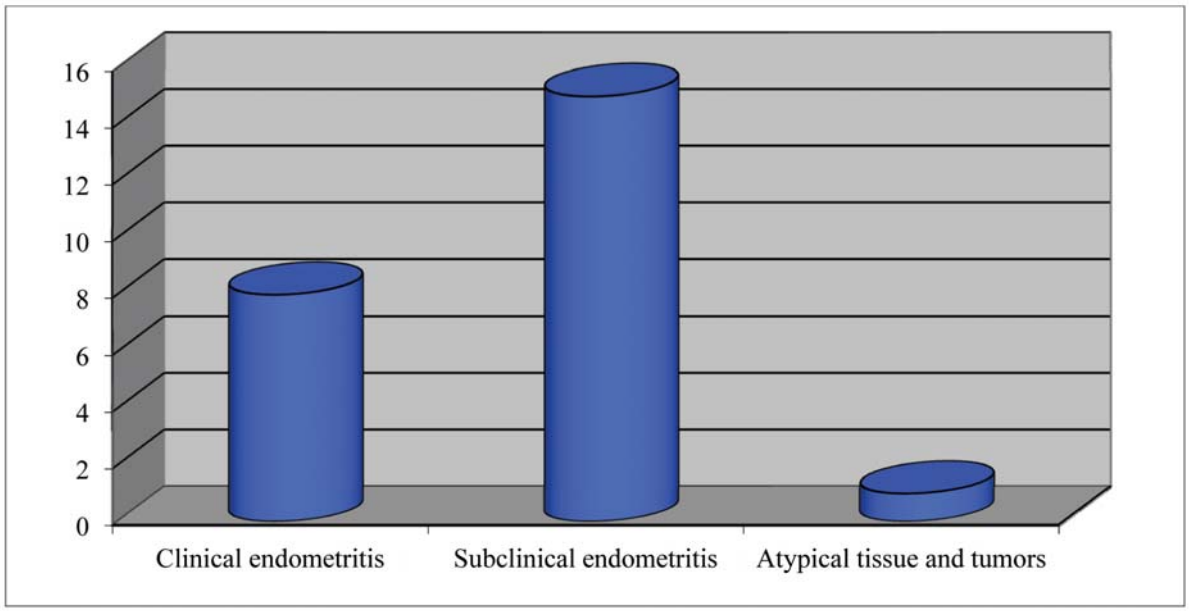


Figure 5. *Display pathological changes in the uterus*

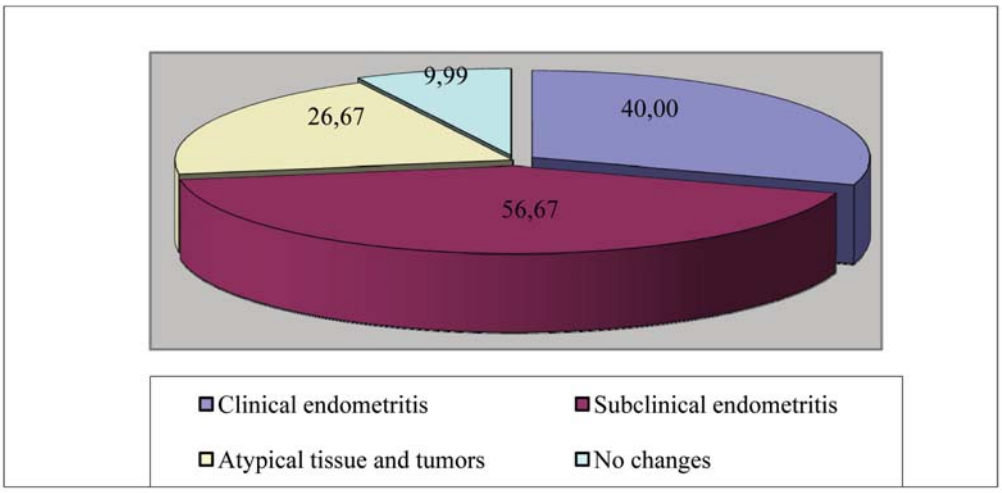


Figure 6. *The structure of pathological changes in the uterus*

Changes in the uterine horns

Table 4. *The frequency of changes in the uterine horns of cows tested*

Change	Number	%	% of total number
1. Clinical endometritis	8^{AY}	33,33	26,67
2. Subclinical endometritis	15^{XA}	62,50	50,00
3. Atypical tissue and tumors	1^{XY}	4,17	3,33
TOTAL	**24**	**100,00**	**80,00**

Statistical significance is shown in capital letters. The same letters A, B, C show the significance ($p \leq 0.05$), and the same letters X, Y, Z, showing statistical significance ($p \leq 0.01$)

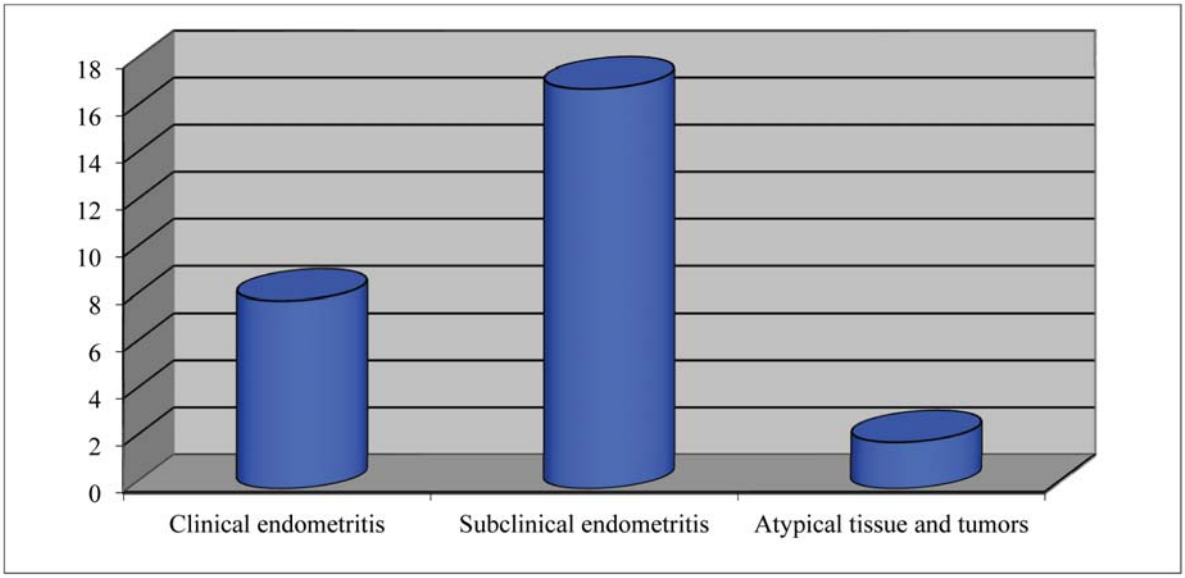


Figure 7. *Display pathological changes in the uterine horns*

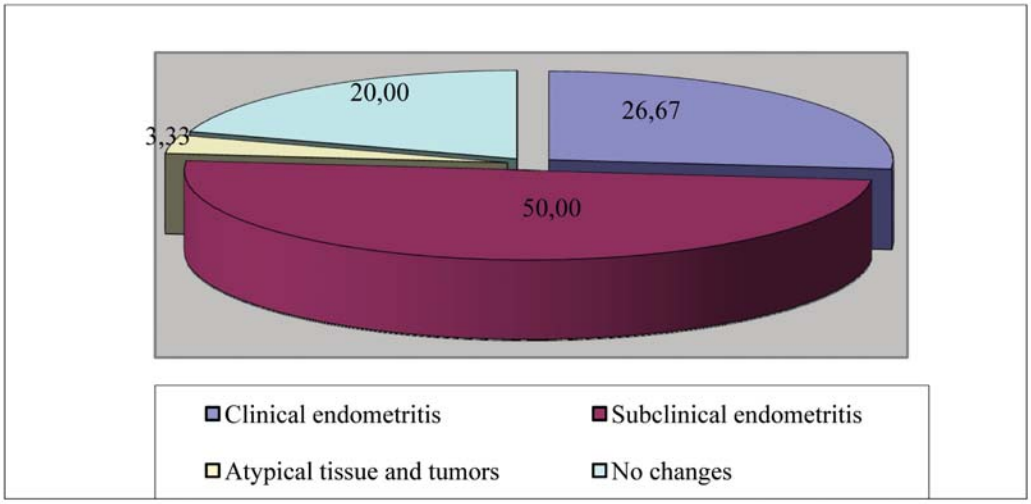


Figure 8. *The structure of pathological changes in the uterine horns*

Table 5. *Display the total change in the examined cows*

Changes in:	Number	% of total
1. Vagina	11^{XY}	36,67
2. Cervix	16^{ZA}	53,33
3. Uterus	27^{XZ}	90,00
4. Uterine horns	24^{YA}	80,00

Statistical significance is shown in capital letters. The same letters A, B, C show the significance ($p \leq 0.05$), and the same letters X, Y, Z, showing statistical significance ($p \leq 0.01$)

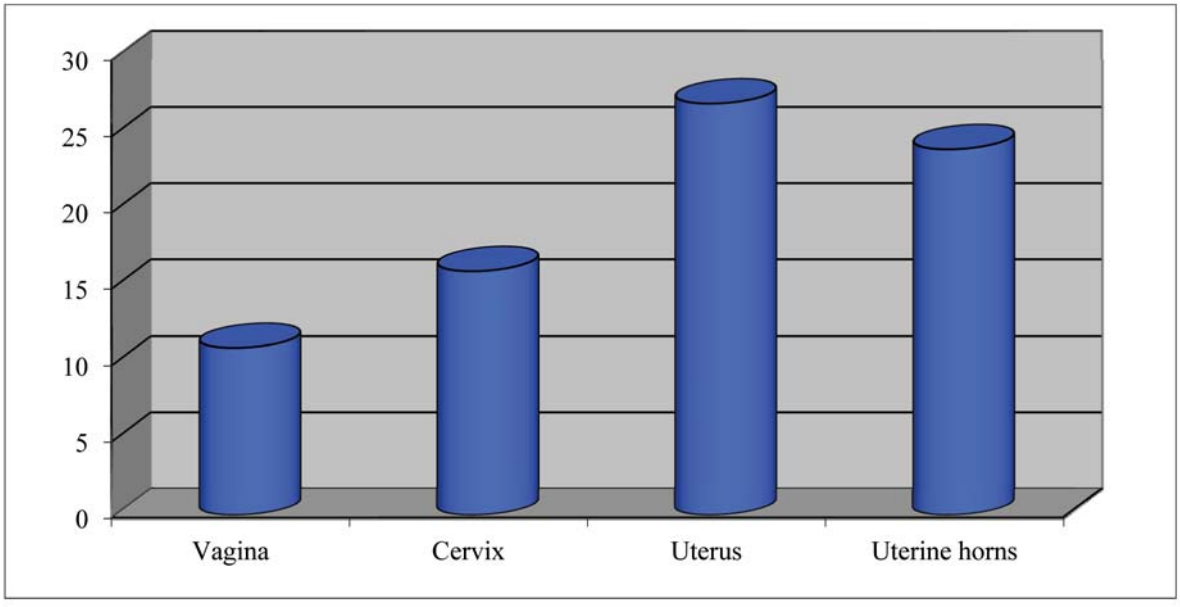


Figure 9. *Display pathological changes in the reproductive organs of a total*

CONCLUSIONS

Based on the results achieved, the following conclusions can be drawn:

1. Through the endoscopic examination of the mucous of the vagina, as well as portio vaginalis, the presence of secret which helps in determining the endometritis type and the presence of distensions and tumor-related changes in the vagina and the portio vaginalis can be determined.
2. Based on the uteroscopic examination, 75% of the changes on the mucous of the vagina with examined cows, is established to be an important factor of sterility. Due to the damage on the cervical musous, in the form of lesions and hematomas, as well as distensions and tumor-related changes, it is considered a valid reason for diagnosed infertility.
3. It is possible to establish diagnosis of symptomless infertility in the period of 50 days from birth by examining the presence of 50% of subclinical endometritises through the use of uteroscopy . This type of infertility requires targeted therapy, which is different from symptomatic treatment.
4. Uteroscopy was proven to be a justified choice in diagnosing and examining subclinical and clinical endometritis in cows with an elongated service period as a result of the experiment on the farm.

REFERENCES

1. Barlund C.S;Carruthers T.D.;Waidner C.L.; Palmer C.W;2008; A comparison of diagnostic tehniques for postpartum endometritis in dairy cattle;Theriogenology, volume 69, issue 6, April, pages 714-723.
2. Bettocchi S.. Nappi L.,Ceci O.,Selvaggi L.;2003 Aug; What does "diagnostic hysteroscopy" mean today? The role of new techniques.Curr Opin Obstet Gynecol: 15 (4) :303-8
3. Cheong S.H.,Nydam D.V.,Galvao K.N.,Crosier R.o.Gilbert, 2011,cow-level and herd – level risk factors for subclinical endometritis in lactating Holstein cowe,Journal of dairy science vol.94.issue 2. Pages 762-770
4. Devine D.A. Linsay E.F., 1984. Hysteroscopy in the cow using a flexible fiberscope. Vet. Rec. 24, 627-628.
5. Kasimanickam, R., T.F. Duffield, R.A. Foster, C.J. Gartley, K.E. Leslie, J.S. Walton and W.H. Johnson. 2005. A comparison of the cytobrush and uterine lavage techniques to evaluate endometrial cytology in clinically normal postpartum dairy cows. Can. Vet. J. 46:255-259.
6. LeBlanc S., 2003. Field Study of the Diagnosis and treatment of clinical endometritis in Dairy Cattle. Cattle Practice 11, iss 4
7. Madoz LV; De La Sota RL; Suzuki K; Heuwisser W; Drillich M; Use of hysteroscopy to evaluate the diagnosis of endometritis in dairy cows Veterinary record British Veterinary assoc, Londres; 2010 vol.167 p.142-143. **ISSN:** 0042-4900
8. Pavlovic M.,Pavlovic V., Vakanjac S.,Endoscopy examination as clinical diagnostic of subfertility in cows;Domestic animal reproduction, Divcibare 2011.
9. Pavlović M., Pavlović V., Zupanc D.: Endoscopical diagnostics of the endometrial pathology in mares. Bilateral Veterinary Medical School meeting Belgrade, Thesaloniki. Kopaonik, 1996.
10. Plontzke J.Madoz L.V.De la Sota R.L.Drilich M.Heuwiseser W.2010,Subclinical endometritis and its impact on reproductive performance in grazing dairy cattle in Argentina,Journal of animal reproductive science,20727691

УТЕРОСКОПСКИ ИСПИТУВАЊА НА ПОРЕМЕТУВАЊАТА ВО МАТКА КАЈ МОЛЗНИТЕ КРАВИ

Павловиќ Милош[1], Павловиќ Војислав[1,] Вакањац Слободанка[1],
Павловиќ Иван[2], Јакиќ- Димиќ Добрила[2]

[1]Оддел за акушерство, гинекологија и в.о, Факултет за ветеринарна медицина, Универзитет во Белград , Србија
[2] Научен ветеринарен институт "Србија", Белград, Србија

АПСТРАКТ

Ендоскопија на матката (утероскопија) е една од многуте методи за дијагностика на болестите на женскиот генитален тракт. Во пракса кај говедата како дијагностичка и терапевтска техника не е многу употребувана. Ги опишува лимитирачките репродуктивни перформанси на многу прецизен начин. Триестет холштајн-фризиски крави беа изложени на утероскопија, сите над 150 и повеќе дена после отелувањето. Различни облици на ендометритиси предизвикуваат намалена фертилност и големи економски загуби. Високо продуктивните грла се со намалена фертилност за време на лактацијата. Овариалната инатктивност е поврзана со забрзана маткина инволуција. Намалените репродуктивни перформанси кај молзните крави обично се предизвикани од пореметувања на матката. Покрај акутниот метритис и хроничниот ендометритис, целта на оваа студија е да го покаже негативното влијание на субклиничкиот ендометритис. Во раниот постпартален период, локалното воспаление на матката од повеќе автори е наречено метритис. Ендоскопското испитување на матката (утероскопијата) кај кравите овозможува добивање на реална слика и ги препознава непалпаторните промени. Ендоскопијата има предност во препознавање на ендометријалните промени и оштетувања. За нејзино изведување потребно е скапа опрема, услови и компетентност кои и не се толку едноставни за работа. Вообичаениот метод за детекција на патолошките состојби поврзани со гинеколошкото испитување со вагинална или ректална експлоатација – палпација обезбедува малку информации за воспалението на ендометриумот. Ендоскопската слика на матката кај кравите може да е од голема важност особено кога се појавуваат општи или нетипични симптоми. На ветеринарите им дава широка интраутерина инспекција и имагинација т.е претстава за здравствените проблеми отколку палпаторните импресии. Ендометритисот кај кравите без исцедок од матката е вообичаено резултат на дистокии или задржување на постелката и вклучува подлабоки слоеви на маткиниот ѕид со различни негови оштетувања. Преку ендоскопското испитување на матката, можат да се дијагностицираат и третираат различни форми на ендометритиси или ткивни оштетувања дури и најмалите делови од ендометријалното ткиво.

Клучни зборови: крава, утероскопија, матка

UDC: 636.2.09:618.11-073.432.19

ULTRASOUND OBSERVATION OF OVARIAN DYNAMIC IN DAIRY COWS WITH TRUE POSTPARTUM ANOESTRUS AFTER TREATMENT WITH DIFFERENT DOSES OF eCG

Atanasov Branko[1], Mickov Ljupco[1], Esmerov Igor[1], Trojacanec Plamen[2], Ilievska Ksenija[2], Adamov Nikola[1], Dovenski Toni[1]

[1]*Institute of reproduction and biomedicine, Faculty of Veterinary Medicine Skopje, Ss. Cyril and Methodius University, R. Macedonia*
[2]*Veterinary institute, Faculty of Veterinary Medicine Skopje, Ss. Cyril and Methodius University R. Macedonia*

ABSTRACT
The most important factors contributing to reproductive inefficiency in dairy cattle are failures to resume ovarian activity early in postpartum period. Static ovaries (no CL, follicles <10 mm, P_4 <0.5 ng/ml) are one of the reasons which play a part in this condition. The aim of this trial was to observe the changes on the ovaries after application of different doses of eCG, in postpartum anestrous dairy cows. Twenty three cows with static (acyclic) ovaries were identified during a routine ultrasound examination, on one dairy farm. The cows were divided into three groups: controls (n=7); Group1 treated with 750IU eCG (n=8) and Group2 treated with 1000 IU (n=8). Following daily measurement of follicular size and CL diameter on day 9 after ovulation, follicular growth rate, reaction time and number of ovulations per cow, were estimated. Results have shown that average size of the follicles (dominant) were 14.6±0.2mm in Group1,15.3±0.1mm in Group2, and 16.3±0.3mm in the control group. The average growth rate of the dominant follicles was 1.29±0.2mm/day, 1.4±0.4mm/day and 0.9±0.2mm/day for Group1, Group2 and the control group, respectively. Resumption of the cyclic activity occurred overall in 47.82% (11/23) of the treated cows, with 25% in Group1; 75% in Group2 and 42.85% in the controls. Cows treated with 1000IU and 750IU eCG responded faster (6.2±0.3 and 8±1.7days respectively) in comparison to the controls (15±0.1days). Average P_4 level on day 9 were 3.3±0.2ng/ml, 3.7±0.7ng/ml, 4.2±0.3ng/ml in Group1, Group2 and control group, respectively. Incidence of multiple ovulations was higher in Group2, in average 2.1±0.5 ovulations, which lead to desirable results for breeding beef cattle. In conclusion, treatment with higher doses of eCG in cows with true postpartum anoestrus, causes resumption of follicular growth and ovulation. However, because of higher ovulation and twining rate, eCG treatment could not be considered as a perfect method of choice for treatment of static ovaries in dairy farming.
Key words: cows, ultrasound examination, eCG, ovaries

INTRODUCTION

A well organized management in the field of reproduction is one of the most important factors for successful production in modern dairy farms. The rapid progress in genetics and management in the dairy industry throughout the world has created a new era in which dairy cows meet the growing demand for dairy products. By reaching this demands dairy cows are exposed to greater milk production which inevitably led to functional disorders of the reproductive and endocrine system represented trough reduced ovary functionality, early embryonic mortality, extended postpartum anoestrus, increased inter-calving period, ovulation disorders, the appearance of cysts, reduced production, as well as increased costs for treatment and culling rates. According to Lucy (2007) there are four primary mechanisms that depress fertility in lactating cows: anovulatory and behavioral anestrus (failure to cycle and display oestrus), suboptimal and irregular estrous cyclicity (this category includes ovarian disease and subnormal luteal function after breeding), abnormal preimplantation embryo development (may be secondary to poor oocyte quality), and uterine/placental incompetence. Dovenski (1997), reported that one on the major reason for fertility decline were static ovaries (true anoestrus). The same authors defined static ovaries as small ovaries with small antral follicles less than 10 mm, absence of functional corpus luteum and concentration of the progesterone < 0.5 ng/ml, and clinically, absence of cyclic activity. Arthur's 1989 indicated that the reason for this condition might be in the inability of the ovaries to respond to the stimulus of the gonadotropins or their insufficient secretion. Short et al. (1990) indicate that one of the main factors for this condition was nutritional imbalance especially in the first few weeks after calving.

The aim of this investigation was to observe the respond of the ovary after single application of different doses of eCG in cows diagnosed with true anestrus.

MATERIALS AND METHODS

This study was conducted on one dairy farm with a total of 350 dairy cows; mainly Holstein-Frisian breed held on tied system and fed with corn silage, haylage, alfa - alfa hay, and premix at the time of our examination. By routine ultrasound examination out of 150 cows, 23 cows were included in the study after meeting the following criteria:

A) being minimum 55 days after calving,
B) having static ovaries as described by Dovenski (1997)
C) having 2.3 average body score condition

Ultrasonography examination:

A linear array 7.5 MHz transrectal transducer and a B-mode real time ultrasound scanner Aloka SSD – 500

were used. The rectum of the cows was emptied before insertion of the lubricated transducer, and the ovaries were first manually located before placing the transducer of the site. Diameters of the follicles were measured by means of electronic calipers located on the ultrasound device. The images were recorded in freeze mode and printed by a printer connected with the ultrasound device.

Body condition scoring:

Body condition scoring - BCS was done on day 0 of the experiment with the BCS chart ranging from 1.0 – 5.0 where 1 means lean and 5 means obese.

Blood samples and progesterone determination

The blood samples were collected on day 0 (ultrasound examination) and day 9 after ovulation from jugular vein into heparinized tubes and centrifuged within 3 hours of collection at 4^0 C, 3000 rpm, for 15 minutes. The plasma samples where than stored at – 20^0 C until determination of progesterone by enzyme-immune assay EIA were done.

Experiment protocol:

After identification and blood progesterone determination, 23 cows were divided into 3 groups. The first group (n=7) served as control where no further treatment was presiding. The Group 1 and Group 2 each including 8 cows (n=16) were subjected to the treatment with 750 IU and 1000 IU of eCG respectively. Ultrasound examination was conducted daily to record the changes in the growing follicles, diagnosing of functional CL's, responded time and ovulations rate.

RESULTS AND DISCUSSION

Total of 23 cows or 15.33 % were identified as having static ovaries and were subjected for further research. The mean serum concentration of P_4 were 0,32 ± 0,01 ng/ml (M±SEM), 0,35 ± 0,01 ng/ml и 0,38 ± 0,02 ng/ml (M±SEM) in Group 1, Group 2 and control groups, respectively.

In Group 1, the average period from calving was 56 ± 1.77 days. The mean BCS was 2.5 ± 0.28. Resumption of cyclic activity occurred in 2/8 cows or 25 %. The average growth rate and the mean diameter of the largest (dominant) follicle were 1.29 ± 0.2 mm/day and 14.6 ± 0.2 mm, respectively. Reaction time was 8 ± 1.7 days. The mean serum concentration of P_4 was 3.3 ± 0.02 ng/ml on day 9 after ovulation. No multiple ovulations were recorded. In the second group (Group 2) the average period from calving was 62 ± 2.55 days and the mean BCS was 2.67 ± 0.30 respectively. 75% (6/8) positively responded to therapy by establishing regular folliculogenesis. The average growth rate was 1.4 ± 0.4 mm/day and the mean diameter of the dominant follicle was 15.3 ± 0.1 mm, respectively. Reaction time was 6.2 ± 0.3 days. The mean serum concentration of P_4 was 3.7 ± 0.07 ng/ml on day 9 after ovulation. Multiple ovulations were recorded in 4 cows or in 50% of the cows. In the control group 3/7 cows or 42.85 % establish regular folliculogenesis. The mean BCS, the average period from calving, the average growth rate, reaction time and the mean diameter of the dominant follicle were 2.68 ± 0.5, 60 ± 1.55 days, 0.9 ± 0.2mm/day 15 ± 0.1 days and 16.3 ± 0.3 mm. The mean serum concentration of P_4 was 4.16 ± 0.04 ng/ml.

Resumption of cyclic activity occurred in 47.82% (11/23) of the treated cows 25% in Group1; 75% in Group2 and 42.85% in the controls. This data shows that lack of cyclicity especially in Group 1 might be as results of negative energy balance (NEB). Grummer (2000) reported that negative energy balance probably does not directly affect ovarian function, but it is more likely that negative energy balance influences endocrine status of the animal which in turn regulates ovarian function. Negative energy balance may prohibit the development of pulsatile GnRH and LH patterns required for the re-establishment of ovulatory cycles (Butler and Canfield, 1989). There were no significant differences ($p>0.5$) in average growth rate and reaction time between the trial groups, but significant differences ($p<0.001$) between the trial groups and the control group.

The presence of multiple ovulations in Group 2 was 50%. It can be assumed that the reason might be in the high administrated dose of eCG (1000 IU) where its dominantly follicle-stimulating activity directly affects follicles and causes stimulation of folliculogenesis (Popovski and K'nchev, 1998). Thus, administration of FSH during the common growth phase in the follicular wave in cows prevents single domination and allows development of multiple dominant follicles (Mihm et al. (1997). Musssard et al., (2007) reported 47% of the cows with follicle co-dominance and multiple ovulations pointing that the reason might be in follicle waves developing in the presence of very low amounts of progesterone.

From the data obtain it can be concluded that treatment with higher doses of eCG in cows with true postpartum anoestrus, causes resumption of follicular growth and ovulation. However, because of higher ovulation rate followed by higher twining rate, eCG treatment isn't suitable method for treatment of static ovaries in dairy farming.

REFERENCES

1. Arthur H. G. Noakes D.E Pearson H. - Veterinary reproduction and obstetrics (1989) Theriogenology – Sixth edition W.B Saunders Toronto.
2. Butler, W. R. and R. W. Canfield. 1989. Interrelationships between energy balance and postpartum reproduction. In Proc. Cornell Nutrition Conference for Feed Manufacturers, October 24-26, 1989, Cornell University, Ithaca.
3. Dovenski T. (1997) - Usporedba ehograma jajnika s razinom progesterona i estradiola u krvi krava tijekom spolnog ciklusa u puerperiju i u jalovih krava; disertacija Veterinarski fakultet Sveuciliste Zagreb
4. Grummer R. Ric. (2000) - Energy status and reproductive function in dairy cattle
5. Lucy MC (2007) - Fertility in high-producing dairy cows: reasons for decline and corrective strategies for sustainable improvement Soc Reprod Fertil Suppl.64:237-54.
6. Mihm M, Good TE, Ireland JL, Ireland JJ, Knight PG, Roche JF (1997). Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers Biol Reprod. 1997 Dec; 57(6):1328-37.
7. Mussard, M. L., C. R. Burke, E. J. Behlke, C. L. Gasser, and M. L. Day. 2007. Influence of premature induction of an LH surge with GnRH on ovulation, luteal function and fertility in cattle. J. Anim. Sci. 85:937-943
8. Popovski K., K'ncev Lj. (1998) Endokrinologija na reprodukcijata prvo izdanie Skopje
9. Short R.E., Bellows R.A., Staigmiller R.B., Berardinelli J.G., Custer E.E.: (1990) Physiological mechanism controlling anestrus and fertility in postpartum beef cattle. J Anim Sci, **68**, 799-816.

УЛТРАЗВУЧЕН МОНИТОРИНГ НА ОВАРИЈАЛНАТА ДИНАМИКА КАЈ МОЛЗНИТЕ КРАВИ СО ВИСТИНСКИ ПОСТПАРТАЛЕН АНЕСТРУС ПОСЛЕ ТЕРАПИЈА СО РАЗЛИЧНИ ДОЗИ НА eCG

Атанасов Бранко[1], Мицков Љупчо[1], Есмеров Игор[1], Тројачанец Пламен[2], Илиевска Ксенија[2], Адамов Никола[1], Довенски Тони[1]

[1] Институт за репродукција и биомедицина, Факултет за ветеринарна медицина, Скопје, Универзитет „Св. Кирил и Методиј“, Скопје, Република Македонија,
[2] Ветеринарен институт Факултет за ветеринарна медицина, Скопје, Универзитет „Св. Кирил и Методиј“, Скопје, Република Македонија,

АПСТРАКТ
Едни од главните фактори кои доведуваат до репродуктивна неефикасност кај молзните крави се невозможноста за воспоставување на повторната циклична активност во раниот постпартум период. Нефункционалните јајници (без CL, фоликули <10 mm, P_4 <0.5 нг/мл) се едни од тие фактори кои допринесуваат до ваква состојба. Целта на оваа студија беше да се набљудуваат промените на јајниците после апликација на различни дози на еЦГ кај крави со дијагностициран вистински постпарум анеструс. Дваесет и три крави со статични јајници беа идентификувани за време на рутински ултразвучен преглед во една краварска фарма на молзни крави. Кравите беа поделени во 3 групи: контролна (n=7), Група 1 третирани со 750 ИЕ еЦГ (n=8) и Група 2 третирани со 1000 ИЕ на еЦГ (n=8). Секојдневно беа мерени големините на фоликулите и дијаметарот на жолтото тело на 9 ден после овулација, при што се оценуваа растот на фоликулите, времето на одговор и бројот на овулации по крава. Резултатите покажаа дека средната големина на доминантните фоликули беа 14.6±0.2 мм во Група 1, 15.3±0.1мм во Група 2, и 16.3±0.3 мм во контролната група. Просечниот раст на фоликулите беше 1.29±0.2 мм/ден, 1.4±0.4 мм/ден and 0.9±0.2 мм/ден во Група 1 Група 2 и контролната група, респективно. 47.82% од кравите или (11/23) воспоставија повторна циклична активност и тоа 25% во Група 1; 75% во Група 2 и 42.85% во контролната група. Кравите третирани со 1000 ИЕ и 750 ИЕ на еЦГ реагираа побрзо (6.2±0.3 и 8±1.7 дена, респективно) во споредба со контролната група (15±0.1дена). Просечната концетрација на прогестеронот на деветиот ден изнесуваше 3.3±0.2 нг/мм, 3.7±0.7 нг/ммl, 4.2±0.3 нг/мм во Група 1, Група 2 и контролната група, респективно. Појавата на мултипна овулација беше повисока во Група 2 во просек 2.1±0.5 овулации, што води кон посакувани резултати во одгледувањето на товните раси. Како заклучок, третманот со повисоки дози на еЦГ кај крави со вистински анеструс, предизвикува продолжување на циклична активност следена со овулација. Но поради високата застапеност на мултипната овулација следена со можност на близност, еЦГ третманот не е препорачлив методи за терапија на статични јајници кај млечните крави.
Клучни зборови: крави,ултразвучно испитување, еЦГ, јајници

UDC: 636.7.09:618.14-007.44

VAGINAL FOLD PROLAPSE AND TRANSMİSSİBLE VENEREAL TUMOR RELATED TO OVARIAN REMNANT SYNDROME İN A BITCH

Turna Yılmaz Özge[1], Uçmak Melih[1], Kırşan İsmail[1]

[1]*Department of Obstetrics and Gyneacology, Faculty of Veterinary Medicine, University of Istanbul, Istanbul, Turkey*

ABSTRACT

A 4-year-old, cross-breed, neutered bitch weighing 24 kg, was presented with one month history of vaginal bleeding and a vaginal mass distinguished two weeks ago. According to the anamnesis the bitch was still mating with male dogs although it had undergone an ovariohysterectomy when it was 2-year-old. Vaginal bleeding and an irregular-shaped, edematous vaginal mass (12×9×7 cm) were detected on clinical examination. The bitch had leukocytosis and erythrocytopenia. Abundant erythrocytes in company with neutrophils, lymphocytes, intermedier cells and transmissible venereal tumor cells were observed in vaginal cytology. Abdominal ultrasonography demonstrated the cystic ovarian tissue (2.42×1.36 cm) next to the right kidney. Chemotherapy was performed to the bitch once a week for six weeks. After chemotherapy treatments the decline in the size and edema of the mass was determined. The vaginal mass was extirpated. The ovarian remnant tissue was also removed via laparotomy on the same day. According to the physical examination on the 4th month, the bitch was found healthy.

Key Words: bitch, ORS, TVT, vaginal fold prolapse

INTRODUCTION

Ovarian remnant syndrome (ORS) is a complication which occurs as a result of failure to remove all of the ovarian tissue during ovariectomy or ovariohysterectomy operation. The clinical signs of this syndrome are similar with proestrus and estrus signs such as bloody vaginal discharge, vulvar swelling, behavioral changes (Wallace, 1991). Canine transmissible venereal tumor (TVT) commonly occurs in dogs which have an uncontrolled sexual behavior in temperate climates (Purohit, 2009). In addition to these cases vaginal fold prolapse is another common case in sexually intact dogs. It is the protrusion of vaginal mucosa into the lumen during proestrus and estrus (Rushmer, 1980). This presentation contains the evaluation of a bitch which diagnosed with ORS, TVT and vaginal fold prolapse concomitantly.

MATERIAL AND METHOD

A 4-year-old, cross-breed, neutered bitch weighing 24 kg, was presented to the clinic of Obstetrics and Gyneacology, Faculty of Veterinary Medicine, University of Istanbul, with one month history of vaginal bleeding and a vaginal mass distinguished two weeks ago. Accompanying complaints were loss of apetite and weakness. The owner indicated that the bitch had continued mating however she had been neutered two years ago.

On physical examination the rectal temperature, pulse and respiratory rates of the patient were within reference ranges. On palpation and inspection of the genital tract vaginal bleeding, edematous vulva and an irregular shaped vaginal mass (12×9×7 cm) protruding through the vulva in a pendulous fashion were identified. Plenty of erytrocytes, neutrophils, lymphocytes, intermedier cells and ovoid cells containing intracytoplasmic vacuols were revealed in the vaginal cytology stained with Diff-Quick stain. According to the hematological findings, leucocytosis and erytrocytopenia were determined while all other parameters were in normal ranges. An anecogenic area (2.42×1.36 cm) were identified behind the right kidney in the ultrasonographic examination of the abdomen.

After the diagnostic evaluation of the bitch it is decided to perform preoperative chemotherapy to decrease the size of the vaginal mass and to obviate bleeding. Systemic antibiotic (%5 Baytril-K®, Bayer, 5mg/kg) and Vit B_{12} (Dodex®, VETAŞ, 150 mcg/kg) prescribed for the treatment of leucocytosis and lethargy. Vincristine® (Koçak Pharma, 0.025mg/kg IV) was performed to the bitch once a week for six weeks. Hematology and vaginal cytology were controlled before each cycle of chemotherapy. One week after the last chemotherapy surgical removal of the residual ovary and vaginal mass was recommended. 6mg/kg intravenous propofol (Pofol®, Dongkook, Korea) and afterwords isoflurane (Isoflurane®, Rhodia Organique, UK) with oxygen were administered for general anesthesia. Residual ovarian tissue was removed with a standart laparotomy operation. The vaginal mass was extirpated without the necessity of episiotomy in the same time.

RESULTS

Based on the anamnesis, vaginal cytological, clinical and ultrasonographic findings of the patient ORS, TVT and vaginal fold prolapse diagnosed. In cytologic examination of the vagina the erytrocytes and ovoid cells disappeared at the third and fifth week of treatment, respectively. After completed chemotheraphy it was detected that the vaginal mass has shrinked compared to past although it was still continuing it's existence. The sutures on the median area were removed on the 10th day. According to the physical examination on the 4th month, the bitch was found healthy.

CONCLUSION

Ovarian remnant syndrome is a surgical error which brings along continued secretion of reproductive hormo-

nes (Ball et al., 2010). The bitches with ORS can not be pregnant but they become more susceptive to the pathologies like ovarian cysts and tumours, stump pyometra, TVT, vaginal fold prolapse, skin diseases caused by hyperestrogenism. In the present case report, the bitch continued her sexual activity after the ovariohysterectomy operation. Because of uncontrolled matings, she got TVT. In addition to this, estrogen secretion from the residual ovary caused vaginal fold prolapse. Ovaries should be removed totally in ovariohysterectomy operation, due to residual ovary related pathologies which it brings alone.

REFERENCES

1. Wallace MS, 1991. The ovarian remnant syndrome in the bitch and queen. Vet Clin North Am Small Anim Pract, 21, 501-507.
2. Purohit G, 2009. Canine Transmissible Venereal Tumor: A Review. The Internet Journal of Veterinary Medicine, 6 (1), http://www.ispub.com/journal/the_internet_journal_of_veterinary_medicine/volume_6_number_1_39/article_printable/canine-transmissible-venereal-tumor-a-review.html.
3. Rushmer RA, 1980. Vaginal hyperplasia and uterine prolapsed. In: Kirk RW. ed. Current Veterinary Therapy VII, Small Animal Prcatise. Philadelphia: WB Saunders, 1222-1224.
4. Ball RL,Birchard SJ, May LR, Threlfall WR, Young GS, 2010. Ovarian remnant syndrome in dogs and cats: 21 cases (2000-2007), JAVMA, 236 (5), 548-553.

ПРОЛАПСУС НА ВАГИНАТА И ТРАНСМИСИВЕН ВЕНЕРИЧЕН ТУМОР ПОВРЗАН СО СИНДРОМ НА ЗАОСТАНАТ ЈАЈНИК КАЈ КУЧКА

Турна Јулмаз Озге[1], Учмак Мелих[1], Киршан Исмаил[1]

[1]Катедра за акушерство и гинекологија, Факултет за ветеринарна медицина, Универзитет во Истамбул, Истамбул, Турција

АПСТРАКТ

Четири годишна стерилизирана кучка, мелез со тежина 24 кг е примена со еден месечно вагинално крварење и пролабирани вагинални делови. Според анамнезата кучката сеуште се парела со мажјаци иако била подлегната на овариохистероктомија на возраст од 2 години. При клиничкиот преглед беше забележано крварење од вагината и едеметозни неправилни делови од вагината со големина од 12×9×7 cm. Кај кучката беше забележана леукоцитоза и еритроцитопенија. Зголемен број на еритроцити заедно со неутрофили, лимфоцити, интермедијарни клетки и клетки на трансмисивен венеричен тумор беа забележани при вагинална цитологија. При абдоминална ултрасонографија беше забележано цистично овариално ткиво со големина од (2.42×1.36 cm) непосредно до десниот бубрег. Еднаш неделно беше извршена хемотерапија во траење од шест недели. После завршената хемотерапија беа забележани намалувања во големината и едематозноста на вагиналните делови. Истите беа отстранети. Истиот ден преку лапаратомија беше отстранет и остатокот од оваријалното ткиво. Според физичкиот преглед после 4 месеци кучката беше прогласена за здрава.

Клучни зборови: кучка, СЗЈ, ТВТ, пролапсус на вагината

UDC: 636.7.09:[618.1-006:618.11-089.87

TRANSMISSIBLE VENEREAL TUMOR IN A BITCH WITH OVARIAN REMNANT SYNDROME

Evkuran Dal Gamze[1], Töre Merih Duygu[1]

[1]*Department of obstetrics and gynaecolog, Faculty of veterinary medicine, Istanbul University, Istanbul, TURKEY*

ABSTRACT

A 6 year-old ovariohysterectomized mix-breed bitch was referred to hospital with a complaint of bleeding tumoral structure which protruded from vulva. A huge, cauliflower-shaped, fragile mass with hemorrhagic discharge were observed in vagina and vulva. Vaginal smear revealed transmissible venereal tumor. Ovarian remnant syndrome was diagnosed by demonstration of the residual ovary in abdominal ultrasonography. Vincristine sulphate with 0.9% NaCl was given as slow infusion once a week according to patient's weekly clinical examination and hemogram findings. The residual ovary was removed via a midline laparatomy after chemoterapy was completed. In conclusion, transmissible veneral tumor may be transplanted to spayed animals with ovarian tissue residues and these animals may play role in spread of the tumor.

Keywords: transmissible venereal tumor, ovarian remnant syndrome, vincristine, dog.

INTRODUCTION

Transmissible venereal tumor (TVT) is a round-cell tumor affecting external genitalia of dogs and other members of the canine family such as coyotes, foxes and jackals (1,2,3,4). TVT is a naturally occuring and coitally transplanted allograft through major histocompatibility complex barriers and is trasmitted by transplantation of viable neoplastic cells (2,3,5,6). Free roaming sexually active dogs are at greatest risk for trasmission of the tumor (1,7).

Although the tumor affects primarily external genitalia, it may also develop at nasal or oral cavities, lips, skin and rectum by licking and sniffing. Metastasis is uncommon but is reported to occur in the regional lymph nodes, tonsils, eyes, brain, pituitary, liver, spleen, kidney, lung, musculature, thoracic and abdominal viscera (1,3,6,7).

Diagnosis of TVT is based on history, findings of clinical and cytological examination (1) and the most effective treatments for TVT are radiation and chemotherapy, particularly with vincristine (1,7).

Ovarian remnant syndrome (ORS) is the presence of functional ovarian tissue in a previously ovariohysterectomized animal. The bitch is usually presented to the veterinarian because of the recurrent estrous cycles. History, findings on clinical examination, hormone analysis, vaginal cytology, and finally laparotomy are the ways for diagnosing ORS (8).

A TVT case in a bitch with ORS was presented in this case report. It is aimed to emphasize the importance of stray dogs which were not sterilized properly in transmission of this tumor.

MATERIALS AND METHODS

Case Description

A 6 year-old ovariohysterectomized mix-breed bitch weighing 20 kg was referred to Obstetrics and Gynaecology Clinic with a complaint of bleeding tumoral structure which protruded from vulvar lips. Diagnosis of TVT was made by history, clinical examination and vaginal cytological findings. A presumptive diagnosis of ORS was made based on history. Findings in vaginal cytology and abdominal ultrasonography confirmed the diagnosis of ORS. Treatment of TVT was performed by chemotherapy with vincristine. The residual ovary was removed via a midline laparatomy after chemoterapy was completed.

RESULTS

Findings on clinical examination, vaginal cytology and abdominal ultrasonography

A pineapple-sized, solid, cauliflower-shaped, fragile and hemorrhagic mass in vagina and vulva, and multifocal ulserations throughout the perineal region were observed in clinical examination (Figure 1). The external genitalia was deformed and had abnormal odor.



Figure 1. *Clinical appearence of the tumor on the first cure of treatment.*

Vaginal cytology was performed by a sterile cotton swab and dyed with Diff-Quick method. Vaginal

cytological findings revealed plenty of transmissible venereal tumor cells, red blood cells, neutrophils and bacteria (Figure 2).

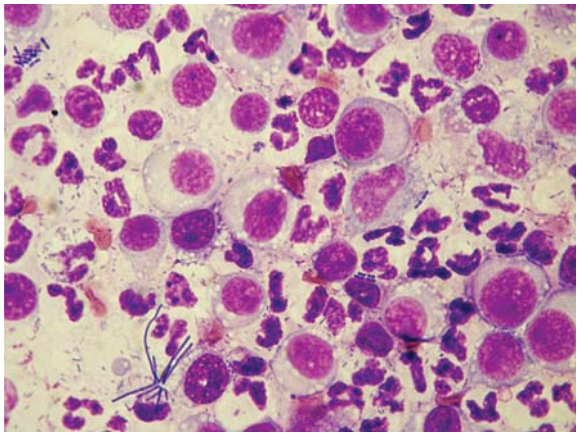


Figure 2. *TVT cells in vaginal cytological examination, Diff-Quick, X100.*

However it was told that the bitch was ovariohysterectomized previously in the history, ORS was suspected. Abdominal ultrasonography was performed and a residual ovary was demonstrated next to right kidney.

Treatment

13 cures of 0,025 mg/kg Vincristine sulphate with 0.9% NaCl was given as slow infusion once a week according to patient's weekly clinical examination and hemogram findings. The chemotherapy was suspended when Red Blood Cell, Hemoglobin and Hematocrit levels in hemogram were decreased and patient's general condition was affected until these parameters had reached reference ranges. A supportive therapy with Vit B_{12} was undertaken during chemotherapy.

After the chemotherapy was completed a midline laparatomy was performed under general anesthesia and the residual ovary was removed. The bitch was in good health condition during one year follow-up.

CONCLUSIONS

ORS is the condition of functional ovarian residue in a previously ovariohysterectomized animal due to iatrogenic conditions as dropping of ovarian tissue, improper clamp placement or elective reasons (8). As animals with ORS can continue to cycle even without the uterus (8) uncontrolled stray dogs may continue mating and may suffer from contagious diseases as in this case.

TVT is a worldwide naturally occuring round cell tumor of dogs (3,5) and remains a big problem in countries where free roaming dogs mate without control (1,7) like in our country.

TVT is usually located on the posterior vagina, at the junction of vestibule and the vagina, and may protrude from the vulva (1). Genital masses are friable with hemorrhagic discharge from their surfaces. Ulseration of the tumor, deformation of external genitalia and abnormal odor may be noted (7). These reports are compatible with clinicial findings in our case.

Monochemotherapy with weekly injections of vincristine is considered to be safe and effective (9) among radiotherapy and many chemotherapeutic agents with their single or combined use. Vincristine chemotherapy was preferred in this case. The tumor was totally regressed after 13 cures. Considering the huge size of tumor in our case, report of Scarpelli et al. (9) about larger tumor volume and treatment during hot and rainy months may help to explain the long duration of our vincristine chemotherapy.

It is concluded that transmissible veneral tumor may be transplanted to previously ovariohysterectomized dogs with ovarian tissue residues and these animals with ovarian remnant syndrome may play role in spread of the tumor.

REFERENCES

1. NAK D., NAK Y., CANGUL I.T., TUNA B. (2005). A clinico-pathological study on the effect of vincristine on transmissible venereal tumour in dogs. J. Vet. Med. A 52: 366–370.
2. MUKARATIRWA S., CHIWOME T., CHITANGA S., BHEBHE E. (2006). Canine transmissible venereal tumour: asessment of mast cell numbers as ındicators of the growth phase. Veterinary Research Communications, 30: 613–621.
3. PARK M.S., KIM Y., KANG M.S., OH S.Y., CHO D.Y., SHIN N.S., KIM D.Y. (2006). Disseminated transmissible venereal tumor in a dog. J Vet Diagn Invest 18: 130–133.
4. BRIDGETT M.V., ELAINE A.O. (2006). The singular history of a canine transmissible tumor. Cell 126: 445-447.
5. GONZALEZ C.M., GRIFFEY S.M., NAYDAN D.K., FLORES E., CEPEDA R., CATTANEO G., MADEWELL B.R. (2000). Canine transmissible venereal tumour: a morphological and ımmunohistochemical study of 11 tumours in growth phase and during regression after chemotherapy. J. Comp. Path. 2000, Vol. 122, 241–248.
6. MOZOS E., MENDEZ A., GOMEZ-VILLAMANDOS C, MARTIN DE LAS MULAS J., PEREZ J. (1996). Natural disease: Immunohistochemical characterization of canine transmissible venereal tumor. Vet Pathol33: 257-263.
7. ROGERS K.S., WALKER M.A., DILLON H.B. (1998). Transmissible venereal tumor: A retrospective study of 29 cases. Journal of the American Animal Hospital Association, 34: 463-470.
8. Miller D.M. (1995). Ovarian remnant syndrome in dogs and cats: 46 cases (1988-1992). J Vet Diagn Invest 7: 572-574.
9. SCARPELLI K.C., VALLADÃO M.L., METZE K. (2010). Predictive factors for the regression of canine transmissible venereal tumor during vincristine therapy. The Veterinary Journal 183: 362–363.

ТРАНСМИСИВЕН ВЕНЕРИЧЕН ТУМОР КАЈ СИНДРОМ НА ЗАОСТАНАТ ЈАЈНИК КАЈ КУЧКА

Евкуран Дал Гамзе[1], Торе Мерих Дујгу[1]

[1]Катедра за акушерство и гинекологија, Факултет за ветеринарна медицина, Универзитет во Истанбул, Турција

АПСТРАКТ
Шестгодишна овариохистеректомирана кучка од мешана раса, беше хоспитализирана со жалба на крварење од туморна структура со протрузија од вулвата. Огромна, во форма на карфиол, фрагилна масасо хморагичен исцедок беше забележана во вагината и вулвата. На вагиналениот брис се покажа дека станува збор за трансмисивен венеричен тумор. Синдром на заостанат јајник беше дијагностициран со ехо на абдомен, со прикажување на резидуален јајник. Винкристин сулфат со 0,9% NaCl беше даден во бавна инфузија еднаш неделно, според неделните прегледи на пациентот и нодот од хемограмот. По хемотерапијата, заостанатиот јајник беше отстранет лапараскопски. Како заклучок, трансмисивниот венеричен тумор може да се трансплантира на стерилизирани животни со остатоци на ткиво од јајник и овие животни може да имаат учество во ширењето на туморот.
Клучни зборови: трансмисивниот венеричен тумор, синдром на заостанат јајник, винкристин, куче

UDC: 636.7.09:618.11-006-076

OVARIAN SEX CORD TUMOR WITH ANNULAR TUBULES IN A DOG

İbrahim Firat [1], Hande Özyogurtcu[1], Sinem Özlem Apaydin[2], Hayri Ekici[2]

[1]Department of Pathology, Faculty of Veterinary Medicine, Istanbul University, Istanbul - Turkey
[2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Istanbul University, Istanbul - Turkey

ABSTRACT
A case of ovarian sex cord tumor with annular tubules (SCTAT) is described in a dog. The tumor was an incidental finding in an ovariohysterectomy specimen, obtained because of pyometra and dilatation of the uterine cervix in a 15-year-old dog. Histologically, the tumor consisted of granulosa cells, arranged in a pattern of tubulary or reminiscent

INTRODUCTION

Sex cord-stromal (gonadostromal) tumors are clearly the most common ovarian neoplasms of the mare, cow, and queen (1,2). Bitches with sex cord-stromal tumors also may manifest signs of hyperestrinism including endocrine alopecia, bone marrow suppresion with consequent anemia, leukopenia fallowing an initial period of leukositosis, trombocytopenia and hemorrhagic diathesis, and cystic endometrial hyperplasia. These changes are reversible by removal of the tumor (2).

The case reported was an incidental finding in a ovariohysterectomy specimen, obtained because of pyometra and dilatation of the uterine in a 15-year-old dog and to our knowledge, this is the first case reported in the dog.

CASE

The tumor (10x8x7 cm) was an incidental finding in an ovariohysterectomy specimen, obtained because of pyometra and dilatation of the uterine cervix in a 15-year-old dog. Histologically, the tumor consisted of granulosa cells, arranged in a pattern of tubulary or reminiscent of Sertoli cells growth. The entity, immunohistochemical assessment of which is ongoing, was diagnosed as sex cord tumor with annular tubules (SCTAT) on the basis of histomorphological evaluation alone.

CONCLUSION

A review of the bibliography shows that sex cord tumors with annular tubules are very rare in human. The sex cord tumor with annular tubules (SCTAT) is a distinctive ovarian neoplasm the predominant component of which has morphologic features intermediate between those of the granulosa cell tumor and those of the Sertoli cell tumor; focal differentiation into either granulosa cell or Sertoli cell tumor may occur. (3, 4).

In conclusion, a case of sex cord tumor with annular tubules (SCTAT) arising in a dog ovary is described. This tumor are considered to originate from granulosa cells, and grow in patterns reminiscent of Sertoli cells. To our knowledge, this is the first reported case of an ovarian sex cord tumor with annular tubules (SCTAT) in a dog.

REFERENCES

1. McEntee K (1990): Ovarian neoplasms. In, Reproductive pathology of domestic mammals. pp. 69-93. San Diego, Academi Pres Inc.
2. MacLachlan NJ, Kennedy PC (2002): Tumors of the urinary system. In, Meuten DJ (Ed): Tumors in Domestic Animals. 4th ed. pp. 547-574, 2121 State Avenue, Ames, Iowa 50014, USA, Iowa State Press, A Blackwell Publishing Company.
3. Young RH, Welch WR, Dıckersın GR, Scully RE (1982): Ovarian Sex Cord Tumor with Annular Tubules. Review of 74 Cases Including 27 with Peutz-Jeghers Syndrome and Four With Adenoma Malignum of the Cervix. *Cancer* 50:1384-1402.
4. Scully RE. (1970): Sex cord tumor with annular tubules: A distinctive ovarian tumor of the Peutz-Jeghers syndrome. *Cancer*; 25:1107-1121.

ОВАРИЈАЛЕН СТРОМАЛЕН ТУМОР СО ПРСТЕНЕСТИ КАНАЛИ КАЈ КУЧЕ

Ибрахим Фират [1], Ханде Озјогуртчу[1], Синем Озлем Апајдин[2], Хајри Екичи[2]

[1] Катедра за патологија, Факултет за ветеринарна медицина, Универзитет во Истамбул, Истамбул Турција
[2]Оддел за акушерство и гинекологија, Факултет за ветеринарна медицина, Универзитет во Истамбул, Истамбул, Турција

АПСТРАКТ
Опишан е случај на овариален стромален тумор со престенести канали ((SCTAT) кај куче. Туморот беше откриен случајно при овариохистеректомија, извршена поради пиометра и дилатација на цервиксот на матката кај 15 годишно куче. Хистолошки, туморот се состоеше од гранулоза клетки, наредени како канали или наликуваа на профилерирани Сертолиеви клетки. Врз база на хистоморфолошката процена, туморот беше идентификуван/дијагностициран како овариален стромален тумор со прстенести канали.

ABSTRACTS

KINETICS OF THE RESIDUE LEVELS OF GATIFLOXACIN IN POULTRY MEAT AT STORAGE

Kyuchukova Ralica and Pavlov Aleksandar

Department of Food Hygiene, Technology and Control, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

ABSTRACT
Studies were carried out on the residue levels of gatifloxacin in poultry meat and internal organs during their storage at minus 18°C. Twelve chickens were treated with the antibiotic orally at a dose of 10 mg/kg BW for 5 days. Six chickens were slaughtered on the day immediately after stopping treatment (day 0) and six chicken – on 6-th day. Breast muscle, gizzard, liver, heart and skin (with fat) were analyzed by microbiological method (test-microorganism E. coli ATCC 25922). It has been estimated that the initial levels of gatifloxacin on both days 0 and 6-th in skin and liver were higher than in meat, stomach and heart. On day six the level of antibiotic in liver and skin was decreased to 38% and 18% respectively. The samples taken on day 0 and day 6 were frozen at minus 18°C and analyzed on days 1, 7, 14, 21, 60, 75 and 90 of the storage. During the storage gatifloxacin residues decreased in all the tissues and the rate of decreasing in samples with higher initial level (day 0) was faster, than in samples with lower levels (day six). The final level of the antibiotic on day 90 was approximately the same for every tissue, regardless of slaughtering day (zero or 6-th).
Key words: gatifloxacin, chicken, residues, storage

SPECIES DISTRIBUTION OF METHICILLIN RESISTANT STAPHYLOCOCCI ISOLATED FROM ANIMALS, ENVIRONMENTAL SAMPLES AND STAFFS

Arzu Funda Bağcıgil[1] , Serkan İkiz[1], Özlem Güzel[2], Çağla Parkan Yaramış[3], Atila Ilgaz.[1]

[1] University of Istanbul, Faculty of Veterinary Medicine, Department of Microbiology, Avcılar, İstanbul
[2] University of Istanbul, Faculty of Veterinary Medicine, Department of Surgery, Avcılar, İstanbul
[3] University of Istanbul, Vocational School of Veterinary Medicine, Avcılar, İstanbul

ABSTRACT
The aim of this study is to investigate the presence of methicillin resistant staphylococci, particularly methicillin resistant *Staphylococcus aureus* (MRSA), from the dogs and cats that brought to the Veterinary Faculty clinics and the staff in those clinics, and risk of the environmental contaminations in the clinics.
For this purpose; swab samples were taken from nasal mucosa of 18 staffs, nasal and oral mucosa of 7 cats and 21 dogs. For determination of environmental contamination 33 swab samples from various surfaces of the clinics were collected. Gram positive cocci were identified following after the determination of methicillin resistance by disc diffusion method. All the isolates examined for the presence of the *mec*A gene by PCR, for molecular typing by RAPD-PCR. Antibiotic susceptibilities of the isolates were determined.
Three (42.9 %), 5 (23.8 %), 19 (57.6 %) and 13 (72.2 %) methicillin resistant coagulase negative staphylococci (MRCoNS) were isolated out of 7 cats, 21 dogs, 33 environmental and 18 staff samples, respectively. While *S. hominis* were isolated predominantly, no MRSA were isolated from the samples. Out of 41 isolates 87.7%, 63.4%, 58.5% of them were resistant to penicillin G, erythromycin and tetracycline, respectively.
Key words: *Staphylococcus* spp., methicillin, *mecA*,

THE EVALUATION OF BLOOD ELECTROLYTES, SYSTOLIC BLOOD PRESSURE AND ELECTOCARDIOGRAPHIC FINDINGS IN CATS WITH AZOTEMIA

M.Ali Sağır[1], Alev Akdoğan Kaymaz[2], Alper Bayrakal[2]

[1]Greenpet Veterinary Clinic;
[2]Departments of Internal Medicine,
Faculty of Veterinary Medicine, Istanbul University, İstanbul, Turkey

ABSTRACT
Early diagnosis of the azotemia is very important in the development of the kidney diseases. So, when the disease is severe, it would threat the animal life. In this study, systolic blood pressure, electrocardiography findings, electrolyte levels and urine protein/creatinine ratio (UPC) were taken into account in cats with azotemia for this purpose.
As a working material, 30 of the cats with different age and gender and with increased blood urea nitrogen were selected for the study group. Fifteen healthy cats constituted the control group. Complete blood count, serum biochemistry (BUN, creatinine, glucose, AST, ALT, t.protein, albumin) and electrolites were measured. Elecrocardiogram and also urine samples for UPC ratio (UPC) were taken from the cats. Results were analysed with independet samples t test and Pearson correlation test..
When the results of the healthy cats were compared to the results of the cats with azotemia, the amount of albumin decreased ($p<0.05$) significantly. The levels of phosphorus ($p<0.01$) and calcium ($p<0.05$) also significantly increased. Electrocardiographic traces taken in cases of hyperkalemia and hypokalemia have been introduced and significant changes have occurred. UPC ratio were also significantly higher in cats with azotemia than the healthy cats ($p <0.05$). There was a negative correlation between blood pressure and UPC ratio ($p<0.05$, $r=-0.34$).
As a result, electrocardiographic findings, blood pressure measurements, electrolytes and UPC ratio were considered to be usefull parameters for the evaluation of azotemia to arrange of the effective treatments and to determine the prognosis in cats with azotemia.
Key words: cat, azotemia, systolic blood pressure, ECG, UPC

RABIES AND CSF CONTROL (SITUATION) IN KOSOVO

Skender Muji[1], Ardita Jahja[1], Bajram Batusha[2]

[1] University of Prishtina, Faculty of Agriculture and Veterinary– Prishtina, Kosovo.
[2] Ministry of Agricuture, Forestry and Rural Development – Prishtina, Kosovo

ABSTRACT
The main objective of the research was to assess and improve the animal health situation in Kosovo as regards rabies and CSF.
The purpose of these study is the control and/or eradication of CSF and rabies from animal populations, in particular from wildlife, in Kosovo, which contribute to an improved human and animal health status, as well as an improved trading status of Kosovo for the export of live animals and animal products. Based on a further enhanced capacity of the veterinary services in Kosovo. At the same time, considering the trans-boundary character of interventions, regional cooperation in the West Balkans will be promoted.
The last case of human rabies was in 1954. In October 2007, two cases of rabies in wild animals (foxes) were reported in the region near the Macedonian border (Štrpca). In both cases, a rabid fox came to premises such as a police station and a holding. Laboratory tests were performed by FLI, Wusterhausen (Germany).
The last outbreak of CSF was reported in 2006. The virus was at that time characterised in cooperation with the European Community Reference Laboratory for CSF. It belonged to genotype 2.3.
For the collection of samples, hunters and private veterinarians (PVPTs) were involved.

RHEOLOGICAL PARAMETERS AND CHANGES IN THE PERIPHERAL LYMPHOCYTE MEMBRANE IN DIABETIC DOGS

Alev Akdoğan Kaymaz[1], Işıl Albeniz[2], Şule Tamer[3]

[1]*Departments of Internal Medicine, Faculty of Veterinary Medicine;*
[2]*Biophysics;*
[3]*Physiology, Istanbul Faculty of Medicine, Istanbul University, Istanbul*

ABSTRACT
It has been reported that several parameters affecting blood flow mechanics, including blood viscosity and erythrocyte deformability are altered in patients with diabetes mellitus. However, there are limited data concerning the contribution of cell membrane protein abnormalities to the increased leukocyte rigidity and blood rheology. Functional alterations of polymorphnuclear leukocytes and lymphocytes subgroups have been implicated in the pathogenesis of immune dysfunction associated with diabetes mellitus (DM). In this study, we aimed to assess the rheological properties of lymphocytes and their membrane protein content in dogs with DM.
After the clinical examination, venous blood samples were obtained from dogs with DM (n=10) and healthy dogs (n=10) with different ages and gender which were brought to the Department of Internal Medicine, Veterinary Teaching Hospital, Istanbul University. Complete blood count and serum fasting glucose, BUN, creatinin, AST, ALT, t.protein and albumin levels were detected. Lymphocyte deformability was assessed with the microfiltration technique by measuring cell rigidity against pressure. Membrane proteins were evaluated with the SDS-PAGE polyacrylamide gel electrophoresis technique. Statistical analyses were performed by Student-t test.
The deformability of peripheral blood lymphocytes were found to be significantly decreased in diabetic dogs compared to healthy group ($p<0.001$). There was no difference in the membrane proteins in both groups.
It has been well established that DM is associated with impairment of microcirculation and immune dysfunction. The findings of the present study suggest that although the membrane protein content seems to be unaltered, decreased lymphocyte deformability may be involved in such circulatory disturbances and possibly in the propensity to infections.
Key words: lymphocyte deformability, membrane proteins, diabetes mellitus, dog

VENTRICULAR SEPTAL DEFECT IN FIVE DOGS

Sinem Ülgen and Utku Bakırel

Istanbul University, Faculty of Veterinary, Department of Internal Medicine, Istanbul, Turkey

ABSTRACT
Ventricular septal defect (VSD) is a common congenital disorders of dogs occured by the formation of defects in development of embryonic ventricular septum. In this cases, 6th month old Golden Retriever male dog (case 1), 1 year old Miniature Schnauzer female dog (case 2), 6 years old Golden Retriever male dog (case 3), 9 month old male Golden Retriever dog (case 4) and 8 month old male German Shepherd dog (case 5) were presented to the veterinary teaching hospital of Istanbul University with common complaints of growth retardation, excercise intolerance ,tachycardia and tachypnea. Case 1 and 3 were sent to our faculty hospital with diagnosis of acute kidney failure. No abnormal laboratory findings were detected in dogs except these cases. Cardiomegaly was determined by X-ray examinations in only one case and echocardiographic examinations were performed to dogs. Ventricular septal defect (VSD) was diagnosed by colour doppler echocardiographic evaluation in the cases. VSDs of all cases were located in the upper ventricular septum below the aortic valve on the left side. Congenital mitral valve disease were detected in three dogs and additionally tricuspital dysplasie was also determined in one of these dogs. Right ventricular dilatation (n=3), left ventricular posterior wall and interventricular septum thickening (n=2), left atrial dilatation (n=2) and left ventricular dilatation (n=1) were detected. Ejection fractions and fractional shortening were ranged between 76-85% and 38-52%, respectively.
These cases were considered worthy to be presented, as congenital disorders are rarely diagnosed and VSD with congental valve disease is seldomly detected in dogs.
Key words: dog, ventricular septal defect

THE URINE BLADDER STONE IN FIVE-MONTH TABBY CAT

Alev Akdoğan Kaymaz[1], Taner Bahçeli[1*], Kürşat Özer[2]

[1]Departments of Internal Medicine;
[2]Surgery, Faculty of Veterinary Medicine, Istanbul University, İstanbul, Turkey

ABSTRACT
It appears that diet may increase the risk of the development of urinary crystals, stones, and urethral plugs in the cats. The development of crystals and stones is mostly dependent upon the urine pH and the concentration of minerals in the urine. Genetics also seems to play an important role. This case is about Cracker that is male, five-month aged, tabby cat. Although he treated a few times for tree months, the complicant of recurring hematuria was presented to the Department of Internal Medicine of Veterinary Teaching Hospital, İstanbul University. In anemnesis, general conditions of the patient were very good, but occasionally hematuria and pollakuria were observed. The patient owner was feeding Cracker by uncooked meat. A sensibility detected during the palpation of abdomen. It were taken the blood and urine samples and x-ray and ultrasonography of the abdomen from the cat were taken. It was found that leucocytes was slightly increased in the complete blood count. In serum biochemistry, BUN, creatinine, ionized calcium and parathormone levels were in normal limits. But, serum calcium (4.5 mg/dl) and phosphorus (10 mg/dl) levels were higher than normal values. There was 3+ positive proteinuria in urine sample taken by cytocentesis. It's found that pH was 6.5 and urine specific gravity was 1.025. Erythrocyte, leucocyte, triple phosphate crystals and kidney epithels were abundant in the urine sediment. There was no isolation in the urine culture. With the ultrasonography, the stone about 7.84x0.5 mm was observed in the urine bladder. This stone was taken by cystotomy and irrigated the bladder. Based on this case, we considered that the formation of this stone in Cracker was connected to the nutrition by uncooked meat from the very beginning.
Key words: cat, bladder stone

COMPARATIVE INVESTIGATIONS ON THE DISTRIBUTION AND NUMBER OF MAST CELLS IN THE PELVIC PART OF THE MALE PIG`S URETHRA AFTER DIFFERENT STAININGS

Kostadinov G.[1], Vodenicharov A.[1], Kostadinova L.[2]

[1]Department of Veterinary Anatomy, Histology and Embryology,
[2]Department of Legislation and Management,
Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria

ABSTRACT
It is known that the mast cells granules content ligands with important biological significance. This determine a considerable interest, which is appear in over last three decades to their species, organs, histo- and immunocytochemical features. The localization and histochemical characteristics of mast cells in the urogenital apparatus, and especially in the male genital organs of domestic animals are poorly investigated. Until now, there are data for mast cells presence in the kidney, testis and urinary bladder of domestic pig. Recently data showed that the mast cell's visualization is depends not only of the fixative type, but and the respective method of staining, as well. However, the species of animal on which such investigation carry out, is also important.
The aim of that study is to establish the localization and some histochemical peculiarities of mast cells in: the pelvic part of urethra, the disseminate pars of prostate (pars disseminate) and *M. urethralis*, after using of different staining methods.
The data obtained showed different distribution of mast cells in above mentioned structures. The prevailing part of them were observed in the connective tissue among glandular lobules and on the border between the pelvic part of urethra and *M. urethralis*, surrounding its ventral and lateral sides. Clusters of mast cells were observed in vicinity to interstitial small and middle blood vessels and among smooth muscle cells of *M. urethralis*. An interest provoke and The obtained results concerning of percentage distribution and the number of mast cells in investigated layers the organ, after toluidine blue and alcian blue – safranin stainigs also provoke an interest.
Key words: mast cells, urethra pelvina, toludine blue, alcian blue-safranin

HOUSING FACTORS EFFECTING ON MEAT TYPE POULTRY BONE STRENGTH

Jahja Ardita[1], Mestani Nuridin[1], Alltane Kryeziu[1]

University of Prishtina, Agriculture and Veterinary Faculty, Prishtina – Kosovo.

ABSTRACT

Bone breakage is serious welfare problem. Meat type poultry and cage layers exhibit a high incidence of bone problems that include bone weakness, deformity and breakage. These problems include economic and welfare issues. Bone breakage is a serious problem of poultry in both intensive and extensive husbandry systems.

The maintenance of bone strength has been an important issue in the debate over cage use. Bone strength depends on adequate mechanical load and restricted movement. Hybrid Ross 308 of broilers was kept in two different housing systems (conventional cages and floor). Also the focus of experiment is gender.

Leg weakness is one of the major welfare problems in rapid growing meat-type chickens (BESSEI, 1993; EUROPEAN COMMISSION, 2000). Sporadic incidences of bone fractures, frequently femoral, are reported in growing broilers. In these incidences the aetiology is mainly undefined, but the presence of poorly organized cortical bone may indicate some form of osteodystrophy.

Physical activity improves bone formation. This has been widely documented in human osteoporosis and rats. Physical activity increases bone apposition while, on the contrary, a reduction in mechanical stress by spaceflight decreases bone density. Improvement of bone apposition via exercise has also been reported in broiler chickens and in laying hens.

The present study aims at investigating the effect of, cage and floor housing as well as gender and diet on the bones of broilers.

ASSESSING THE WELFARE OF LAYING HENS IN CONVENTIONAL CAGE HOUSING SYSTEMS

Prodanov Mirko[2*], Sekulovski Pavle[2], Ilieski Vlatko[1]

[1] Center for animal welfare Faculty of Veterinary Medicine, University "Ss. Cyril and Methodius,,- Skopje, Republic of Macedonia
[2]Food Institute, Faculty of Veterinary Medicine, University "Ss. Cyril and Methodius,,- Skopje, Republic of Macedonia

*e-mail: m.prodanov@fvm.ukim.edu.mk

ABSTRACT

The aim of this survey was to conduct a science-based assessment of the welfare of laying hens in commercial production systems in Republic of Macedonia. The survey was carried out on a selection five different flocks form three laying hens farms. Two of the flocks were housed in modified enriched cages, while three of the flocks were in conventional cages. The performed welfare assessment was designed in accordance with the established welfare principles, criteria and measures by the Welfare Quality Project, used twelve define welfare criteria. According to the criteria a welfare assessment protocol for laying hens was created and the ongoing assessment was conducted according to the overall assessment on the farm, the farm records and assessment of 100 individual birds form each flock. The initial data showed that although two of the flocks declared to have enriched cages, the cages were modified in such way so they could house more birds and thus making it function as conventional cages. The results showed relatively good scores in the criteria good feeding and good health, but showed low scores on the criteria good housing and appropriate behavior. The low scores obtained in this survey were due to the lack of interest of the farmers to invest in the new cages that will increase the cost of the production thus increasing the cost of the eggs. According to the new legislation the farmers all the conventional cages will have to be replaced with enriched cages or other alternative housing systems, and accent is put on improving the welfare of laying hens. For further surveys, it is necessary to involve more laying hen farms for in order to gain more reliable study for animal welfare in the laying hen farms. Also added value will be introduction of welfare assessment protocols and involving evaluation of the scores for expressions of normal behaviors.

EFFECTS OF DIFFERENT TRANSPORT TEMPERATURES ON IN VITRO DEVELOPMENT OF QUEEN OOCYTES

Ozen Banu Ozdaş[1], Alper Baran[1], Asiye Izem Sandal[1], Gul Bakirer[1], Cagatay Tek[2], Sinem Ozlem Enginler[2], Mehmet Can Gunduz[2], Guven Kasikci[2] and Kemal Ak[1]

[1]Istanbul University,Veterinary Faculty, Department of Reproduction and Artificial Insemination Avcılar/Istanbul
[2] Istanbul University,Veterinary Faculty, Department of Obstetrics and Gynecology, Avcılar/Istanbul

ABSTRACT
Domestic cats are chosen as a model for in vitro culture studies. So many investigators have problems in transporting ovaries to laboratories from place to place. In this study effects of different transport temperatures on the way to laboratory on in vitro maturation of oocytes was investigated. Ovaries were collected from 12 ovariectomised queens of 2-3 age, 4 of which were at oestrus and 8 at anoestrus. One ovary of each pair was brought to the laboratory in PBS at 4˚C and the other at 37˚C. Two main groups as oestrus and anoestrus were established and each were divided into further 2 subgroups as 4˚C and 37˚C. Total 162 oocytes were used. Oocytes were collected in TCM-199 medium and matured for 24 hours under 5% CO2 at 38.5˚C. Matured oocytes were fertilized with fresh semen. Denuded zygotes were divided into groups and in vitro cultered for 5 days in SOF medium under 5% CO2, 5% O2 and 90% N2 gas mixture. At the 48th hour of culture, the best cleavage was 44.4% at 37˚C oestrus group, and the lowest was 18% (15/66) in the oestrus group at 4˚C. These rates were 22% (9/50) and 28% (8/28) respectively for the anoestrus group. At the 5th day of culture, in 37˚C oestrus group 7 embrio stayed at 4-8 blastomere stage and 1 embrio reached 16-32 blastomere stage. This result was significant at $p<0.001$ level when compared to the other groups. In anoestrus group at 37˚C, 3 of 15 cells stayed at 4-8 blastomere stage and 3 reached 8-16 blastomere. In 4˚C anoestrus group only 3 have reached 4-8 blastomere and no significant difference among the results was observed. It is concluded that oestrus cat ovaries are beter transported at 37˚C but anoestrus ovaries could be carried at 4˚C.
Key words: Queen, transport, temperatures, oocyte, in vitro fertilization

UTERINE PROLAPSE IN A COCKATIEL RELATED TO CHRONIC EGG LAYING

Sinem Özlem Enginler, Gamze Evkuran, Esra Çalışkan, Hayri Ekici

Faculty of Veterinary Medicine, University of Istanbul, Department of Obstetrics and Gynaecology, Avcılar, 34320, Istanbul, Türkiye

ABSTRACT
A 6 years old cockatiel that laid three eggs in two consecutive days was admitted to the clinic with the complaint of bleeding from the cloaca generate our case. A necrotic tissue that was hanging out and shattered by the cockatiel was noticed in our examination. The prolapsed tissue was determined as uterus (shell gland). The cockatiel underwent inhalation anesthesia by using %2 isoflurane by mask. Oxytocin was administered to the prolapsed tissue and uterine canal opening by a swab in order to reduce the mass. The tissue was regularly flushed with serum physiologic and crystal penicilline combination during the surgery. The canal opening lubricated by a swab to avoid laceration. The necrotic tissue was dissected and 5/0 vicryl was used to suture the mouth of the canal opening by simple split suturing. After revision of the tissue, the rest of the healthy prolapsed tissue replaced to its normal position by a cotton swab. The cloaca was sutured by purse-string suture. For postoperative care an antibiotic was prescribed for a week. The sutures at the cloaca were removed one week later and at the last examination of the cockatiel no abnormality was detected.
In conclusion, it is indicated that such cases can be treated operatively.
Key Words: Cockatiel, prolapse of uterus, surgical approach

STUDY OF THE CRITERIA FOR DEVELOPMANT OF STAFF IN THE FIELD OF VETERINARY SCIENCE BEFORE AND AFTER THE LAW OF DEVELOPMENT OF THE ACADEMIC STAFF IN REPUBLIC OF BULGARIA

Kostadinova Laska

Trakia University, Faculty of Veterinary Medicine, Stara Zagora 6000, Bulgaria

e-mail: lassy_2002@abv.bg

ABSTRACT
Many are the factors and measures that define the image and the prestige of given university, but one mostly important is the amount and the quality of the academic staff. The issue of the development of the academic staff in the universities in Bulgaria has been a subject of many discussions within the scientific society, governmental institutions, political and public organizations. After the 90's political and democratic changes in the country the problem becomes much more important and relevant.
The aim of the study is related to research on the measures of awarding of scientific degrees and titles by the staff, according to the old regulatory and the new law approved for the development of the academic staff from 2010. There has been a tracking of the basic positive and some negative aspects of both laws – The Law of the scientific degrees and titles and the Law of the development of the academic staff in Republic of Bulgaria in the organizational, legal and financial aspect. Conclusions are made according to their advantages and disadvantages.
Key words: scientific workers, academic staff, human resources, staff development.

INDEX OF AUTHORS

Acevski S. 28,59,86
Adamov M. 234
Adamov N. 229, 234, 265, 282
Ajdzanovic V. 223
Ajmone Marsan P. 226
Ak K. 298
Akdoğan Kaymaz A. 294, 295, 296
Albeniz I. 295
Ali Sağır M. 294
Angelovski Lj. 116, 143, 176, 179, 182
Angjelovski B. 30
Anil H. 193
Antonijević B. 108
Apaydin S. Ö. 290
Arnaudova-Matey A. 185
Arsova G. 154, 179
Atanaskova Petrov E. 73
Atanasoff A. 90
Atanasov B. 229, 265, 282

Babic J. 102
Bahçeli T. 296
Bakırel U. 295
Bakirer G. 298
Baran A. 298
Batusha B. 294
Bauer M. 106
Bayrakal A. 294
Biasizzo M. 106
Binev R. 207
Blagoevska K. 212, 223, 229, 265
Blagoevski A. 212
Bordonaro S. 226
Buncic S. 97

Carta A. 226
Celeska I. 28, 73, 83
Chaprazov Z. 239
Chervenov M. 262
Ciampolini R. 226
Ciani E. 226
Cliquet F. 30
Consorzio B. 226
Crceva Nikolovska R. 179
Crepaldi P. 226
Cvetkovikj A. 30
Cvetkovikj I. 28, 30, 38, 59
Çalışkan E. 298
Ćurčić M. 108
ÇilİplikçioğluG. 136

D'andrea M. 226
Daskalov H. 120
Daskalova A. 120, 204
Demirel Y. 136
Dimeski Z. 77
Dimitrieska-Stojkovik E. 123, 127, 132, 139, 149, 154, 159, 165, 170
Dimitrov R. 239
Djadjovski I. 28, 30, 59, 86
Dobrosavljević I. 17, 21
Dodovski A. 25, 212
Dovc P. 199
Dovenski T. 265, 282
Đurić M. 48

Ekici H. 290, 298
Elezovic M. 34
Enginler S. O.
Esmerov I. 212, 229, 234, 282
Evkuran Dal G. 287
Evkuran G. 298

Farkas R. 38
Firat İ. 290
Funda Bağcıgil A. 293

Georgiev B. 262
Giadinis N.D. 255
Giorgobiani M. 62
Gjurovski I. 30
Gombač M. 71
Gunduz Can M. 298
Güzel Ö. 293

Hajrulai-Musliu Z. 123, 127, 132, 139, 149, 154, 179
Horvat J. 11
Hristovski M. 71

İkiz S. 293
Ilgaz A. 293
Ilic Ž. 21
IlieskiV. 212, 216, 223, 247, 297
Ilievska K. 40, 64, 73, 282
Ivanov V. 90

Jahja A. 294, 297
Jakić-Dimić D. 272
Janevski A. 86
Janković S. 108
Jankuloski D. 116, 143, 176, 182
Jevšnik M. 106
Josheski M. 77
Jovcevski S. 34
Jovcevsi St. 34

Kacheva D. 262
Kasikci G. 298
Kirandjiski T. 28, 30
Kirbiš A. 106
Kirovski D. 11, 48
Kırşan İ. 285
Kistanova E. 262
Kochevski Z. 38
Kompan D. 226
Kostadinov G. 296
Kostadinova L. 296, 299
Krstevski K. 25, 28, 30, 59, 86
Krstić V. 15
Kryeziu A. 297
Kukovska V. 34
Kunej T. 199
Kyuchukova R. 189, 293

Lacalandra G.M. 258
Lalev D. 44
Lasagna E. 226

Macciotta N. 226
Makaradze L. 62
Matassino D. 226
Matekalo-Sverak V. 102
Mehmedov T. 185
Mestani N. 297
Mickov Lj. 212, 234, 265, 282
Mihajlović J. 159
Milijasevic M. 102
Miljković B. 17, 21,
Milosevic V. 223
Mircheva T. 44
Mirtskhulava M. 62
Mitev Y. 207
Miteva Ch. 207
Mitrov D. 25, 28, 59, 86
Modesto P. 226
Mojsova S. 116, 143, 176, 182
Mrenoshki S. 28, 30, 38, 59
Muji S. 294

Naletoski I. 25, 28, 38, 59
Napolitano F. 226
Naumoska M. 165
Nicassio M. 258
Nikolić D. 108
Nikolov G. 90
Nikolovski A. 179
Nikolovski G. 73
Nikolovski M. 265

Ogorevc J. 199
Ozdaş O. B. 298
Özer K. 296
Özlem Enginler S. 298
Özyogurtcu H. 290

Parkan Yaramış Ç. 293
Pavlov A. 204, 293
Pavlovic I. 34, 272
Pavlovic M. 34, 272
Pavlović V. 272
Pavlovski D. 67, 83
Pendovski L. 223, 234, 247
Petkov V. 247, 265
Petkovic D. 34
Petrova B. 90
Petrović Z. 108
Peycheva M. 111
Pogačnik M. 71
Popovska-Percinik F. 212, 223, 247
Portolano B. 226
Prodanov M. 116, 143, 176, 182, 297
Prodanov R. 149, 179
Prodanović R. 48
Prpar S. 199

Radeski M. 30, 212, 216
Radičević T. 108
Radojčić M. 21
Ratkova M. 116, 143, 176, 182
Russenov A. 239

Sabev M. 262
Šamanc H. 11, 48
Sandal A. I. 298
Sekovska B. 56
Sekulovski P. 116, 123, 127, 132, 139, 143, 176, 179, 182, 297
Seničar M. 71
Sinem Hande Ö. 290
Sladojević Ž. 48
Stamatova-Yovcheva K. 239
Stamenkovic V. 34
Stefanov I. 243
Stefanov R. 262
Stefanović S. 108
Stefanovska J. 38
Stoilova N. 111
Stojanovska-Dimzoska B. 123, 127, 132, 139, 149, 154
Stojković G 149, 159, 165, 170
Stojkovski V. 123, 127, 212, 229
Stoyanchev T. 146
Švara T. 71

Tamer Ş. 295
Tansel U. Ş. 136
Taushanova P. 262
Tek C. 298
Todoroska M. 207
Todorova I. 52
Todorovic A. 123, 127, 132, 139
Tomeska-Mickova S. 265
Töre Merih D. 287
Torkar K. 106
Tosevska – Apostolova M. 56
Tososka Lazarova D. 247
Trajanoska B. 77
Trajkovska V. 170
Trenkoska-Spasovska P. 40, 64
Trifunovic S. 223
Trojacanec P. 40, 64, 73, 282
Turna Yılmaz Ö. 285

Uçmak M. 285
Ulčar I. 83
Ülgen S. 295
Uzunov R. 123, 127, 132, 139, 154
Uzunova K. 207

Vadnjal S. 106
Vakanjac S. 272
Vashin I. 146
Velev R. 73, 159
Velhner M. 17, 21
Velkovski D. 86
Vodenicharov A. 243, 296
Vujanac I. 11, 48

Yankovska T. 111
Yovchev D. 239

Zapryanova D. 44
Zhelyazkov G. 90
Žižek S. 71

Sponsors:

ALKALOID SKOPJE
Health above all
www.alkaloid.com.mk

УНИВИГО
ДОО Експорт - Импорт Скопје

VETERINA

АЛИМАН - КОМЕРЦ
ул. Антон Попов бб
1000 Скопје, Р. Македонија
гел. 02/2550 712, 2550 713
факс: 2550 714

BI EL
engeneering
Strumica

ВИН СКОПЈЕ
ВИН Скопје доо
Франце Прешерн 61,
1000 Скопје, Македонија
телефон: +389 2 2032397
факс: +389 2 2032138
e-mail: vin@on.net.mk
Virbac ANIMAL HEALTH
Allflex
KRUUSE
Anigen Animal Genetics Inc.

LIDIAVET
MERIAL
ROYAL CANIN

General Sponsors:

VITA-VET
SKOPJE
++389 2 / 2614-681 ; 2636-588
Str. Tajmiska No.32, 1000 Skopje, R. Macedonia

BIOTEK
SOLUTIONS