ISSN 1820-9955
UDK 619

Scientific Veterinary Institute „Novi Sad” Novi Sad
Naučni institut za veterinarstvo „Novi Sad” Novi Sad

Archives of Veterinary Medicine
Arhiv veterinarske medicine

Arch. vet. med.	Vol. 14	No. 1	Pg. 1-132	Novi Sad, 2021.

ISSN 1820-9955 UDK 619

Scientific Veterinary Institute „Novi Sad" Novi Sad
Naučni institut za veterinarstvo „Novi Sad" Novi Sad

Archives of Veterinary Medicine
Arhiv veterinarske medicine

Arch. vet. med.	Vol. 14	No. 1	Pg. 1-132	Novi Sad, 2021.

CIP – Каталогизација у публикацији
Библиотека Матице српске, Нови Сад

619

Archives of Veterinary Medicine = Arhiv veterinarske medicine / Editor in Chief Sava Lazić. – Vol. 1, br. 1 (2008) –.– Novi Sad : Scientific Veterinary Institute "Novi Sad", 2014 –.– 25 cm

Published twice a year.

ISBN 1820-9955

COBISS.SR-ID 235692807

EDITORIAL TEAM

Published twice a year. Circulation: 100 copies

Address:
Scientific Veterinary Institute "Novi Sad",
21000 Novi Sad, Rumenački put 20
Tel. 381 (0)21 48 95 321
E-mail: arhiv@niv.ns.ac.rs
Account No: 355-1006444-18 Vojvođanska banka,
ID No.: 08608857,
PIB 100236555

Original research paper

UDC: 636.594:637.54
https://doi.org/10.46784/eavm.v14i1.256

THE EFFECT OF ORGANIC SELENIUM ON HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF BLOOD AND THE QUALITY OF PHEASANT BREAST MEAT

Saša Obradović[1*], Branko Petrujkić[2], Vladan Đermanović[3], Vladimir Živković[4], Bojan Stojanović[3], Nenad Đorđević[3], Milanka Radulović[5]

[1]Faculty of Agriculture, Kruševac, University of Niš, Republic of Serbia
[2]Faculty of Veterinary Medicine, University of Belgrade, Republic of Serbia
[3] Faculty of Agriculture, University of Belgrade, Republic of Serbia
[4] Institute for Animal Husbandry, Belgrade-Zemun, Republic of Serbia
[5]State University of Novi Pazar, Department of Biomedical Sciences, Novi Pazar, Republic of Serbia

Abstract

The aim of this study was to determine the effect of supplementing food with various concentrations of organic selenium (0.2 and 0.3 mg/kg diet) on the biochemical and haematological parameters of blood and the quality of breast meat of 45 pheasants. The pheasants were divided into three groups and fed mixtures containing organic selenium supplementation in the concentrations of 0.2 mg/kg (1st group) and 0.3 mg/kg (2nd group) and a mixture without selenium in a control group (K). After 60 days of the experiment, it was determined that the average values of selenium content in the breast meat and blood serum of the 2nd group of pheasants which were fed 0.3 mg/kg of organic selenium were significantly higher ($p < 0.05$) than the same parameters of the pheasants from K group. The pheasants from the 2nd group also had better sensory traits of meat and they had the highest difference of the sum of the ranks of meat acceptability. The difference was by 15 points higher than that in the K group and 7 points higher than in the meat of the pheasants from the 1st group that fed 0.2 mg/kg of selenium. The addition of organic selenium supplementation to the diet for the 2nd group of pheasants (0.3 mg/kg) increased the water retention capacity in breast

[1*] Corresponding Author: sasaobradovic1@gmail.com

meat by 0.75% compared to the K group, namely by 0.58% in comparison to the 1st group. The average values of chemical parameters of meat (pH, water, fat, proteins and ash content), haematological parameters of pheasant blood (number of erythrocytes, leucocytes and platelets, haemoglobin and haematocrit values) and biochemical parameters of blood serum (glucose, enzymes: aspartate transaminase and alanine aminotransferase, total protein concentration, total cholesterol albumin, triglycerides, calcium, potassium and sodium) were within the limits of reference values for pheasants and very uniform without significant variations among experimental groups.

Key words: pheasant, breast meat quality, biochemical and haematological profile

EFEKAT DODAVANJA ORGANSKOG SELENA NA HEMATOLOŠKE I BIOHEMIJSKE PARAMETRE KRVI I KVALITET GRUDNOG MESA FAZANA

Saša Obradović[1], Branko Petrujkić[2], Vladan Đermanović[4], Vladimir Živković[3], Bojan Stojanović[4], Nenad Đorđević[4], Milanka Radulović[5]

[1]Poljoprivredni fakultet, Kruševac, Univerzitet u Nišu, Republika Srbija
[2]Fakultet veterinarske medicine, Univerzitet u Beogradu, Republika Srbija
[3]Institut za stočarstvo, Beograd-Zemun, Republika Srbija
[4]Poljoprivredni fakultet, Univerzitet u Beogradu, Republika Srbija
[5]Državni univerzitet u Novom Pazaru, Departman za biomedicinske nauke, Novi Pazar, Republika Srbija

Kratak sadržaj

Cilj rada je bio da se ispitaju biohemijski i hematološki parametri krvi, kao i kvalitet grudnog mesa 45 fazana podeljenih u tri grupe i hranjenih smešama sa dodatkom organskog selena u koncentracijama od 0.2 mg/kg (I grupa) i 0.3 mg/kg (II grupa) u hrani i smešom bez selena u kontrolnoj grupi (K). Nakon 60 dana trajanja ogleda utvrđeno je da su prosečne vrednosti sadržaja selena u belom mesu i sadržaja selena u krvnom serumu fazana II grupe fazana hranjenih sa 0.3 mg/kg organskog selena značajno veće ($p > 0.05$) u odnosu na iste parametre fazana grupe K. Fazani II grupe su takođe imale bolje senzorne osobine mesa i ostvarile su najveću razliku sume ran-

gova prihvatljivosti mesa, koja je bila veća za 15 bodova od K grupe i 7 bodova od mesa fazana hranjenih sa 0.2 mg/kg selena. Dodavanje organskog selena u hranu fazana II grupe (0.3 mg/kg) je imalo pozitivan efekat na povećanje sposobnosti zadržavanja vode u grudnom mesu za 0.75% u odnosu na K grupu, odnosno za 0.58% u poređenju sa I grupom. Prosečne vrednosti hemijskih parametara mesa (pH, sadržaj vode, masti, proteina i pepela), hematoloških parametara krvi fazana (broj eritrocita, leukocita i trombocita, vrednosti hemoglobina i hematokrita) i biohemijskih parametara krvnog seruma (glukoze, enzima: aspartat aminotransferaza i alanin aminotransferaza, koncentracije ukupnih proteina, albumina ukupnog holesterola, triglecirida, kalcijum, kalijum i natrijum) bile su u granicama referentnih vrednosti za fazane i vrlo ujednačenih vrednosti bez signifikantnih razlika između ispitivanih grupa.

Ključne reči: fazan, kvalitet belog mesa, biohemijski i hematološki profil

INTRODUCTION

Pheasant farming is a branch of agriculture that is developing worldwide. Due to its favourable nutritional constituents, pheasant meat is the food that is increasingly used in human nutrition. At the same time, pheasant hunting is a very popular sport. Bird and poultry meat is particularly important for human nutrition, primarily because of its high quality proteins, minimum amount of fat, essential vitamins and minerals.

Biochemical and haematological blood parameters are significant for successful farm production, because the health condition of farmed birds, their nutritive status, the content of certain nutrients as well as symptoms of disease can be assessed using these parameters. Avian haematology is also a useful diagnostic tool in veterinary medicine and haematological values can be used as physiological indicators. Several papers dealing with haematological and biochemical parameters of pheasant blood have been published so far (Šperanda et al., 2005; Lloyd and Gibson, 2006; Tucak et al., 2008; Kececi and Ramazan, 2011).

Biochemical parameters and shaped blood elements of poultry have been examined in numerous papers, while the available literature data related to the issue of the effect and content of selenium in the blood of pheasants is scarce. Selenium (Se) is an essential microelement and an integral part of glutathione peroxidase enzyme (GPx) which plays a crucial role as antioxidant enzyme in antioxidant defence of free radicals in bird and mammal cells (Coles, 1977; Dukes, 1993; Edens, 2001).

The physiological role of selenium is complex. It is primarily important for the development of numerous metabolic processes in the organisms of birds and mammals. It also has antioxidant role in health preservation and improving production performances. According to Surai (2000), selenium and vitamin E deficiency causes various diseases in about 60 species of domestic, wild and laboratory animals and humans. In modern food production for domestic animals, selenium supplementation is mandatory, and lately, significant research efforts have been made to increase selenium concentration in food, in order to obtain functional animal products (meat, eggs, milk) enriched with selenium.

Selenium deficiency and loss of GPx enzyme activity causes cell membrane damage, free radical accumulation and cell decay. The antioxidant effect of selenium protects poultry from atherosclerosis, degenerative processes of the pancreas and kidneys, reproductive disorders, tumorigenesis and at the same time selenium also has immunostimulatory effect (Dukes, 1993; Kang et al., 2000; Dlouha et al., 2008). The intake of selenium through food results in increased content of its concentration in tissues and eggs (Edens, 1997; Surai and Dvorska, 2002; Edens and Kymberly, 2004). Selenium deficiency in poultry causes numerous pathological changes and diseases, such as the pancreas atrophy, kidney damage, exudative diathesis, decreased fertilization and weakened immunity. Selenium is also an activator of thyroid hormones which are responsible for thermogenesis of poultry. The concentration of selenium in the organism of birds under heat stress can be significantly reduced (Silva and Gloria, 2002; Skrivan et al., 2008). Poultry and bird production depends on numerous stress factors (overpopulation, heat stress, diseases), which is why selenium, as an integral part of many selenoproteins, actively participates in their prevention by activating antioxidant mechanisms. The concentration of selenium in the tissue differs and primarily depends on the amount of selenium ingested with food whereby the highest amount of selenium of 30 - 40% is found deposited in skeletal muscles and the liver and the rest of it is in the heart, pancreas and kidneys (Surai, 2000; Surai and Dvorska, 2002).

Breast muscles (mm. pectoralis) known as breast meat, consist of larger superficial muscle (m. pectoralis superficialis) and a smaller deep muscle (m. pectoralis profundus) and they are extremely well-developed in all birds because they enable the movement of the wings and flying. Breast muscles account for one quarter of the total body weight of birds and a half of total edible proteins. Poultry and bird breast meat is rich in proteins, low in fat and cholesterol compared to the meat of other domestic animals which makes it a high quality dietary product (Barroeta, 2006). If poultry meat contains selenium

in the concentration higher than prescribed, then the meat can be considered a functional food because it has beneficial effect on improving human health and lowering the risk of diseases.

Poultry and bird meat is significant for human nutrition because of its high content of biologically valuable proteins, fats, vitamins, minerals and essential fatty acids (Franco and Lorenzo, 2013). The smell, appearance and taste are very important sensory traits of meat, and these traits can significantly affect the quality and acceptability of the product by consumers. The smell and taste have the greatest impact on acceptability of meat, so the final judgment on its acceptability is mostly based on these traits. Sensory traits ranking by evaluators represent a simple method for practical comparison and assessment of meat samples or other products (Baltić, 1993; Baltić and Teodorović, 1997). Consumers want the meat with minimal water loss during preparation and cooking, and that is why the ability to retain water is an essential characteristic of meat quality (Surai, 2000; Edens et al., 2000; Surai, 2007).

There are few papers in the available literature dealing with the effects of organic selenium on breast meat quality, biochemical and haematological profile of blood of the pheasants which are fed different amounts of organic selenium. For that reason, the main goal of this paper is to examine the effect of organic selenium on selected haematological, biochemical parameters and selenium content in blood serum as well as the effect of selenium on chemical and sensory quality of pheasant breast meat.

MATERIAL AND METHODS

The experiment was conducted on 45 common pheasants (Phasianus colchicus), which were 42 days old, both male and female, weighing 385 ± 75 g on average. They were divided into three groups each containing 15 pheasants. The experiment lasted for 60 days and during that period the pheasant barns and nutrition were adapted to the floor way of rising. We used pelleted complete feed mixtures for formulated for nutritional needs of pheasants (NRC, 1994). The organic selenium contained in Alkosel® preparation (Lallemand, Fra) was added to the premixes, and after appropriate mixing, the premix with selenium was applied to the complete feed mixtures and mixed again with the complete mixture. The control group (K) was fed the feed without selenium supplementation, while the feed of experimental 1st group contained organic selenium in a concentration of 0.2 mg/kg, and the feed of experimental 2nd group contained organic selenium in the concentration of 0.3 mg/kg. The raw material and chemical composition of pheasant food is shown in Table 1.

Table 1. Ingredients and nutrient content of complete feed mixture for pheasants

Components	%	*The feed composition of all three groups:* Proteins 24.25%; Cellulose 6.05%; Fats 4.63%; Ash 7.35%; Dry matter 88.20%; Ca 1.02%; Total phosphorus 0.82%; ME 12.75 MJ/kg; Lysine 1.35%; Methionine + Cystine 0.90%.
Maize	40	
Wheat bran	3	
Soybean meal	24	
Sunflower meal 33%	4.3	
Alfalfa meal	3	*Composition of premix in 1kg of complete mixture:* Vitamin A (IU/kg) 15000; Vitamin D3 (IU/kg) 3000; Vitamin E (mg/kg) 32; Biotin (mg/kg) 0.20; Vitamin C (mg/kg) 15; Folicacid (mg/kg) 1.20; Niacin (mg/kg) 30; Pantothenicacid (mg/kg) 15; Vitamin B6 (mg/kg) 3.20; Vitamin B2 (mg/kg) 7; Vitamin B1 (mg/kg) 2.10; Vitamin B12 (mg/kg) 0.03; Cholinechloride (mg/kg) 500; Fe (mg/kg) 40; Mn (mg/kg) 80; Cu (mg/kg) 8; Zn (mg/kg) 60; J (mg/kg) 0.80; Co (mg/kg) 0.45; Antioxidant BHT (mg/kg) 100.
Yeast	3.5	
Soybean grits	12	
Sunflower meal 42%	5	
Lysine	0.1	
Methionine	0.2	
Limestone	1.6	
Mono-Ca-phosphate	1.5	
Salt	0.3	
Additive Pelletin	0.5	
Premix	1	

Diet I (K group): basal diet without organic Se;
Diet II (1st group) – Diet I + organic Se at 0.2 mg/kg diet;
Diet III (2nd group) –– Diet I + organic Se at 0.3 mg/kg diet;

Throughout the entire experiment, the consumption of food and water was ad libitum. An average sample of complete mixtures for pheasant nutrition was analysed for the basic chemical composition at the beginning of the experiment (the amount of dry matter, crude ash, crude proteins, crude fats and crude cellulose), by applying standard analytical methods of chemical food examination (AOAC, 1990). The content of calcium (Ca) was determined by volumetric method (SRPS ISO 6490-1/2001). Phosphorus (P) content was determined using spectrophotometric method (SRPS ISO 6491/2002), while the content of metabolic energy and amino acids was obtained by calculation based on their content in nutrients (INRA-AFZ, 2004).

Control measurements of body weight were performed on an electronic scale with an accuracy of ± 0.5 g at the beginning and the end of the experiment. This was used as a basis for calculating average body weight. Throughout the experiment, the health condition of pheasants and their mortality were

monitored. By the method of random sampling at the end of this 60-day long experiment, seven pheasants were taken from each group. They were then individually measured before slaughtering, and after primary slaughter processing and water cooling, chilled carcasses were cut into basic pieces in a manner regulated by Ordinance on the Quality of Poultry Meat (Official Gazette SFRJ No. 1/81 and 51/88), and the brest muscle was separated and measured on an automatic scale with an accuracy of ± 0.5 g.

The water in the breast meat was analysed using standard examination methods. The total water content was determined by drying the samples to constant weight (SRPS ISO 1442/1998), total ash by burning and annealing the sample at the temperature of 500 °C to 600 °C (SRPS ISO 936/1999), pH of the meat were determined by a pH meter (SRPS ISO 2917: 2004), total proteins by the Kjedahl method based on nitrogen content (SRPS ISO 937/1992), while the total fat was determined by Soxlett extraction with pre-drying of the sample (SRPS ISO 1443/1992). The content of selenium in breast meat and blood serum of pheasants was determined by atomic absorption spectrophotometry using hydride technique (SRPS EN 14627:2008). The ability to retain water in breast meat was determined by measuring the content of total moisture, 24 and 48 hours after the time of meat cooling at 4 °C. The quality of meat sensory traits was examined by the method for determination of the difference in meat acceptability using Rank test, assessed by 7 evaluators (Baltić, 1993; Baltić and Teodorović, 1997; SRPS EN ISO 8587/2006). The evaluators assessed the sensory characteristics of the meat (the smell, taste, juiciness, softness and appearance). The obtained differences in the acceptability of the meat are the differences in the overall impression of all evaluators. Before the examination, all the samples were grilled for about 15 minutes and after that the evaluators graded the samples labelling them the most acceptable, less acceptable and the least acceptable.

At the end of the experiment and before slaughtering, seven pheasants from each group, their blood were taken by puncturing the ulnar vein. The sterile tubes used for haematological tests contained the anticoagulant Sodium-Ethylenediaminetetraacetic acid (Na-EDTA). The blood for biochemical examinations was put in special tubes without anticoagulants, and the serum was separated by centrifugation at 3000 rpm for 10 minutes and after that the analysis of the selected serum parameters was performed. Determination of biochemical parameters was performed on a multiparametric biochemical analyzer Hitachi 750 (Tokyo, Japan) with tests from Boehringer Mannheim (Germany), while we used an analyzer ISE, Nova 5 (USA) and a set tests by Randox for the determination of serum electrolytes (Ca, Na, K). The number of erythrocytes and leukocytes was determined in the Thoma-Zeiss chamber, the number of platelets in the Neubauer chamber, the value of hematocrit was

determined by the Wintrobe method. Haemoglobin concentration was determined using the Sahli method.

The collected data were processed using statistical program Statistica 10 (StatSoft, USA). The following analyses were performed: statistical analysis of the obtained results, the analysis of variance by a standard procedure with testing of the statistical significance of the differences between certain groups and examined parameters by applying LSD test.

RESULTS

No health issues or pheasant deaths were recorded during the experiment. The results of chemical investigation of breast meat are shown in Table 2. Addition of organic selenium to pheasant diet resulted in relative higher values of breast muscle weight, electrochemical reactions of meat, water and fat content. However, statistically significant differences between treatments ($p > 0.05$) were not recorded. The obtained results of breast meat weight indicate that the mass was the lowest in pheasants of the K group amounting 221.50 g, and the highest was in 2nd group of pheasants with 229.32 g.

Table 2. Chemical composition of breast muscle and selenium concentration in meat

Group / Chemical composition of breast muscle meat	K group n = 7	1st group n = 7	2nd group n = 7
Mass (g)	221.50 ± 28	224.60 ± 18	229.42 ± 32
pH	6.10 ± 16	6.02 ± 32	6.19 ± 42
Moisture (%)	72.63 ± 23	72.38 ± 52	72.49 ± 55
Fat (%)	1.08 ± 56	1.10 ± 23	1.11 ± 36
Total protein (%)	25.14 ± 14	25.15 ± 11	25.11 ± 87
Ash (%)	1.18 ± 41	1.20 ± 09	1.20 ± 23
Selenium (mg/kg)	0.121 ± 13	0.129 ± 03	0.135 ± 33*

*$p < 0.05$; ** $p < 0.01$

The results of selenium content in breast meat samples shown in Table 2. indicate that the average selenium content was the lowest in the K group with 0.121 mg/kg. It was slightly higher in the 1st group with 0.129 mg/kg and the highest in the 2nd group of pheasants with 0.135 mg/kg. Statistical analysis of the data on selenium content in breast meat showed a statistically significant

difference ($p < 0.05$) among control K group which did not have organic selenium in feed and the 2nd group of pheasants that had organic selenium in their food in the concentration of 0.3 mg/kg.

The ability of water retention represents a very important trait of meat quality because yield, juiciness, taste and meat texture depend on it. The results of breast meat moisture loss are shown in Table 3. The best relative values of water retention in breast meat were achieved by the 2nd group both after 24 h (1.17%) and after 48 h (1.93%) from the time of meat cooling. Therefore, in comparison with the K group it had better efficiency by 0.75%. Similar results were achieved by the 1st group, which had better water retention in meat by 0.58% compared to K group. The worst results were recorded in the K group which had relative value of water retention of 1.79% after 24 h, and 2.68% after 48 h.

Table 3. Moisture loss in pheasant breast meat

Group/ Water loss in meat	**K group** n = 7		**1st group** n = 7		**2nd group** n = 7	
	g	%	g	%	G	%
Water loss after 24 h	1.30	1.79	1.10	1.52	0.85	1.17
Water loss after 48 h	1.95	2.68	1.70	2.10	1.40	1.93
Difference %	-	-	-	0.58	-	0.75

A very important indicator of meat quality, besides chemical composition, are its sensory traits which were examined using the method of Rank test in this experiment, or meat acceptability by consumers whereby lower sum of ranks represents higher meat acceptability. The differences in assessment of the acceptability of pheasant's breast meat are displayed in Table 4. When it comes to ranking of breast meat samples, the samples of the 2nd group (32 points) were assessed as the most acceptable, and this group had the highest difference in the sum of ranks of 15 points. It was followed by the 1st group of pheasants (39 points) with the difference in the sum of ranks of 7 points in comparison to the K group, and as the least acceptable were the breast meat samples of the K group (47 points).

Table 4. Total acceptability assessment of pheasant's breast meat (Rank test)

Group	K group n = 7	1st group n = 7	2nd group n = 7
	The sum of ranks		
	47	39	32
K group	-	8	15
1st group	-	-	7
2nd group	-	-	-

Haematological examinations of shaped blood elements (Table 5) show similar values and insignificant differences ($p > 0.05$) between the examined groups of pheasants. The number of erythrocytes (RBC- Red Blood Cells) varied within relatively narrow limits in all the groups from 2.15 x 10^6/mm^3 in the 1st group to 2.32 x 10^6/mm^3 in the 2nd group of pheasants. Similar results were also recorded for the average values of the number of leukocytes (WBC - White Blood Cells), which ranged from 21.30 x 10^3/mm^3 in the 1st group to 21.50 x 10^3/mm^3 in the 2nd group of pheasants. These are the figures that vary within normal physiological values for this species of birds. The other examined parameters in all groups, such as number of platelets (PLT), haemoglobin (MCH- Mean Corpuscular Hemoglobin) and haematocrit (HCT – Haematocrit-Packed cell volume) also had uniform median values without statistical significance both between control and experimental groups, and between groups with different treatments with organic selenium. The platelet number was the lowest in the 2nd group with 28.40 x 10^3/mm^3, followed by K group with 28.60 x 10^3/mm^3, and the highest was in the 1st group where organic selenium was added at the concentration of 0.2 mg/kg and it was 29.20 x 10^3/mm^3. Haematocrit had average values of 38.3% in K group, 37.10% in the 1st group and 38.20% in the 2nd group of pheasant blood. The concentration of haemoglobin had the lowest average value of 125 g/L in the 1st group, followed by 129 g/L in K group and the highest average value of 130 g/L was determined in the pheasants blood of the 2nd group.

Table 5. Haematological parameters of pheasants

Haematological parameters	K group n = 7	1st group n = 7	2nd group n = 7
RBC (x 106/mm3)	2.27 ± 0.15	2.15 ± 0.28	2.32 ± 0.45
WBC (x 103/ mm3)	21.40 ± 2.65	21.30 ± 1.30	21.50 ± 2.20
PLT (x 103/ mm3)	28.60 ± 40	29.20 ± 70	28.40 ± 50
MCH (g/l)	129.00 ± 1.80	125.00 ± 2.30	130.00 ± 1.50
HCT (%)	38.30 ± 0.73	37.10 ± 0.24	38.20 ± 0.35

*$p < 0.05$; ** $p < 0.01$

Table 6. Biochemical parameters of pheasant blood

Parameters	K group n = 7	1st group n = 7	2nd group n = 7
GLU (mM/L)	18.50 ± 0.65	18.10 ± 0.80	17.60 ± 0.30
AST (U/L)	250.00 ± 1.52	263.00 ± 1.41	257.00 ± 2.15
ALT (U/L)	9.20 ± 0.48	10.00 ± 0.16	8.90 ± 0.27
TP (g/l)	36.50 ± 1.23	35.80 ± 0.98	37.0 ± 1.10
ALB – Albumin (g/l)	14.50 ± 0.14	14.20 ± 0.21	14.80 ± 0.11
CHOL (mM/L)	3.53 ± 0.12	3.51 ± 0.16	3.40 ± 0.23
Triglycerides (mM/L)	0.93 ± 0.24	0.84 ± 0.15	0.87 ± 0.20
Ca (mM/L)	2.28 ± 0.10	2.30 ± 0.25	2.30 ± 0.15
K (mM/L)	3.90 ± 0.21	3.82 ± 0.15	3.92 ± 0.38
Na (mM/L)	149.45 ± 1.54	147.20 ± 0.20	151.10 ± 0.80
Se (µg/ml)	0.137 ± 0.12	0.141 ± 0.05	0.145 ± 0.24*

*p < 0.05; ** p < 0.01

According to the results shown in Table 6, the highest average value of glucose (GLU) concentration was recorded by K group, which amounted 18.50 mmol/L, followed by 18.10 mmol/L in the 1st group and the lowest value of 17.60 mmol/L was determined in the 2nd group. No statistically significant differences were found between the analysed groups ($p > 0.05$). The highest values of the examined blood serum enzymes were found in pheasants of the 1st experimental group. Those pheasants consumed organic selenium from food at the concentration of 0.2 mg/kg of mixture with the average AST value of 263.00 U/L, and the value of alanine aminotransferase (ALT) was 10.00 U/L.

The selenium added to the food resulted in insignificant increase in the values of aspartate aminotransferase (AST) and ALT in the 1st experimental group of pheasants but with no statistical significance compared to the other two experimental groups ($p > 0.05$).

Total proteins and albumin concentrations in our experiments had the highest values in blood serum of the 2nd group of pheasants whose feed contained 0.3 mg/kg of selenium. The analysis of variance did not show any significant differences between treatments ($p > 0.05$). Cholesterol and triglyceride concentrations ranged within normal physiological limits and without determined statistic differences between average values of the examined groups of pheasants. The part of our examinations related to monitoring of the concentrations of selected cation electrolytes of pheasant serum (Ca, K and Na) is shown in Table 6. The concentrations of examined electrolytes in blood serum were rather uniform in all pheasant groups, so statistical analysis showed no significant differences between median values of the examined groups of pheasants ($p > 0.05$).

The most reliable criterion for selenium status in animals is considered to be the determination of selenium concentration in the blood and tissues of birds and animals. In our research the average content of selenium in blood serum of the 2nd group of pheasants was 0.145 μg/mL and it was statistically significantly higher ($p < 0.05$) than the average content of selenium in blood serums of K group of pheasants where its value amounted 0.137 μg/mL. There was no statistical significance between the average content of selenium in blood serum of K group and the 1st group of pheasants despite the fact that the average value of selenium in blood serum was higher than in the K group of pheasants (0.141 μg/mL).

DISCUSSION

No deaths of the examined pheasants were registered during the experiment. As this was an older age category of experimental pheasants, it cannot be confirmed with certainty, based on our data on mortality, that selenium from feed does not affect mortality, because the literature shows that the biggest losses are in chickens and that mortality rates in older categories are much lower (Ristić, 2005).

The relative uniformity of chemical composition of breast meat in terms of the content of the analysed parameters between the examined groups of pheasants is in accordance with the research of Cvrtila et al. (2007). In our experiments, no statistically significant differences were determined between the treatments ($p > 0.05$) of the examined chemical parameters of meat. Tucak et al. (2008) state that the body weight of adult pheasants raised in an avi-

ary ranging from 969 to 1,144 g and the breast meat weight varied between 248 and 295 g. These authors determined higher values of protein, fat and ash in breast meat of pheasants in comparison to our results. In our experiment, the protein and fat concentration in pheasant meat were uniform, whereas the protein content was higher than the fat content. The results we obtained are in accordance with other researchers who stated that wild populations and farm-raised pheasants are distinguished by higher protein and lower fat content in meat, which represents a characteristic of this bird species (Hofbauer et al., 2010; Franco and Lorenzo, 2013).

Organically bound selenium in the form of selenomethionine has a strong antioxidant effect in poultry organisms, directly affecting the increase in the concentration of GPx in the liver and decrease in the concentration of lipid peroxidase, resulting in the meat of exceptional quality with high content of selenium. Several researchers have confirmed the positive effect of increasing the concentration of selenium in musculature of broilers whose feed was treated with organic selenium compared to control group without supplement or with inorganic selenium supplementation in food (Edens and Kymberly, 2004; Payne and Southern, 2005; Dlouha et al., 2008).

Our research confirmed the positive antioxidant effect of selenium on the musculature of the breast meat because the 1st group and the 2nd group of pheasant samples contained less water loss during storage. Meat quality can successfully be preserved during storage by adding selenium antioxidants to the feed of domestic animals, because selenium is deposited in musculature cells, which improves the ability of the cells to retain water (Surai, 2000). According to Edens (2001), selenium in the muscle tissue reduces fat peroxidation during meat storage and in that way it affects the preservation of meat quality. Organic selenium in the form of selenomethionine has a positive effect on meat quality because it increases the activity of glutathione peroxidase (GSH-Pk) and keeps lipid peroxidase at a low level, which results in cell membrane stability and the ability to bind water (Edens et al., 2000; Edens and Kymberly, 2004).

Based on the Rang test results obtained in these research studies, it can be concluded that the addition of organic selenium to feed or diet had a positive effect on acceptability of pheasant meat. According to many authors, the compounds that are the carriers of smell and taste have the greatest effect on food acceptability (Baltić, 1993; Ivanović et al., 2012). The supplementation of organic selenium in feed has a positive effect on the appearance, juiciness, smell and colour of meat (Surai and Dvorska, 2002), while the increased juiciness is the result of better water retention in meat (Džinić et al., 2006).

Determination of haematological parameters is a reliable indicator of the physiological condition of animal organisms and the impact of certain

supplements on blood chemistry. These parameters can be affected by different factors such as nutrition, diseases, gender, age, physical activity and ambient conditions (Kaneko, 1989; Jovanović, 1990). Recognizing the change in the number of leukocytes is of particular significance, whether it is an increase or decrease in their number, it is always a certain pathological phenomenon in the organism (Harr, 2002). In the conditions of temperature stress, organic selenium has a positive effect on reducing the effect of this negative ambient factor, so the level of leukocytes in blood does not change, but increases food consumption and the level of glutathione peroxidase, which has a defensive role (Mahmoud and Edens, 2003). Our data are in accordance with the research of Šperanda et al. (2005) who established that selenium (0.2 mg organic selenium/kg diet) did not have any effect on erythrocytes and leukocytes count values. The obtained average values of haematological parameters were in accordance with normal physiological values and the results published by other authors (Schmidt et al.2007; Hauptmanova et al. 2006; Kececi and Ramazan, 2011). Based on the results obtained in these studies, it can be concluded that the application of organic selenium as food additive did not affect the haematological blood parameters of the examined pheasants.

The part of the examination related to the analysis of biochemical parameters of pheasant blood serum (glucose, AST and ALT enzymes, total protein concentration, total cholesterol, albumin, triglycerides, Ca, K and Na) which are shown in Table 6, indicates that the majority of these parameters had the values which ranged within normal physiological limits and which were reported by other authors (Šperanda et al., 2005; Loyd and Gibson, 2006; Suchy et al., 2010). The variations in the concentration of some blood constituents in birds and animals indicate the changes in physiological and biochemical homeostasis of an organism. By analysing these parameters, it is relatively easy to determine the health status of animals and the deficiency of nutrients.

Glucose is a very important energetic material of all living systems, considering that all cells have enzymes for their catalytic decomposition. The normal range of glucose concentration for most birds is between 11.1 and 27.8 mmol/L (Coles, 1977). The results we obtained in our research are in accordance with the data of the other authors (Loyd and Gibson, 2006; Šperanda et al., 2005) and indicate that by adding selenium normal glucose homeostasis is maintained.

Significant biochemical parameters of blood serum are the activity of the AST and ALT enzymes which are found in bird organisms in the liver, blood, cerebrospinal fluid and urine. The liver is very sensitive to increased concentrations of nutrients and toxins. Any increase in the activity of these enzymes in blood serum indicates pathological changes primarily in the hepatobiliary tract, liver damage, heart and skeletal muscles (Kaneko, 1989; Dukes, 1993; Moss et al., 1997).

The concentrations of total proteins are of great significance in the diagnosis of liver and kidney disease, metabolic and nutritional disorders which lead to the loss of protein in the organism reduced protein synthesis and increased decomposition (Kaneko, 1989; Dukes, 1993). The results we obtained in our research are in line with the data of other authors (Šperanda et al., 2005) who determined that selenium results in a partial increase of total proteins and albumin, but does not cause statistically significant differences.

Cholesterol is found in all organism cells, and main organs where about 90% of it is synthesized are the liver and intestines. Triglycerides play important roles in bird organisms, serving as metabolic fuel and components of cell membranes (Jovanović 1990; Dukes, 1993). According to available literature data, organic selenium supplementation in pheasant diet does not lead to disorder of normal physiological cholesterol and triglycerides levels in serum (Šperanda et al. 2005), which is in accordance with the results obtained in this experiment. Cholesterol and triglyceride levels determined in this research were lower than the data presented by Kecci and Ramazan (2001) and Šperanda et al. (2005), but they had similar values to those found by Lloyd and Gibson (2006).

The physiological effect of blood electrolytes is of great importance for the health, productivity and maintenance of normal homeostasis of an organism. Serum electrolyte concentrations can indicate disorder in the functioning of certain organs and systems especially in the functioning of intestinal tract, kidneys and cell homeostasis (Kaneko, 1989; Dukes, 1993). The obtained concentrations of pheasant serum electrolytes were within normal physiological limits for this species of birds. They match the data stated by Šperanda et al. (2005), Lloyd and Gibson (2006).

The significance of selenium as pheasant food supplement and its content in the blood is very scantily described in the available literature. We achieved significantly higher concentration of selenium in the blood of pheasants by adding organic selenium to their feed at the concentration of 0.3 mg/kg of food compared to the blood of K group whose food did not contain selenium. Similar results were presented by Payne and Southern (2005) and Marković et al. (2008). At the same time, in comparison with inorganic selenium which was added in the feed in the same concentration, Marković et al. (2008) determined a higher concentration of selenium in the blood of broiler groups where organic selenium was added to the food. Based on our research and obtained values of selenium in the blood serum of the examined pheasants, it can be concluded that organic selenium added in concentrations of 0.2 and 0.3 mg/kg in the food led to an increase in its value in the blood serum. Therefore, the concentration of 0.3 mg/kg in food in the 2nd group of pheasants caused

significant difference ($p < 0.05$) compared to the K group of pheasants which were fed without selenium supplement in food.

By the analysis of the majority of examined biochemical indicators of pheasant blood serum, apart from selenium concentration, we did not observe other statistically significant differences between the values for the K group and the pheasant groups whose food contained organic selenium, which is in accordance with Šperanda et al. (2005). The measured parameters of blood serum, apart from selenium concentration, were within the limits of reference values for pheasants, which indicates that selenium supplementation in pheasant food does not affect the examined parameters of pheasants blood serum.

CONCLUSION

Based on all of the above mentioned, we can generally conclude that the examined impact of different doses of organic selenium in pheasant feed or diet, applied in the concentrations of 0.2 mg/kg and 0.3 mg/kg, did not have any noticeable effect on the values of biochemical parameters of blood serum and haematological blood parameters. The addition of organic selenium in pheasant feed did not have any statistical significance on chemical quality of pheasant breast meat muscle. The results of the research of the effect of organic selenium from the feed at the concentration of 0.3 mg/kg on its content in the breast meat and blood serum of pheasants indicate statistical significance ($p > 0.05$) compared to the parameters of the control group of pheasants which were fed without selenium supplement. The justification of this research is reflected in obtaining required concentrations of selenium in pheasant meat and the production of nutritionally important selenized meat for human consumption.

Author's Contribution:

S.O., B.S. and N.Đ. made contributions to conception and design of the article and draft ed the manuscript; B.P. made substantial contributions to the basic idea; M.R. carried out the chemical analyses; V.Đ. was involved in draft ing of the manuscript; S.O. collected the samples and experimental data; V.Ž. did statistical analysis. All the authors have read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

REFERENCES

1. AOAC, Association of Official Analytical Chemists. 1990. Official Methods of Analysis, ed by Kenneth Helrich, AOAC, Arlington, Virginia, USA,15th ed, 246. ISBN 0-935584-42-0.
2. Baltić, M., Teodorović, B.V. 1997. Higijena mesa riba, rakova i školjki, Veterinarski fakultet Beograd, 250. ISBN 56-81043-01-3.
3. Baltić, Ž.M. 1993. Kontrola namirnica, Institut za higijenu i tehnologiju mesa, Beograd.
4. Barroeta A.C. 2006. Nutritive value of poultry meat: Relationship between vitamin E and PUFA. World's Poultry Science Journal, 63, 277-284. doi:10.1017/S0043933907001468.
5. Coles, H. Brian. 1977. Avian medicine and surgery, Wiley-Blackwell, 2nd edition (January 15, 1997), Blackwell Science Ltd, a Blackwell Publishing company, 408. ISBN 10:0632033568.
6. Cvrtila Ž., Hadžiosmanović M., Kozačinski L., Zdolec N., Filipović I., Severin K., Mašek T. 2007. Kemijski sastav mesa fazanskih kopuna. Meso, 9, 3, 148-151. doi:10.31727/m.
7. Dlouha G., Ševčikova S., Dokoupilova A., Zita L., Heindl J., Skrivan M. 2008. Effect of dietary selenium sources on growth performance, breast muscle selenium, glutathione peroxidase activity and oxidative stability in broilers. Czech Journal Animal Scinces, 53, 6, 265-269. doi:10.17221/361-CJAS.
8. Dukes H.H. 1993. Physiology of Domestic Animals, Editor: Swenson J. Melvin, Reece O. William, Ithaca, USA, 11th Edition, 962, ISBN 10:0801428041.
9. Džinić N., Tomović V., Petrović Lj., Perić L. 2006. Uticaj dodataka selena različitog porekla u hranu za piliće na kvalitet m. pectoralis. Tehnologija mesa, 47, 5-6, 199-203.
10. Edens, F.W. 1997. Potential for organic selenium to replace, selenite, in poultry diets. Zootecnica International, 20, 28-31. https://zootecnicainternational.com/online-magazine.
11. Edens F.W., Carter T.A., Parkhurst C.R., Sefton A.E. 2000. Effect of selenium source and litter type on broiler feathering. Journal of Applied Poultry, 9, 407-413. doi:10.1093/japr/9.3.407.
12. Edens F.W. 2001. Involvement of Sel-Plex in physiological stability and performance of broiler chickens. In Proceedings of Altech's 12th Annual Symposium , Biotechnology in the Feed Industry, Eds. Lyon T.P. and Jacques K.A., Nottingham University Press, Nottingham, UK, 349-376. ISBN-10:1897676360.

13. Edens F.W., Kymberly M. Gowdy. 2004. Selenium sources and selenoproteins in practical poultry production. Nutritional Biotechnology in the Feed and Food Industries. In Proceedings of Alltech's Twentieth Annual Symposium, Edited by TP Lyons and KA Jacques, Typeset by Nottingham University Press, Nottingham, England, 35-56. ISBN 1-904761-27-5.
14. Franco D., Lorenzo J. M. 2013. Meat quality and nutritional composition of pheasants (Phasianus colchicus) reared in an extensive system. British Poultry Science, 54, 594-602. doi.org/10.1080/00071668.2013.828195.
15. Harr E. Kendal. 2002. Clinical chemistry of companion avian species: A Review. Veterinary clinical pathology, 31, 140-151. doi:10.1111/j.1939-165x.2002.tb00295.x.
16. Hauptmanova K., Maly M., Literak I. 2006. Changes of haematological parameters in common pheasant throughout the year. Veterinarni Medicina Czech, 51, 1, 29–34. doi.org/10.17221/5514-VETMED.
17. Hofbauer P., Smulders F. J. M., Vodnansky M., Paulsen P., El-Ghareeb W. R. 2010. A note on meat quality traits of pheasants (Phasianus colchicus). European Journal of Wildlife Research. 56, 809–813. doi: 10.1007/s10344-010-0396-7.
18. INRA-AFZ 2004: http://www.feedtables.com
19. Ivanović, S., Teodorović, V., Baltić, Ž. M. 2012. Kvalitet mesa (biološke i hemijske opasnosti), Naučni institut za veterinarstvo Srbije, Beograd, 356. ISBN 978-86-81761-51-9.
20. Jovanović, M. 1990. Fizologija domaćih životinja, Medicinska knjiga, Beograd-Zagreb, 687. YU ISBN 86-311-0056-0.
21. Kaneko, J. 1989. Clinical Biochemistry of Domestic Animals, 4th Edition, Academic Press, San Diego, 932. ISBN 9780080529196.
22. Kang B.P.S., Mehta U., Bansal M.P. 2000. Hyperlipidemia and Type-1-5'-monodeiodinase activity: Regulation by selenium supplementation in rabbits. Biological Trace Element Research, 77, 231-239. doi: 10.1385/BTER:77:3:231.
23. Kececi T., Ramazan C. 2011. Haematological and biochemical values of the blood of pheasants (Phasianus colchicus) of different ages. Turkish Journal Veterinary Animal Sciences. 2011; 35, 3, 149-156. doi:10.3906/vet-0910-135.
24. Lloyd, S., Gibson, J.S. 2006. Haematology and biochemistry in healthy young pheasants and red-legged partridges and effects of spironucleosis on these parameters. Avian Pathology, 35, 335–340. doi: 10.1080/03079450600821794.
25. Mahmoud K. and Edens F.W. 2003. Influence of selenium sources on age-related and mild heat stress-related changes of blood and liver glutathione redox cycle in broiler chickens (gallus domesticus). Comparative bioche-

mistry and physiology. Part B, Biochemistry & molecular biology, 136, 921-934. doi: 10.1016/s1096-4959(03)00288-4.
26. Marković R., Jovanović B.I., Baltić Ž. M., Šefer D., Petrujkić B, Sinovec Z. 2008. Effects of selenium supplementation as sodium selenite or selenized yeast and different amounts of vitamin E on selenium and vitamin E status of broilers. Acta Veterinaria (Beograd), 58. 4, 369-380. doi:10.2298/AVB0801063S.
27. Moss, D.W., Handerson, A.R., Kachmar, J.F. 1997. Enzimi. U Osnovi kliničke hemije, udžbenik. Tietz W. Norbert, Ortomedics, Beograd, 360-444. ISBN 0-7216-8862-4.
28. National Research Council NRC. 1994. Nutrient Requirements of Poultry. The National Academies Press, Washington, DC, 9th edition, 176. ISBN 978-0-309-04892-7.
29. Payne R.L., and Southern L.L, 2005. Comparison of inorganic and organic selenium sources for broilers. Poultry Science, 84, 898-902. doi: 10. 1093/ps/84.6.898.
30. Rulebook on quality of poultry meat. Official Gazette SFRJ. 1981, No 1.
31. Rulebook on quality of poultry meat. Official Gazette SFRJ. 1988, No 51.
32. Ristić Z. 2005. Monografija Fazan, Biblioteka Memorija, Sombor, 592. ISBN 86-83485-09-9.
33. Schmidt E.M.S., Paulillo A.C., Dittrich R.L., Santin E., da Silva P.C.L., Beltrame O., de Oliveira E.G. 2007. The effect of age on hematological and serum biochemical values on juvenile ring-necked pheasants (Phasianus colchicus). International Journal of Poultry Science, 6, 459-461. doi:10.3923/ijps.2007.459.461.
34. Silva C.M.G., Gloria M.B.A. 2002. Bioactive amines in chicken breast and thigh after slaughter and during storage at 4°C and in chicken-based meat product. Food Chemistry, 78, 2, 241-248. doi:10.1016/S0308-8146(01)00404-6.
35. Skrivan M., Dlouha G., Mašata O., Ševčikova S. 2008. Effect of dietary selenium on lipid oxidation, selenium and vitamin E content in the meat of broiler chickens. Czech Journal of Animal Science, 53, 7, 306-311. doi:10.17221/358-CJAS.
36. Šperanda M., Florijančić T., Bošković I., Bogut I., Gutzmirtl H., Senčić Đ., Antunović Z., Bodakoš D. 2005. Utjecaj organskog selena na rast i biokemijske pokazatelje u serumu fazanskih pilića. Krmiva, 47, 6, 295-301. doi:org/10.33128/k.
37. SRPS EN 14627:2008. Hrana za životinje. Određivanje selena atomskom apsorpcionom spektrometrijom sa hidridnom tehnikom (HGAAS) posle mikrotalasne digestije

38. SRPS EN ISO 8587/2006. Sensory analysis – Methodology – Ranking.
39. SRPS ISO 1442/1998. Meso i proizvodi od mesa. Određivanje sadržaja vlage.
40. SRPS ISO 1443/1992. Meso i proizvodi od mesa. Određivanje sadržaja ukupne masti.
41. SRPS ISO 2917/2004. Meso i proizvodi od mesa. Merenje pH vrednosti (referentna metoda).
42. SRPS ISO 6490-1/2001. Hrana za životinje. Određivanje sadržaja kalcijuma, volumetrijska metoda.
43. SRPS ISO 6491/2002. Hrana za životinje. Određivanje sadržaja fosfora, spektrometrijska metoda.
44. SRPS ISO 936/1999. Meso i proizvodi od mesa. Određivanje ukupnog pepela.
45. SRPS ISO 937/1992. Meso i proizvodi od mesa. Određivanje sadržaja azota (referentna metoda).
46. Suchy P., Strakova E., Kroupa L., Steinhauser L., Herzig I. 2010. Values of selected biochemical and mineral metabolism indicators in feathered game. Acta Veterinaria Brno. 79,9, 9-12. doi:10.2754/avb201079S9S009.
47. Surai P.F. 2000. Organic selenium: benefits to animals and humans, a biochemist's view. In Proceedings of Alltech's 16th Annual Symposium, Biotechnology in the Feed Industry, The Future of Food, Eds. Lyons, T.P. and Jacques, K.A., Nottingham University Press, Nottingham, UK, 205–260. ISBN 10:1897676751.
48. Surai P. F. and Dvorska, J. E. 2002. Effect of selenium and vitamin E content of the diet on lipid peroxidation in breast muscle tissue of broiler breeder hens during storage. In Proceedings of Australian Poultry Science Symposium, editors R.A. Pym, World Poultry Science Association, Volume 14, 187-192.
49. Surai P.F. 2007. Selenium in poultry nutrition 2. Reproduction, egg and meat quality and practical applications. World's Poultry Science Journal, 58, 4, 431-450. doi:10.1079/WPS20020032.
50. Tucak Z., Skrivanko M., Posavcevic S., Periskić M., Bošković I., Jumić V. 2008. Influence of keeping pheasants in captivity vs. nature on the biological value of meat and its use in human nutrition. Collegium Antropologicum, 32 3: 959–962.

Received: 24.03.2021.
Accepted: 10.06.2021.

Original research article

UDC: 637.56:597.555.3
https://doi.org/10.46784/eavm.v14i1.260

QUALITY OF CARP MEAT (*Cyprinus carpio*) PRODUCED IN A POND WITH THE ADDITION OF PURIFIED WASTEWATER ORIGINATING FROM THE SLAUGHTERHOUSE

Miloš Pelić[1*], Milica Živkov Baloš[1], Nenad Popov[1], Suzana Vidaković Knežević[1], Nikolina Novakov[2], Nikola Puvača[3], Dragana Ljubojević Pelić[1]

[1] Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia
[2] Faculty of Agriculture, University of Novi Sad, Novi Sad, Republic of Serbia
[3] Faculty of Economics and Engineering Management, Department of Engineering Management in Biotechnology, University Business Academy, Novi Sad, Republic of Serbia

Abstract

The aim of the study was to investigate production parameters and nutritive quality of carp meat produced in a fish pond fed with fresh well water mixed with purified slaughterhouse wastewater. The quality of carp meat was analysed in view of seasonal variations in chemical composition of fillets. Our research idea was that purified slaughterhouse wastewater would provide essential nutrients for carp and positively affect the quality of fish meat, which was confirmed in this experiment. The total carp production at the end of study period was 3,270 kg/ha. The research revealed a feed conversion ratio (FCR) of 1.5 kg of feed per one kg weight gain and a survival rate of 87%. Statistically significant difference ($p < 0.05$) between meat protein content during spring and autumn season was established. Protein content in meat was higher during spring season, whereas higher fat content was established during autumn sampling season. The application of purified slaughterhouse wastewater provided high level of nutrients resulting in high meat yield per area unit and good chemical composition of meat. Such production model is meaningful in both economic and ecological aspect.

Key words: carp, slaughterhouse, wastewater, meat quality, spring, autumn

[1*] Corresponding Author: milosp@niv.ns.ac.rs

KVALITET MESA ŠARANA (Cyprinus carpio) PROIZVEDENOG U RIBNJAKU SA DODATKOM PREČIŠĆENE OTPADNE VODE POREKLOM IZ KLANICE

Miloš Pelić[1], Milica Živkov Baloš[1], Nenad Popov[1], Suzana Vidaković Knežević[1], Nikolina Novakov[2], Nikola Puvača[3], Dragana Ljubojević Pelić[1]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija
[2] Poljoprivredni fakultet, Univerzitet u Novom Sadu, Republika Srbija
[3] Fakultet za ekonomiju i inženjerski menadžment, Departman za inženjerski menadžment u agrobiznisu, Novi Sad, Republika Srbija

Kratak sadržaj

Cilj ovog rada je ispitivanje proizvodnih parametara i nutritivnog kvaliteta mesa šarana proizvedenog u ribnjaku koji je punjen bunarskom vodom sa dodatkom prečišćene vode iz klanice. Ispitani su hemijski parametri kvaliteta fileta šarana kao i uticaj godišnjeg doba kada je izvršeno uzorkovanje. U ovom istraživanju ideja je bila da će prečišćena otpadna voda iz klanice obezbediti hranjive materije za šarana i da će pozitivno uticati na kvalitet mesa, što je ogledom i potvrđeno. Utvrđeno je da je ukupna proizvodnja šarana na kraju ogleda bila 3,270 kg/ha. Konverzija je bila oko 1.5 kg hrane za kg prirasta, a stopa preživljavanja 87%. Sadržaj proteina u mesu je bio viši u proleće u odnosu na jesen i razlika je bila statistički značajna ($p < 0.05$). Sadržaj masti u mesu je bio viši u jesenjem uzorkovanju. Korišćenjem prečišćene otpadne vode iz klanice za uzgoj šarana, je obezbeđen visok nivo hranjivih materija što je rezultiralo visokim prinosom mesa po jedinici površine i dobrim hemijskim sastavom mesa. Ovakav vid proizvodnje je značajan sa ekonomskog i ekološkog aspekta.

Ključne reči: šaran, klanica, otpadna voda, kvalitet mesa, proleće, jesen

INTRODUCTION

In some regions, wastewater from slaughterhouses is still released into the rivers, channels, lakes and other water bodies without adequate pre-treatment. This causes substantial hazard for the environment. Such wastewaters contain high quantity of organic matter that represents an excellent source of nutrients

for carp species. Vo (2001), Thi Phong Lan et al. (2007) and Pelić (2020) reported that such wastewaters can be used in aquaculture as a source of both water and important nutrients for fish. The use of purified wastewater in fishponds is an innovative recycling method implicating conversion of soluble nutrients into biomass, that is, fish. It can be considered a completely novel approach to providing sustainability of meat industry and environment protection (Pelić, 2020). Carp is an omnivorous fish species that feeds on natural food available in the pond, which makes it particularly suitable for farming in such systems. Fish meat is a valuable source of nutrients, which is essential for variable and healthy human diet. An optimal ratio of proteins, fats, carbohydrates, minerals and vitamins contributes to a high nutritive value of fish meat (Ćirković et al., 2011). It contains up to 85% water, 16 to 22% proteins, 1 to 20% fat and 0.8 to 2% minerals (Ljubojević et al., 2013a; 2013b). The amounts of nutrients in fish meat are highly variable depending on a wide range of factors such as genetic, species, age, diet, gender, health condition, season of the year, water quality, farming system, etc. (Marković et al., 2016; Ljubojević Pelić et al., 2018). Proteins from fish are characterized by good amino-acid composition with substantial amounts of free amino acids (Buchtová et al., 2010). Fish proteins contain all amino-acids essential for human body and can be used as the sole source of protein in the diet (Vladau et al., 2008). Fish meat contains much less connective tissue comparative to the meat of endothermic animals, which contributes to its better digestibility preferred by specific consumer categories (Vladau et al., 2008). Fish contains negligible amounts of carbohydrates in the form of glycogen and high water content (60-86%) (Ćirković et al., 2011). Fish meat fat content is highly variable. Accordingly, based on their fat content, fishes are categorized into lean (< 5% fat), semi-fat (5 - 10% fat) and fat (> 10% fat) (Ljubojević et al., 2013b). Being a most valuable source of polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that can only be effectively synthesized by aquatic organisms, consumption of fish meat is the only way to intake the aforementioned fatty acids by humans (Pal et al., 2018). Thus, regular use of fish in the diet is highly recommended.

Fish consumption is beneficial and associated with lower risk of dementia and Alzheimer disease, and protective effects against some cancers are suggested (Connor and Connor, 2010). Regular intake of fish decreases the risk from cardiovascular diseases, especially myocardial infarction, atherosclerosis, hypertension and other (Kris-Etherton et al., 2002). Positive effects of fish in human diet are established in view of prenatal development, preservation of functions of nervous system, eyes and skin (Allen and Harris, 2001). Specific flavor, taste and good digestibility contribute to overall acceptability of carp meat.

Considering the aforementioned, investigating the options of fish farming by using purified wastewater is highly supported. The lack of available literature data on production parameters and meat quality of fish reared in ponds filled with purified wastewater encouraged our research towards determining production parameters and meat quality of carps farmed under the described conditions. To that end, carp fillets were analysed for basic quality parameters. Moreover, the carp fillet samples were examined during two sampling periods in order to identify potential seasonal effects on meat quality.

MATERIAL AND METHODS

A 1 ha fish pond with average depth of 1.3 m was set up as a part of the meat industry „Đurđević" in Pećinci providing an integrated production structure including slaughterhouse, wastewater purification system and fish pond. The process of water purification was performed in a wastewater treatment system. The purification procedure includes physical, biological and chemical processes. First, untreated wastewater was mechanically treated. Afterwards, water flows into the aeration tank and undergoes biological processing. Finally, the wastewater is discharged into the secondary wastewater treatment tank and chemically treated. The pond was built at the high-quality arable land and in line with basic principles and requirements described by Ćirković et al. (2002). The pond is supplied with both well water and purified slaughterhouse wastewater under continuous aeration. The ratio of water from the well and purified water from the slaughterhouse was about 2:1. In the first year, two-year old carps purchased from commercial ponds were introduced with a stocking density of 2,500 individuals/ha. The average initial weight of carps was 200 g. Fish were fed mixture of grains and edible offal (spleen, liver) of slaughtered animals supplemented with complete carp-feed mixtures. Edible offal was heat-treated (cooking) at a temperature of 90 °C. The offal has been finely ground and mixed in a cutter with the addition of cereals until obtaining a doughy consistency. The relationship between fresh feed (grains and edible offal) and complete mixtures in spring was 1:1. The ratio in fresh feed used was 30% for grains and 70% for edible offal. At the end of the first year, the survival rate was 74%. Production parameters were calculated according to fish biomass and feed conversion ratio described Abdelghany and Ahmad (2002). The first sampling was carried out in April next year, and the second one in the same year in October, that is, at the end of experimental period. In the second year, the survival rate was 87%. During the last three months, only grains and complete mixtures were added in the ratio 1:1. The samples of three-year-old

common carp were transported to the laboratory in a transport cooler. All samples were filleted before analysis.

Chemical composition of fish was examined applying standard SRPS ISO methods. Gravimetric method was utilized to determine the moisture content (SRPS ISO 1442:1998) and total fat (SRPS ISO 1443:1992); total protein content was determined combustion method (AOAC Official Method 992.15). Ash content was determined by combustion at 550±25 °C applying standard method SRPS ISO 936:1999. Salt concentration was determined applying standard method SRPS ISO 1841-1:1999. Hydroxyproline level was measured spectrophotometrically applying SRPS ISO 3496:2002. Subsequently, collagen content and collagen content in total protein were calculated. Energy value was expressed per 100 g of carp fillet, and was calculated according to the equation below using conversion factors indicated in the Appendix 13 of the Rulebook on declaration, labelling and advertising of food ("Official Gazette of RS", No. 19/2017, 16/2018, 17/2020 and 118/2020): Energy value (kcal/g) = 4 × carbohydrate content + 4 × protein content + 9 × fat content.

Statistical data analysis

The data were analysed using Excel (Microsoft Excel 2007, Microsoft Corporation, Redmond WA, USA) software package and Data Analysis. Student's t-test was applied to compare the arithmetic mean values of the results obtained for samples collected in spring and autumn. The data were expressed as mean values ± SD. A p-value of $p < 0.05$ was considered a statistically significant difference. Besides, Pearson correlation coefficient was used as well.

RESULTS

Production parameters were determined during harvesting at the end of study period. Total carp production per area unit was 3,270 kg/ha. Feed conversion calculated as the amount of supplementary feed spent for 1 kg weight gain was around 1.5 kg. Survival rate was high, reaching 87%. At spring sampling, in April in the second year of the study, the carp body weight ranged from 695 to 1,675 g (average weight 820 g), whereas values at autumn sampling in October in the second year of the study ranged between 1,710 and 2,700 g. At the end of experimental period in October in the second year of the study, the average live body weight of three-year old common carp was 2,020 ± 252.3 g.

Chemical composition of carp fillet is shown in Table 1. Higher protein content (17.99 g/100g) in fillets was determined in spring period. The differ-

ence between spring and autumn protein content was statistically significant ($p < 0.05$). The obtained results revealed higher fat content in the fillets during autumn period (5.19 g/100g) as compared with the spring season; however, the difference is not statistically significant ($p > 0.05$). Salt content was significantly higher at spring sampling ($p < 0.05$). The differences in energy value of the fillets analyzed in spring and autumn period were not statistically significant ($p > 0.05$).

Table 1. Chemical composition of carp fillet produced in the integrated system, sampled during spring and autumn season

Parameters (%)	Spring	Autumn	p-value
Moisture content	74.95 ± 1.37	76.16 ± 1.27	0.03
Protein content	17.99 ± 0.40	17.67 ± 0.29	0.03
Fat content	4.57 ± 1.35	5.19 ± 1.55	0.29
Collagen	1.04 ± 0.43	1.07 ± 0.13	0.84
Collagen content in total protein	5.80 ± 2.44	6.05 ± 0.78	0.73
Salt	1.26 ± 0.25	0.52 ± 0.26	< 0.05
Energy value (kcal)	113.06 ± 11.27	117.38 ± 13.09	0.38

The results were expressed as mean values ± SD ($n = 7$); $p < 0.05$

DISCUSSION

To our knowledge and according to the available literature the data on production performance and meat quality of carps reared in purified wastewater are pretty sparse, which suggests the innovative character of our study. The obtained results on production parameters correspond with the production parameters of carps sampled in conventional fishponds in the Republic of Serbia. Total carp production per area unit recorded in this study was 3,270 kg/ha. In fishponds with semi-intensive production system, where fish is fed naturally available feed resources supplemented with grains, the production performance is up to 1,500 kg/ha (Marković et al., 2016). Rearing fish in good production conditions and the use of high-quality concentrated extruded feed would offer possibility of increasing production performance and yields that may reach over 3,000 kg/ha (Stanković et al., 2011).

Adequate knowledge about fish meat quality is of importance in view of its role and value in human diet (Kris-Etherton et al., 2002, Connor and Connor,

2010). Our research revealed that moisture content in carp meat samples collected in spring season (74.95 ± 1.37%) was significantly lower (statistical significance p = 0.03) as compared to values measured in autumn period (76.16 ± 1.27%). Protein content determined in spring period (17.99 ± 0.40%) was higher than that observed in autumn (17.67 ± 0.29%), which is considered statistically significant (p = 0.03). Fat content in spring (4.57 ± 1.35%) was lower than that in autumn season (5.19 ± 1.55%); however, the difference was not statistically significant (p = 0.29). Higher contents of fat in autumn period could be attributed to the increased body-size of the fish. Also, nutrition as well as the types of fish feed play a very important role in the fat content in the body of fish (Stanković et al., 2011; Trbović et al., 2013; Marković et al., 2016). Some earlier researches have proved high correlation between the body-size of the carp and fat content in the meat (Kocour et al., 2007). A negative correlation was observed between fat and moisture contents in carps. Pearson correlation coefficients for the aforementioned parameters were 0.97 and 0.96 in spring and autumn season, respectively, which indicates a high negative correlation. Collagen contents determined in spring and autumn were 1.04 ± 0.43% and 1.07 ± 0.13%, respectively, with no statistically significant differences (p = 0.84). Collagen content in total protein was lower in spring season (5.80 ± 2.44) as compared to autumn period (6.05 ± 0.78%), yet without statistically significant difference (p = 0.73). Calculated energy value was higher in autumn (117.38 ± 13.09 kcal) than in spring (113.06 ± 11.27 kcal) without statistically significant difference (p = 0.38). The difference in calculated energy values in spring and autumn periods is expected, having in mind higher fat content in carp fillets sampled in October as compared to those sampled in April. According to the results reported by Yeganeh et al. (2012), fat and protein contents in samples of both "wild" carp and farmed carp decreased from summer to spring, whereas moisture contents increased during the same period. Over a 6-month research conducted between May and October, Swapna et al. (2010) did not establish any significant seasonal variations in fat contents in fish meat. On the other hand, Guler et al. (2008) reported highest fat content in carp fillets during winter months. This difference was explained by well-known seasonal variation in fat content associated with the changes of environmental temperature. According to the results obtained by Rasoarahona et al. (2004), fat content in carp muscle tissue was lower during warm months and higher during colder period of the year. It is to be emphasized that chemical parameters in wild fish are highly determined by the conditions of aquatic environment, which is well established source of nutritive matter. In farmed fish, diet containing commercially available feed-mixtures provides the abundance of nutrients thus determining the composition of meat. Protein content in meat is mainly determined

by intrinsic factors such as species and size of the fish rather than by the diet itself. Fat content in the meat of farmed carp reported by Yeganeh et al. (2012) was in accordance with the results obtained in our study. With respect to protein contents determined in spring and autumn periods, Yeganeh et al. (2012) did not report any statistically significant seasonal differences. The obtained results suggest a favorable ratio of nutritive matters in farmed carp, which could be attributed to high level of available nutrients in purified slaughterhouse wastewater. The results obtained in our study are in compliance with the results reported by other authors, who have conducted a number of researches on carp meat quality in diverse production systems. Chemical composition of carp meat was closely similar to fish reared in semi-intensive farming system in a commercial fishpond, where carps were fed concentrated feed mixtures and their meat had protein content of 17.30 ± 0.39% and fat content of 3.41 ± 1.37% (Ljubojević et al., 2013c). Such protein content is significantly higher and fat content significantly lower as compared to the cage-farmed carps of the same body weight, which were fed with corn. The protein and fat contents established in these carps were 16.23 ± 0.54% and 9.79%, respectively (Ljubojević et al., 2013c). A range of earlier researches suggested substantial variability in the quality of carp meat depending on the age, farming system and diet of the fish (Marković et al., 2016). The reported fat contents in carp meat ranged between 2.3 and 16.8%, while somewhat lower variability was observed with respect to protein contents, which was within a range from 14 to 18% (Vladau et al., 2008; Trbović et al., 2009; Ćirković et al., 2011).

Protein content in fillets of carp from semi-intensive production system fed diet supplemented with corn and wheat reported by Ćirković et al. (2011) was 15.59%, whereas our results obtained in samples collected in spring and autumn season were 17.99% and 17.67%, respectively. Interestingly, significantly lower protein content was measured in fillets sampled during autumn season (17.67%) as compared with the results obtained in spring sampling period (17.99%). This could be attributed to the effects of diet, that is, higher percentage of grains in fish diet in autumn period, having in mind that no edible offal were added in the last three months. Ljubojević et al. (2015) observed that decreased protein content in fish feed results in an increase in fat content and decrease in moisture and protein contents in fish muscle tissue. Increased amount of carbohydrates in carp diet leads to a more extensive deposition of visceral and muscle fat (Trbović et al., 2013). The fat content in fish meat varies according to the species, season of the year, water temperature and diet (Guler et al., 2008, Ćirković et al., 2011). Elevated fat content in carp meat negatively affects its sensory characteristics because increased fat amount affects the meat consistency and texture.

CONCLUSION

The use of purified slaughterhouse wastewater in carp fish-pond positively affected the production parameters and resulted in production of carp characterized by adequate meat quality corresponding with that of the carp reared in conventional production systems.

The results of this research and their comparison with earlier researches on the quality of carp meat strongly suggest that the use of purified slaughterhouse wastewater results in a production of good-quality carp meat.

The use of slaughterhouse wastewater in fish production represents a novel approach to the sustainability of meat industry and environment protection. The application of this concept within a slaughterhouse is crucial in the aspect of environment protection, having in mind the requirements and standards aimed at minimizing the environment pollution imposed by EU. In that respect, such requirements have to be fulfilled, and Serbian legislation on environment protection must be harmonized with the EU regulations.

ACKNOWLEDGEMENT

This work was funded by Ministry of Education, Science and Technological development of Republic of Serbia by the Contract of implementation and financing of scientific research work of NIV-NS in 2021, Contract No: 451-03-9/2021-14/200031 from 05/02/2021.

Author's Contribution:

MP and DLJP made substantial contributions to basic idea, conception and design, acquisition of samples and data, analysis of the data and interpretation of results; NP, NN, NP and SVK was involved in drafting of the manuscript, revising it critically for important intellectual content, and DLJP and MŽB gave the final approval of the manuscript to be published.

Competing interest

The authors declare that they have no competing interests.

REFERENCES

1. Allen K. G. D., Harris M. A. 2001. The Role of n-3 Fatty Acids in Gestation and Parturition. Experimental Biology and Medicine, 226, 498-506. doi:10.1177/153537020122600602.
2. Abdelghany A. E., Ahmad, M. H. 2002. Effects of feeding rates on growth and production of Nile tilapia, common carp and silver carp polycultured in fertilized ponds. Aquaculture Research, 33, 415-423. doi: 10.1046/j.1365-2109.2002.00689.x.
3. Buchtová H., Svobodová Z., Kocour M., Velíšek J. 2010. Chemical Composition of Fillets of Mirror Crossbreds Common Carp (*Cyprinus carpio* L.). Acta Veterinaria Brno, 79, 551–557. doi:10.2754/avb201079040551.
4. Connor W.E., Connor S.L. (2010) **N**-3 Fatty Acids from Fish and Plants: Primary and Secondary Prevention of Cardiovascular Disease. In: Bendich A., Deckelbaum R. (eds) Preventive Nutrition. Nutrition and Health. Humana Press. https://doi.org/10.1007/978-1-60327-542-2_10.
5. Ćirković M., Jovanović B., Maletin S. 2002. Ribarstvo, Univerzitet u Novom Sadu, Poljoprivredni fakultet, ISBN - 86-7520-013-7.
6. Ćirković M., Trbović D., Ljubojević D., Đorđević V. 2011. Meat quality of fish farmed in polyculture in carp ponds in Republic of Serbia. Tehnologija mesa, 52, 106-121.
7. Guler G. O., Kiztanir B., Aktumsek A., Citil O. B., Ozparlak H. 2008. Determination of the seasonal changes on total fatty acid composition and ω3/ω6 ratios of carp (*Cyprinus carpio* L.) muscle lipids in Beysehir Lake (Turkey). Food Chemistry, 108, 2, 689-694. doi:10.1016/j.foodchem.2007.10.080.
8. Kocour M., Mauger S., Rodina M., Gela D., Linhart O., Vandeputte M. 2007. Heritability estimates for processing and quality traits in common carp (*Cyprinus carpio* L.) using a molecular pedigree. Aquaculture, 270, 43–50. doi:10.1016/j.aquaculture.2007.03.001.
9. Kris-Etherton P. M., Harris W. S., Appel L. J. Nutrition committee. AHA scientific statement. 2002. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation, 106, 2747–2757. doi:10.1161/01.CIR.0000038493.65177.94.
10. Ljubojević D., Ćirković M., Novakov N., Jovanović R., Janković S., Đorđević V., Mašić Z. 2013a. Productivity and Meat Nutrient in Fish: The Diet Effect. Kafkas Universitesi Veteriner Fakultesi Dergisi, 19, 43-49. doi:10.9775/kvfd.2012.7088.
11. Ljubojević D.; Ćirković M.; Đorđević V.; Puvača N., Trbović D.; Vukadinov J., Plavša N. 2013b. Fat quality of marketable fresh water fish speci-

es in the Republic of Serbia. Czech Journal of food sciences, 31, 445-450. doi:10.17221/53/2013-CJFS.
12. Ljubojević D., Ćirković M., Đorđević V., Trbović D., Vranić D., Novakov N., Mašić Z. 2013c. Chemical composition, cholesterol content and fatty acid profiles of common carp (Cyprinus carpio) from free-catch, semi-intensive and cage system. Tehnologija mesa, 54(1), 48-56.
13. Ljubojević D., Radosavljević V., Puvača N., Živkov Baloš M., Đorđević V., Jovanović R., Ćirković M. 2015. Interactive effects of dietary protein level and oil source on proximate composition and fatty acid composition in common carp (*Cyprinus carpio* L.). Journal of Food Composition and Analysis, 37, 44- 50. doi:10.1016/j.jfca.2014.09.005.
14. Ljubojević Pelić D. 2018. Accession of carcass quality of common carp (Cyprinus carpio L.). Journal of Agronomy, Technology and Engineering Management, 1, 119-123.
15. Marković Z., Stanković M., Rašković B., Dulić Z., Živić I., Poleksić V. 2016. Comparative analysis of using cereal grains and compound feed in semi-intensive common carp pond production. Aquaculture international, 24, 1699-1723. doi:10.1007/s10499-016-0076-z.
16. Pal J., Shukla B. N., Maurya A. K., Verma H. O., Pandey G., Amitha A. 2018. A review on role of fish in human nutrition with special emphasis to essential fatty acid. International Journal of Fisheries and Aquatic Studies, 6, 2, 427-430.
17. Pelić M.M. 2020. Examination of the Effect of Using Wastewater from Slaughterhouses on Health and Production of Carp Meat (*Cyprinus carpio*) Safe for Human Consumption. PhD Thesis, Univerzitet u Beogradu-Fakultet veterinarske medicine.
18. Rasoarahona J. R., Barnathan G., Bianchini J. P., Gaydou E. M. 2004. Annual evolution of fatty acid profile from muscle lipids of the common carp (*Cyprinus carpio*) in Madagascar inland waters. Journal of agricultural and food chemistry, 52(24), 7339-7344. doi:10.1021/jf048993y.
19. Rulebook on declaration, labeling and advertising of food. Official Gazette of RS, No. 19/2017, 16/2018, 17/2020 and 118/2020.
20. SRPS ISO 1442/1998. - Meat and meat products — Determination of moisture content.
21. SRPS ISO 1443/1992. - Meat and meat products — Determination of total fat content.
22. SRPS ISO 1841-1:1999 – Meat and meat products — Determination of chloride content — Part 1: Volhard method.
23. SRPS ISO 3496:2002 - Meat and meat products — Determination of hydroxyproline content.

24. SRPS ISO 936:1999. - Meat and meat products — Determination of total ash.
25. Stanković M. B., Dulić Z. P., Marković Z. Z. 2011. Protein sources and their significance in carp (*Cyprinus carpio* L.) nutrition. Journal of Agricultural Sciences, 56, 75-86. doi: 10.2298/JAS1101075S.
26. Swapna H. C., Rai A. K., Bhaskar N., Sachindra, N. M. 2010. Lipid classes and fatty acid profile of selected Indian fresh water fishes. Journal of Food Science and Technology, 47, 4, 394-400. doi:10.1007/s13197-010-0065-6.
27. Thi Phong Lan N., Dalsgaard A., Cam P. D., Mara D. 2007. Microbiological quality of fish grown in wastewater-fed and non-wastewater-fed fishponds in Hanoi, Vietnam: influence of hygiene practices in local retail markets. Journal of Water and Health, 5, 2, 209-218. doi:10.2166/wh.2007.014b.
28. Trbović D, Vranić D, Đinović J, Borović B, Spirić D, Babić J, Spirić A. 2009. Fatty acid profile and cholesterol content in muscle tissue of one year old common carp (*Cyprinus carpio*) during growth. Tehnologija mesa, 50. 5/6, 276-286.
29. Trbović D., Marković Z., Milojković-Opsenica D., Petronijević R., Spirić D., Djinović-Stojanović J., Spirić A. 2013. Influence of diet on proximate composition and fatty acid profile in common carp (*Cyprinus carpio*). Journal of food composition and analysis, 31, 1, 75-81. doi:10.1016/j.jfca.2013.04.002.
30. Vladau V. V., Bud I., Stefan R. 2008. Nutritive value of fish meat comparative to some animals meat. Bulletin of University of Agricultural Sciences and Veterinary Medicine. Animal Sciences and Biotechnologies, 65, 1/2, 301–305.
31. Vo Q. H. 2001. Wastewater reuse through aquaculture in Hanoi: status and prospects. In *Working Paper No. 30*, Wastewater Reuse in Agriculture in Vietnam: Water Management, Environment and Human Health Aspects, Proceedings of a Workshop held in Hanoi, Vietnam 14 March 2001, ed. L. RaschidSally, W. van der Hoe, M. Ranawaka, International Water Management Institute, Colombo, Sri Lanka, pp.20–23.
32. Yeganeh S., Shabanpour B., Hosseini H., Imanpour M., Shabani A. 2012. Comparison of farmed and wild common carp (*Cyprinus carpio*): Seasonal variations in chemical composition and fatty acid profile. Czech Journal of Food Sciences, 30, 6, 503-511. doi:10.17221/455/2011.

Received: 05.04.2021.
Accepted: 27.06.2021.

Original research article UDC: 597.555.3:616-005.98
https://doi.org/10.46784/eavm.v14i1.261

CARP EDEMA VIRUS DISEASE IN SERBIA – A DISEASE OUT OF CONTROL

Vladimir Radosavljević[1*], Dimitrije Glišić[1], Jelena Maksimović-Zorić[1], Ljubiša Veljović[1], Ksenija Nešić[1], Vesna Miličević[1]

[1]Scientific Veterinary Institute of Serbia, Belgrade, Republic of Serbia

Abstract

A poxvirus named carp edema virus (CEV) is the causative agent of carp edema virus disease (CEVD), which is an emerging disease of global concern that may cause high rates of morbidity and mortality in ornamental koi and common carp. Common carp (*Cyprinus carpio*) is the most important fish species for warm-water aquaculture in Serbia. CEVD was first detected in Serbia in 2017. During the 2017-2020 period, an increasing number of CEVD outbreaks in carp farms was reported. The carp were collected from farms in different regions of Serbia from 2017 to 2020. The fish were sampled for disease diagnosis because they exhibited lethargy and anorexia, which eventually led to mortality. Mortality started with clinical signs of hypoxia and the fish swam slowly and were unresponsive. The gills were pale and covered with a thick mucus layer. In later stages of the disease, the lesions in the gills turned into a necrotizing form. A moderate to high amount of opportunistic freshwater bacteria were isolated from the gills of the diseased fish. By performing real-time PCR, CEV was detected in 38 samples of the diseased carp taken from 21 carp farms. These outbreaks further confirm the spread of CEVD and the need for practitioners to be vigilant in the event of an outbreak of this disease. To prevent further spreading of the disease, it is very important to introduce CEV testing before moving fish. To avoid further transmission of the virus to common carp populations in Serbia, the testing of CEV should become part of fish disease surveillance programs. Fish health service should be aware of the presence of CEV in Serbia which may result in high losses in carp aquaculture. Action should also be taken to prevent transmission of CEV to carp populations in open waters.

Key words: Carp edema virus disease, CEV, *Cyprinus carpio*

[1*] Corresponding Author: vladimiradosavljevic@yahoo.co.uk

EDEMSKA BOLEST ŠARANA U SRBIJI - BOLEST VAN KONTROLE

Vladimir Radosavljević[1], Dimitrije Glišić[1], Jelena Maksimović-Zorić[1], Ljubiša Veljović[1], Ksenija Nešić[1], Vesna Miličević[1]

[1]Naučni institut za veterinarstvo Srbije, Beograd, Republika Srbija

Kratak sadržaj

Poksvirus nazvan virus edemske bolesti šarana (CEV) je uzročnik edemske bolesti šarana, nove bolesti koja može prouzrokovati visok morbiditet i mortalitet šarana i ukrasnih koi šarana. Šaran (*Cyprinus carpio*) je najvažnija vrsta za toplovodnu akvakulturu u Srbiji. Edemska bolest šarana (EBŠ) je prvi put utvrđena u Srbiji 2017. godine. Tokom perioda od 2017 do 2020. godine uočen je veliki broj slučajeva pojave ove bolesti na šaranskim ribnjacima. Uzorci šarana su prikupljani sa ribnjaka u različitim regionima Srbije u periodu od 2017. do 2020. godine. Ribe su uzorkovane jer su pokazivale letargiju i anoreksiju, što je na kraju dovodilo i do uginuća, sa kliničkim znacima hipoksije. Škrge obolelih jedinki su bile blede i prekrivene gustim slojem sluzi. U uznapredovalim slučajevima, škržne lezije su postajale nekrotične. Iz škrga obolelih riba je izolovan umeren do veliki broj oportunističkih bakterija. Primenom PCR u realnom vremenu, CEV je otkriven u 38 slučajeva pojave bolesti u 21-om šaranskom ribnjaku. Novi slučajevi pojave bolesti potvrđuju širenje EBŠ u Srbiji. S obzirom da ribe mogu ostati nosioci ovog patogena, potrebno je primeniti odgovarajuće biosigurnosne mere na šaranskim ribnjacima u kojima je dokazana pojava ove bolesti. Da bi se sprečilo dalje širenje bolesti, veoma je važno uvesti CEV testiranje pri prometu prijemčivih vrsta riba. Da bi se izbeglo dalje širenje virusa na populacije šarana u Srbiji, kontrola prisustva CEV trebalo bi da postane deo programa nadzora bolesti riba. Nadležne službe u oblasti zdravstvene zaštite riba moraju biti svesne prisustva ovog oboljenja u Srbiji, s obzirom da ono može dovesti do značajnih gubitaka u akvakulturi šarana.

Ključne reči: edemska bolest šarana, CEV, *Cyprinus carpio*

INTRODUCTION

Common carp (*Cyprinus carpio*) is the most important fish in Serbian aquaculture, with annual production of approximately 11,000 tons (Marković et al. 2011). In order to maintain and intensify the production, one of the main

goals is to prevent the occurrence and spreading of the diseases, which could limit the sustainability of fish production. With that goal, annual control of listed fish diseases, namely koi herpesvirus (KHV) and spring viremia of carp virus (SVCV) has been carried out. However, the occurrence and spread of a new disease which may significantly affect the health of carp is a disturbing fact which requires attention of all the parties involved in the production chain. Carp edema virus disease (CEVD) is an emerging disease considered to be a potential risk for the carp aquaculture and for global food security as well (Kurita et al. 2009; Way et al. 2017), with new data about its spread, economic and biological properties being published rapidly over the past few years (Rehman et al. 2020; Machat et al. 2021).

The first outbreak of a disease caused by carp edema virus (CEV) was reported in 1974 in Japan, and for long time this disease was detected exclusively in ornamental koi carp (Murakami et al. 1976), but recently it was confirmed as a causative agent of a disease in koi and common carp in Europe, USA and many Asian countries (Way et al. 2017; Marsella et al. 2021).

In common carp, the disease was initially detected in the United Kingdom and in the Netherlands in 2012 (Way and Stone 2013; Haenen et al. 2014). After the initial detections, the CEVD was reported in The Czech Republic and Poland in 2013 (Vesely et al. 2015; Matras et al. 2017), followed by Austria and Italy in 2014 (Lewisch et al., 2015; Pretto et al. 2015), Hungary in 2016 (Adamek et al. 2018a), Lithuania and Croatia in 2018 (Adamek et al. 2018b; Zrnčić et al. 2020), and Slovakia in 2019 (Matějíčková et al, 2020)

Figure 1. CEVD in common carp in Europe (map of Europe originated from https://d-maps.com/continent.php?num_con=5&lang=en)

In the majority of cases, farmed common carp were involved. However, there are also reports of mortality events involving wild fish. For the first time in Europe, CEV was detected in wild common carp in Italy (Marsella et al. 2021). A wide temperature range from 6 to 24 °C has been recorded for the reported cases, although most of the outbreaks occurred at the temperature between 19 and 24 °C (Divya et al. 2019). The disease generally has the characteristics of an acute infection at water temperatures between 15 and 25 °C, but it also occurs at a lower temperature (6 – 10 °C) when affecting common carp, resulting in a chronic infection characterized by a lower mortality (Lewisch et al. 2015; Way et al. 2017; Toffan et al. 2020).

Diseased fish become lethargic or show sleepy behavior and eventually die due to anoxia. The juvenile carp usually congregate near the surface of a pond or water inlet, whereas the older fish tend to stay at the bottom of the pond. The most frequently observed external lesions consist of increased skin mucous production, enophthalmos, erosion or hemorrhages at the base of the fin and gill necrosis (Way et al. 2017). Gross lesions in gills (hypertrophy, hyperplasia and lamellae clubbing) and subsequent necrosis of secondary gill lamellae may lead to the death of the infected fish (Machat et al. 2021).

Carp edema virus disease (CEVD) is caused by carp edema virus (CEV), which belongs to the subfamily *Chordopoxvirinae* in the *Poxviridae* family. CEV has double-stranded DNA genome and has a strong affinity to infect the gill epithelial cells. Phylogenetic analysis of the partial core protein p4a has revealed the existence of three genogroups: I, IIa, and IIb showing 6 – 10% genetic diversity (Matras et al. 2017; Soliman et al. 2019).

Genogroup I consists exclusively of viral samples obtained from common carp collected mostly from European aquaculture countries, namely: The United Kingdom, Germany, Poland, Hungary and Balkan countries.

Genogroup IIa contains viruses isolated predominantly, but not exclusively from koi carp showing clinical signs of the disease with Asian and European isolates of the virus being included.

Genogroup IIb includes viral isolates from koi and common carp and was classified by phylogenetic analyses between the two aforementioned genogroups. The genogroup IIb is discovered in various carp samples in Poland (Matras et al. 2017).

The CEV spread is most probably the result of lack of availability of effective diagnostic and preventive measures in the global trade. The detection of the virus is difficult at subclinical level in specimen. Therefore, prevention of the disease is difficult and needs extra care at different stages such as transportation, transfer of fish from one pond to another, etc. It is likely that the establishment of an effective monitoring system and development of a highly specific diagnostic method could be helpful for the control of disease outbreaks

(Rehman et al. 2020). Adequate measures for the prevention and disease control need to be taken in order to minimize the chances of disease outbreak such as avoiding transport and restocking of susceptible fish in different farming systems when the water temperature becomes suitable for virus replication (Sunarto et al. 2014). Also, CEV could have direct immunosuppressive effect on the host (Adamek et al. 2018a; Lewisch et al. 2015; Way et al. 2017), enabling secondary infections through impairment of gills (Adamek et al., 2018a). It is also mandatory to observe and isolate the susceptible fish to avoid further spread of CEV in stock.

Matras et al. (2019) showed that European perch (*Perca fluviatilis*) and five cyprinid species: tench (*Tinca tinca*), roach (*Rutilus rutilus*), bleak (*Alburnus alburnus*), crucian carp (*Carassius carassius*) and Prussian carp (*Carassius gibelio*) may serve as asymptomatic virus vectors, after cohabitation with CEV diseased carp. Adamek et al. (2016) suggested potential CEV persistence in carp and its stress-related reactivation. To prevent the introduction of pathogen in the stock, new fish should be kept in a separate area for at least 30 days at constant water temperature, and it should be taken care (feeding and daily routine maintenance). Further preventive measures include maintaining the fish health and providing quality water and feed while decreasing overcrowding and stress condition (Rehman et al. 2020).

Several methods can be used for CEV diagnostics, but the definitive proof of infection relies on the detection of viral DNA in tissues of the fish. The virus may prove difficult to detect at sub-clinical levels in apparently healthy survivors (Wey et al. 2017). Several cell lines were used for CEV isolation, but they were not successful (Swaminathan et al. 2016).

Currently, no treatment is available for CEVD. Different research showed that the mortality rate reduces the number CEV-infected carp when infected fish are immersed in 0.5% salted water.

After the first occurrence of carp edema virus disease (CEVD) in 2017 (Radosavljevic et al. 2018), an increasing number of CEV positive carp farms was detected. In this paper we report the increase in the incidence, disease in severity and mortality of CEVD at a carp farms during a four-year period, from 2017-2020.

MATERIAL AND METHODS

Fish samples

The carp were collected from 21 fish farms in different regions of Serbia from 2017 to 2020. The carp were sampled for disease diagnosis because the

fish exhibited lethargy and anorexia, which eventually led to mortality. The total of 38 samples (the gill and kidney tissues of five individuals were pooled to form one sample) were examined including one-year-old (C1), two-year-old (C2) or three-year-old (C3) carps (5 fish from each group). External examination and sampling were performed for further investigation. After gill clipping and skin scraping, the presence of external parasite infection was inspected with an optical microscope. For bacterial culture, the organ surface was sterilized using a heated blade and punctured to perform parenchymal swab sampling. Bacterial cultures of the gills, liver, spleen, and kidney were kept for 48 h using tryptic soy agar plates at 20 °C. The gill and kidney tissue were sampled for virus isolation and DNA extraction.

Virus isolation

The post-mortem collected pooled gills and anterior kidney were homogenized in Earle's salt-based Minimal Essential Medium (MEM) (Sigma-Aldrich). The homogenate was inoculated in an array of cell lines (including CCB, EPC, BF-2, RTG-2, CHSE) for three passages, according to standard procedures.

Molecular detection by PCR

The samples were tested for CEV according to real-time PCR protocol described by Way et al. (2017). The sample RNA was extracted from the homogenates of the gill and anterior kidney tissues of diseased fish with the QIAamp Cador Pathogen Mini Kit (Qiagen), according to the manufacturer's instructions. The DNA was then stored at −20 °C before PCR amplification. For detection of CEV p4a DNA, a probe-based qPCR assay developed by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in Weymouth, UK (Adamek et al., 2017; Matras et al., 2017), was performed using the primers CEFAS_qF: AGTTTTGTAKATTGTAGCATTTCC, CEFAS_qR: GATTCCTC-AAGGAGTTDCAGTAAA and the double-labelled probe [FAM]-AGAGTTTGTTTCTTGCCATACAAACT-[BHQ1]. The amplification program included an initial denaturation at 95 **°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s.**

RESULTS

The total of 190 fish from 21 carp farms were examined, and 38 outbreaks of CEVD in common carp of various age were noticed in 6 regions in Serbia (Table 1, Figure 2).

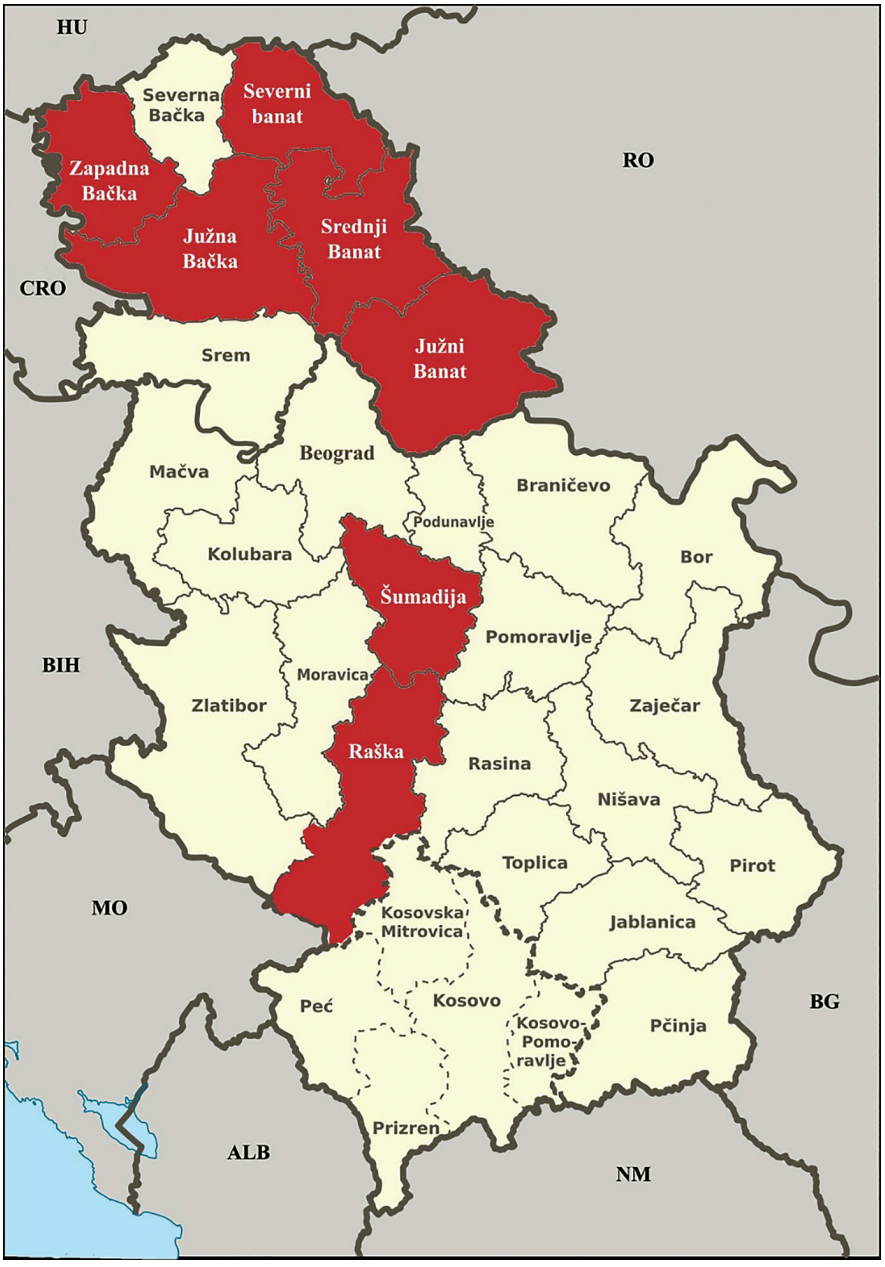


Figure 2. A map of Serbia showing districts with CEVD positive carp farms in red (map originated from: https://commons.wikimedia.org/wiki/File:Serbia_administrative_divisions_-_de_(districts).svg

During CEVD outbreaks, no behavior changes or clinical signs of disease were noticed in other fish species present in the same pond with carp. Most outbreaks occurred between late spring and early summer, when water temperature was between 14 °C and 25 °C. But, in a few cases, the occurrence of the disease with mortalities was noticed in autumn and early spring when temperature was just 7 - 12 °C (Table 1). The highest mortality rates were recorded during an outbreak at the beginning of May (with water temperature of 18 °C) and during three outbreaks in September (water temperature 21 °C and 22 °C) (Table 1). Various clinical signs of the disease were observed in CEV positive carps (Figure 3).

Figure 3. External examination and gross pathology of the carp: A) Lethargic behavior with reduced food intake; B) Overproduction of mucus on skin; C) Secondary bacterial and fungal gill and skin infections; D) Overproduction of mucus on gills; E) Pale swollen gills, sometimes with irregular discoloration and gill necrosis; F) Fibrinous peritonitis.

Usually, fish farmers reported an increasing mortality of the fish that were unresponsive, gasping for air and swimming slowly.

Dominant clinical signs noticed during CEVD episodes includes:

1. Lethargic behavior with reduced food intake (Figure 3A).
2. Various cumulative mortality - mostly low < 20%, but in severe cases > 50%.
3. Overproduction of mucus on gills and skin (Figures 3B, 3D).
4. Pale swollen gills, sometimes with irregular discoloration and gill necrosis, but never as drastic as with KHVD (Figure 3E).
5. No pathologies in internal organs, except fibrinous peritonitis (Figure 3F).
6. Various secondary bacterial and fungal gill and skin infections (motile aeromonads, pseudomonads, Flavobacteriae, *B. mucoides*, *Saprolegnia* spp, *Branchiomyces* spp.) (Figure 3C).

Table 1. CEVD outbreaks in Serbia between 2017 and 2020 in one-year-old (C1), two-year-old (C2) or three-year-old (C3) carps

Isolate no.	Year	Sample name	Location District	Facility no.	Case details Month/water T % mortality, age of fish
1	2017	3405	Južnobački	1	May beg. 14 °C / Low ≤ 20% / C1
2	2017	3406	Južnobanatski	2	May beg. 15 °C / Low ≤ 20% / C1
3	2017	3407	Zapadnobački	3	May beg. 16 °C / Low ≤ 20% / C1
4	2017	2775	Južnobanatski	4	May beg. 17 °C / Low ≤ 20% / C1
5	2017	3386	Raški	5	May beg. 18 °C / High ≥ 50% / C1
6	2017	3394	Severnobanatski	6	May beg. 16 °C / Low ≤ 20% / C1
7	2017	3900	Srednjebanatski	7	May end 18 °C / Low ≤ 20% / C1
8	2018	2820	Šumadijski	8	April beg 8 °C / Low ≤ 20% / C1
9	2018	2885	Srednjebanatski	7	April end 9 °C / Low ≤ 20% / C2
10	2018	4600	Južnobački	9	July beg. 23 °C / Low ≤ 20% / C3
11	2018	4698	Srednjebanatski	10	July beg. 25 °C / Low ≤ 20% / C2
12	2018	4997	Južnobanatski	4	July beg. 24 °C / Low ≤ 20% / C1
13	2019	3578	Srednjebanatski	7	April beg. 8 °C / Low ≤ 20% / C1 & C2
14	2019	3659	Severnobanatski	11	April beg. 7 °C / Low ≤ 20% / C1 & C2
15	2019	3861	Južnobački	12	April beg. 16 °C / Low ≤ 20% / C1
16	2019	4550	Severnobanatski	13	May end 18 °C / Low ≤ 20% / C1 & C2
17	2019	4556	Srednjobanatski	10	May end 17 °C / Low ≤ 20% / C1
18	2019	4592	Južnobanatski	14	May end 17 °C / Low ≤ 20% / C2
19	2019	4683	Srednjobanatski	10	June beg. 18 °C / Low ≤ 20% / C1
20	2019	4706	Srednjobanatski	15	June beg. 17 °C / Low ≤ 20% / C1 & C2
21	2019	6771	Zapadnobački	16	Sept beg. 19 °C / Med 20 – 50% / C1

Isolate no.	Year	Sample name	Location District	Facility no.	Case details Month/water T % mortality, age of fish
22	2019	6844	Severnobanatski	11	Sept beg. 19 °C / Low ≤ 20% / C2
23	2019	6874	Zapadnobački	17	Sept beg. 22 °C / High ≥ 50% / C1 & C2
24	2019	6977	Južnobački	18	Sept end 21 °C / High ≥ 50% / C1
25	2019	7022	Zapadnobački	19	Sept beg. 19 °C / Low ≤ 20% / C1
26	2019	7168	Srednjebanatski	7	Sept beg. 18 °C / Low ≤ 20% / C1
27	2019	7279	Srednjebanatski	7	Sept end 20 °C / Low ≤ 20% / C1
28	2019	7331	Srednjebanatski	15	Sept end 19 °C / Low ≤ 20% / C1
29	2019	7427	Zapadnobački	19	Oct beg. 16/ °C / Low ≤ 20% / C1
30	2019	7724	Zapadnobački	19	Oct end 14 °C / Low ≤ 20% / C1
31	2020	2653	Zapadnobački	3	April end 12 °C / Low ≤ 20% / C1
32	2020	2902	Severnobanatski	11	May beg. 15 °C / Low ≤ 20% / C1
33	2020	2939	Severnobanatski	11	May end 16 °C / Low ≤ 20% / C3
34	2020	2973	Srednjebanatski	20	May end 16 °C / Low ≤ 20% / C2
35	2020	3316	Zapadnobački	19	May end 15 °C / Low ≤ 20% / C1
36	2020	3573	Zapadnobački	16	June beg.19 °C / Low ≤ 20% / C1
37	2020	9299	Zapadnobački	16	Oct end 13 °C / Low ≤ 20% / C2
38	2020	10231	Zapadnobački	21	Nov end 7 °C / Low ≤ 20% / C2

Besides these, various clinical signs were noticed in individuals, including sunken eyes, hyperemia or hemorrhages at fin base, fibrinous peritonitis, the perianal region, and the abdominal body surface, gill hemorrhages, skin erosions and ulcerations. In majority of the cases, the bacteria were not isolated from internal organs (liver, kidney and spleen), but always present on gills and skin lesions of the affected fish.

By performing real-time PCR, CEV was detected in samples from 38 outbreaks of disease from 21 carp farms.

No cytopathic effect was observed in five cell lines (CCB, EPC, BF-2, RTG-2, and FHM) following inoculation of filtrate from gill and kidney tissue homogenate of CEV positive carp, after 10 days of inoculation and even after three blind passages.

DISCUSSION

The present study revealed that CEV was widespread in Serbian common carp farms and the virus was detected during 38 outbreaks on 21 carp farms. After first detection of CEV in 2017 (Radosavljevic et al. 2018), a significant increase of CEVD cases was detected in 2019 and 2020. The number of CEV positive carp farms is probably underrated, since the disease was detected in

all carp farms after sampling initiated by the owner, following occurrence of unexplained mortality in carp population.

The clinical signs and lesions observed in carp during the disease episodes in Serbia were consistent with previous reports (Way & Stone 2013; Lewisch et al. 2015; Machat et al. 2021). The prominent clinical signs present during CEVD episodes in all the cases reported here are increased gill and skin mucous production with gill edema. Gills are the most vulnerable organ, displaying lamellae hypertrophy and hyperplasia, with consequent gaseous exchange impairment (Toffan et al. 2020).

Water temperature is a significant factor for development of CEVD. The disease in common carp has been reported at water temperatures between 7 and 15 °C (Lewisch et al. 2015), and also at lower water temperatures between 6 and 9 °C (Way and Stone, 2013). Important predisposing factor is rapid temperature change and primary rapid rise in temperature. A majority of detected CEVD outbreaks in Serbia occurred when water temperature ranged between 13 and 24 °C, but in 5 cases, the disease occurred between 7 and 12 °C. This corresponds with findings of other authors (Lewisch et al. 2015; Divya et al. 2019; Toffan et al. 2020). Although underestimated and less frequently recognized, it is known that CEV infection can cause juvenile mortality up to 75 – 100% (Toffan et al. 2020). The cumulative mortality rate in majority of the cases described here (34 out of 38) was below 20%, but in three outbreaks, the mortality was higher than 50%, and in one outbreak around 35%.

In 17 outbreaks, co-infection with bacteria or fungi was present, as reported by other authors (Way et al. 2017; Machat et al. 2021). These isolated bacteria and fungi are known to be opportunistic pathogens for carp, and they had probably contributed to increase the severity of the disease.

No cytopathic effect was observed in five cell lines following inoculation of filtrates of gill and kidney homogenate of CEV positive carp, after three blind passages. The results are in accordance with earlier reports, where the virus was not isolated on various cell lines (Oyamatsu et al. 1997; Jung-Schroers et al. 2015; Swaminathan et al. 2016).

Having in mind an obvious increase in the number of CEVD cases in common carp and spread to new locations, adequate measures which will enable timely detection of the causative agent in infected fish and possible vectors should be adopted as soon as possible, since there are still many unsolved questions regarding potential asymptomatic vector species, modes of transmission, virus isolation, etc.

The emergence of infectious diseases is usually triggered by ecological changes, often associated with human interventions, such as transfer of organ-

isms, environmental degradation, agricultural practices or technology (Jones et al. 2008). Introduction of new pathogens into naive populations can cause mass mortalities, as happened with common carp after introduction of KHV and with Prussian carp after introduction of CyHV-2. Usually, the first data about occurrence of new disease comes from fish in aquaculture, and the acquired data should be used as valuable information for prediction of the impact of the disease on susceptible free-living fish populations. Based on the presented information, it is obvious that there is a need for adequate legislation in order to control and prevent further spread of the virus. The implementation of control measures which will slow the spread of the disease is needed urgently, having in mind that CEV is detected in wild common carp populations in Europe and USA (Lovy et al. 2018; Marsella et al. 2021), and in asymptomatic fish hosts (Matras et al. 2019), which can significantly contribute to further spread of the disease.

CONCLUSION

CEVD is slowly becoming one of the biggest threats to carp aquaculture, due to increasing mortality in infected fish, and also due to reduced growth and the fact that secondary bacterial and fungal infections are regularly found in diseased fish, causing additional pathologies and mortality. Fish health service should be aware of the presence of CEVD, which may result in substantial losses in carp aquaculture. Based on the great importance of carp aquaculture in Serbia, a detailed surveillance and control program for CEVD is warranted, especially due to absence of international regulations regarding this disease. Internal movements of carp between aquaculture facilities are of particular concern because large numbers of live carp are moved and, since infections in aquatic animals are frequently subclinical, they would not be directly noticed before, during and immediately after the movement of the infected fish. Having in mind that movement of live aquatic animals always carries a risk of transferring aquatic animal pathogens, the controls should also involve internal fish movements between carp farms. In addition, disease quarantine inspections of imported/exported fish should be performed with greater precision. The quarantine procedure for CEVD should be performed in such way that carp is quarantined for a minimum of 30 days in the water with a constant temperature (between 15 and 25 °C). In order to avoid the spread of CEV infections, testing carp for CEV should become part of fish disease surveillance program.

ACKNOWLEDGEMENT

This work was funded by Serbian Ministry of Education, Science and Technological Development (contract number 451-03-9/2021-14/200030).

Author's Contribution:

VR and VM made contributions to concept and design of the study, they collected data and drafted the manuscript. DG and VM carried out the molecular diagnostic tests and prepared the alignment of nucleotide sequences and conducted the molecular genetic analysis. LJV carried out the data analysis. VM and KN revised the manuscript critically and together with VR prepared the final draft of the manuscript. All the authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

REFERENCES

1. Adamek M., Jung-Schroers V., Hellmann J., Teitge F., Bergmann S.M., Runge M., Kleingeld D.W., Way K., Stone D.M., Steinhagen D. 2016. Concentration of carp edema virus (CEV) DNA in koi tissues affected by koi sleepy disease (KSD). Diseases of Aquatic Organisms, 119, 3, 245–251. doi: 10.3354/dao02994.
2. Adamek M., Matras M., Jung-Schroers V., Teitge F., Heling M., Bergmann S.M., Reichert M., Way K., Stone D.M., Steinhagen D. 2017. Comparison of PCR methods for the detection of genetic variants of carp edema virus. Diseases of Aquatic Organisms, 126, 1, 75–81. doi: 10.3354/dao03152.
3. Adamek M., Teitge F., Jung-Schroers V., Heling M., Gela D., Piackova V., Kocour M., Steinhagen D. 2018a. Flavobacteria as secondary pathogens in carp suffering from koi sleepy disease. Journal of fish diseases, 41, 11, 1631–1642. doi: 10.1111/jfd.12872.
4. Adamek M., Baska F., Nienius D., Radosavljevic V., Zrnčić S., Brnić D., Oraić D., Steinhagen D. 2018b. Erste Nachweise des carp edema virus in Ungarn, Litauen, Kroatien und Serbien bestätigen eine sehr weite Verbreitung des Virus in Karpfenbeständen in Europa. In XVII Gemeinschaftstagung der deutschsprachigen Sektionen der EAFP zum Thema Fischkrankheiten, 3-5. October, Fribourg/Schweiz, P-10.

5. Divya P., Vertika B., Kirty S., Jyotirmaya M., Pramoda Kumar S. 2019. A review of current understanding on carp edema virus (CEV): A threatful entity in disguise. International Journal of Fisheries and Aquatic Studies, 7, 5, 87–93.
6. Haenen O, Way K, Stone D, Engelsma M. 2014. Koi sleepy disease found for the first time in koi carps in the Nether-lands. Tijdschrift Voor Diergeneeskunde, 139, 26–29.
7. Jones K. E., Patel N.G., Levy M.A., Storeygard A., Balk D., Gittleman J.L., Daszak P. 2008. Global trends in emerging infectious diseases. Nature, 451, 990–993. doi:10.1038/nature06536.
8. Jung-Schroers V., Adamek M., Teitge F., Hellmann J., Bergmann S.M., Schütze H., Kleingeld D.W., Way K., Stone D., Runge M., Keller B., Hesami S., Waltzek T., & Steinhagen D. 2015. Another potential carp killer? Carp Edema Virus disease in Germany. BMC Veterinary Research, 11, 114. doi: 10.1186/s12917-015-0424-7.
9. Kurita J., Yuasa K., Ito T., Sano M., Hedrick R.P., Engelsma M.Y., Haenen O.L.M., Sunarto A., Kholidin E.B., Chou H.Y., Tung M.C., Peña L., Lio-Po G., Tu C., Way K., Iida T. 2009. Molecular epidemiology of koi Herpesvirus. Fish Pathology, 44, 2, 59–66. doi:10.3147/jsfp.44.59.
10. Lewisch E., Gorgoglione B., Way K., & El-Matbouli M. 2015. Carp Edema Virus/Koi sleepy disease: An emerging disease in central-east Europe. Transboundary and Emerging Diseases, 62, 1, 6–12. doi:10.1111/tbed.12293.
11. Lovy J., Friend S.E., Al-Hussinee L., Waltzek T.B. 2018. First report of carp edema virus in the mortality of wild common carp *Cyprinus carpio* in North America. Diseases of aquatic organisms, 131, 177-186. doi: 10.3354/dao03296.
12. Machat R., Pojezdal L., Piackova V., Faldyna M. 2021. Carp edema virus and immune response in carp (*Cyprinus carpio*): Current knowledge. Journal of fish diseases, 44: 371– 378. doi: 10.1111/jfd.13335.
13. Marković Z., Stanković M., Dulić Z., Živić I., Rašković B., Spasić M.., Poleksić V. 2011. Akvakultura i ribarstvo u Srbiji – stanje i potencijal. In *Conference Proceesings*, V. International Conference "Aquaculture & Fishery", June 1-3. 2011, Faculty of Agriculture, Belgrade-Zemun, Serbia, 36-40.
14. Marsella A., Pretto T., Abbadi M., Quartesan R., Cortinovis L., Fiocchi E., Manfrin A., Toffan A. 2021. Carp edema virus-related mortality in wild adult common carp (*Cyprinus carpio*) in Italy. Journal of fish diseases, 44, 939–947. doi: 10.1111/jfd.13353.
15. Matějíčková K., Pojezdal Ľ., Pokorová D., Reschová S., Piačková V., Palíková M., Veselý T., Papežíková I. 2021. Carp oedema virus disease outbreaks in

Czech and Slovak aquaculture. Journal of fish diseases, 43, 971– 978. doi: 10.1111/jfd.13179.

16. Matras M., Borzym E., Stone D., Way K., Stachnik M., Maj-Paluch J., Palusinska M., Reichert M. 2017. Carp edema virus in Polish aquaculture – evidence of significant sequence divergence and a new lineage in common carp *Cyprinus carpio* (L.). Journal of Fish Diseases, 40, 319–325. doi: 10.1111/jfd.12518.
17. Matras M., Stachnik M., Borzym E., Maj-Paluch J., Reichert M. 2019. Potential vector species of carp edema virus (CEV). Journal of Fish Diseases, 42, 959–964. doi:10.1111/jfd.13000.
18. Murakami Y., Shitanaka M., Toshida S., Matsuzato T. 1976. Studies on mass mortality of juvenile carp: about mass mortality showing edema. Bulletin Hiroshima Fresh Water Fisheries Experimental Station, 19–33.
19. Oyamatsu T., Hata N., Yamada K., Sano T., Fukuda H. 1997. An etiological study on mass mortality of cultured colorcarp juveniles showing edema. Fish Pathology, 32, 81–88. doi: 10.3147/jsfp.32.81.
20. Pretto T., Abbadi M., Panzarin V., Quartesan R., Manfrin A., Toffan A. 2015. Carp edema virus (CEV): first detection in Italy. In EAFP 17th International Conference on disease of fish and shellfish, Las Palmas de Gran Canaria, September 7–11., Poster P-119, p 343.
21. Radosavljevic V., Adamek M., Milicevic V., Maksimovic-Zoric J., Steinhagen D. 2018. Occurrence of two novel viral pathogens (CEV and CyHV-2) affecting Serbian cyprinid aquaculture and ichthyofauna. Journal of Fish Diseases, 41, 5, 851–854. doi:10.1111/jfd.12789.
22. Rehman T., Yin L., Latif M.B., Zhou Y., Wang K., Geng Y., Huang X., Chen D., Fang J., Chen Z., Guo H., Lai W., Ouyang P. 2020. Current findings on carp edema virus, control challenges, and future outlook. Aquaculture International, 28, 2015–2026. doi:10.1007/s10499-020-00573-6.
23. Soliman H., Lewisch E., El-Matbouli M. 2019. Identification of new genogroups in Austrian carp edema virus isolates. Diseases of Aquatic Organisms, 136, 2, 193–197. doi:10.3354/dao03408.
24. Sunarto A., McColl K. A., Crane M. S., Schat K. A., Slobedman B., Barnes A. C., Walker P. J. 2014. Characteristics of cyprinid herpesvirus 3 in different phases of infection: implications for disease transmission and control. Virus research, 188, 45–53. doi:10.1016/j.virusres.2014.03.024.
25. Swaminathan, T. R., Kumar, R., Dharmaratnam, A., Basheer, V. S., Sood, N., Pradhan, P. K., Sanil N. K., Vijayagopal P., Jena J. K. 2016. Emergence of carp edema virus in cultured ornamental koi carp, Cyprinus carpio koi, in India. Journal of General Virology, 97, 12, 3392–3399. doi:10.1099/jgv.0.000649.

26. Toffan A., Marsella A., Abbadi M., Abass S., Al-Adhadh B., Wood G., Stone D. M. et al. 2020. First detection of koi herpesvirus and carp oedema virus in Iraq associated with a mass mortality in common carp (*Cyprinus carpio*). Transboundary and Emerging Diseases, 67, 523–528. doi:10.1111/ tbed.13428.
27. Vesely T, Pokorova D, Reschova S, Piačková V. 2015. Detection of carp edema virus in common carp (*Cyprinus carpio*) and koi carp in the Czech Republic. In EAFP 17th International Conference on disease of fish and shellfish, Las Palmas de Gran Canaria 7–11 September, European Association of Fish Pathologists, Poster P-122, p. 346.
28. Way K., Haenen O., Stone D., Adamek M., Bergmann S. M., Bigarré L., Diserens N., El-Matbouli M., Gjessing M. C., Jung-Schroers V., Leguay E., Matras M., Olesen N. J., Panzarin V., Piačková V., Toffan A., Vendramin N., Veselý T., & Waltzek T. 2017. Emergence of carp edema virus (CEV) and its significance to European common carp and koi *Cyprinus carpio*. Diseases of Aquatic Organisms, 126(2), 155–166. https://doi.org/10.3354/ dao03164
29. Way K. and Stone D. 2013. Emergence of carp edema virus-like (CEV-like) disease in the UK. Finfish News, 15, 32–35.
30. Zrnčić S., Oraić D., Zupičić I. G., Pavlinec Ž., Brnić D., Rogić Ž. A., Sučec I., Steinhagen D., & Adamek M. 2020. Koi herpesvirus and carp edema virus threaten common carp aquaculture in Croatia. Journal of Fish Diseases, 43, 6, 673–685. doi: 10.1111/ jfd.13163.

Received: 21.04.2021.
Accepted: 22.07.2021.

Original research article UDK 636.52/.58.09
https://doi.org/10.46784/eavm.v14i1.266

EFFICACY OF P 547/17 FORMULATION ON *DERMANYSSUS GALLINAE* EGGS AND LARVAE

Aleksandar Pavlićević[1], Ivan Pavlović[21*]

[1]„AVES MIT" LLC, Subotica-Bajmok, Republic of Serbia
[2]Scientific Veterinary Institute of Serbia, Belgrade, Republic of Serbia

Abstract

High reproductive potential and short development cycle, in addition to other factors, enable *Dermanyssus gallinae* to produce adverse effects in the poultry industry. Not all development stages have the same significance, nor are they equally sensitive to the methods and formulations used for *Dermanyssus gallinae* control. Laboratory tests were conducted by exposing eggs, larvae and protonymphs to P 547/17 formulation of inert oils (Pulcap). The testing was carried out with 20% oil-in-water emulsion with short exposure (1 min), and with 10%, 20%, 50% and 100% oil-in-water emulsion with continuous exposure. In the first control group, water was used (with continuous exposure), while in the other control group eggs, larvae and nymphs were not treated. We determined that in all cases, eggs were laid in high percentage (89-100%). In addition to this, in tested liquids, larvae were present in high percentage. They changed into protonymphs (8-89%). In the conditions of full exposure, where parasitic stages cannot leave the emulsion, P 547/17 in time has complete efficacy on development stages. In short egg exposure, when there is dirt, or on absorbent surface, P 547/17 emulsion cannot control them. However, this drawback is not essential in practical conditions. When applied correctly, P 547/17 formulation is efficient in *Dermanyssus gallinae* control.

Key words: P 547/17 formulation, efficacy, *Dermanyssus gallinae*, eggs, larvae

[1*] Corresponding author: dripavlovic58@gmail.com

EFIKASNOST FORMULACIJE P 547/17 NA JAJA I LARVE *DERMANYSSUS GALLINAE*

Aleksandar Pavlićević[1], Ivan Pavlović[2]

[1]„AVES MIT" LLC, Subotica-Bajmok, Republika Srbija
[2]Naučni institut za veterinarstvo Srbije, Beograd, Republika Srbija

Kratak sadržaj

Velika reproduktivna moć i kratak razvojni ciklus, pored ostalih činioca, omogućavaju štetnost *Dermanyssus gallinae* u živinarstvu. Svi razvojni stadijumi nemaju isti značaj, niti su isto osetljivi prema metodama i formulacijama koje upotrebljavamo u kontroli *Dermanyssus gallinae*. Laboratorijska ispitivanja izvršena su izlaganjem jaja, larvi i protonimfa formulaciji inertnih ulja P 547/17 (Pulcap). Kratka ekspozicija (1 min) korišćena je za ispitivanje 20% vodene emulzije, dok je neprekidna ekspozicija korišćena za vodene emulzije 10%, 20%, 50% i 100% P 547/17. U prvoj kontrolnoj grupi za izlaganje koristili smo vodu (u neprekidnoj ekspoziciji), a u drugoj su jaja, larve i nimfe bile netretirane. Utvrdili smo da se jaja u svim slučajevima izležu u visokom stepenu (89-100%). Osim toga, u ispitivanim tečnostima larve egzistiraju u velikom procentu i presvlače se u protonimfe (8-89%). U uslovima pune izloženosti, gde parazitski stadijumi ne mogu napustiti emulziju, P 547/17 vremenom ostvaruje potpunu efikasnost na razvojne stadijume. Kod kratke izloženosti jaja, kada su prisutne nečistoće ili upijajuće površine, emulzija P 547/17 ih ne može kontrolisati. Međutim, utvrđeni nedostaci nemaju presudnog značaja u praktičnim uslovima. Sa pravilnom upotrebom, formulacija P 547/17 je efikasna u kontroli *Dermanyssus gallinae*.

Ključne reči: P 547/17 formulacija, efikasnost, *Dermanyssus gallinae*, jaja, larve

INTRODUCTION

Poultry red mite *Dermanyssus gallinae* (De Geer, 1778) is the most significant poultry ectoparasite. Its significance in poultry industry arises from its prevalence, degree of control, overall impact on the human and poultry health and the environment, as well as the level of direct and indirect damage.

High reproductive power and short development cycle result in massive numbers of mites, which, in addition to other factors, have harmful effects of *D. gallinae* in the poultry industry. High intensity *D. gallinae* infestations can reach the numbers of 25,000 to 50,000, and in exceptional cases even up to 250,000 mites per hen (Van Emous, 2005). The development of *D. gallinae* has five stages: eggs, larvae, protonymphs, deutonymphs, and adults (Wood, 1917) (Figure 1 and 2). *D. gallinae* reproduction occurs in the gonadotropic cycle, and is conditioned by blood consumption (Pritchard et al., 2015). During 12–24 hours after blood consumption, the female will start to lay eggs in hidden places. The size of eggs is usually 400x270 μm. Females usually lay up to 9 eggs in one batch, with several batches in their lifetime (Nordenfors, 2000). Temperature conditions required for laying eggs range from 5-45 °C, while the maximum numbers of eggs are recorded at 25 °C and 70% humidity (Nordenfors et al., 1999).

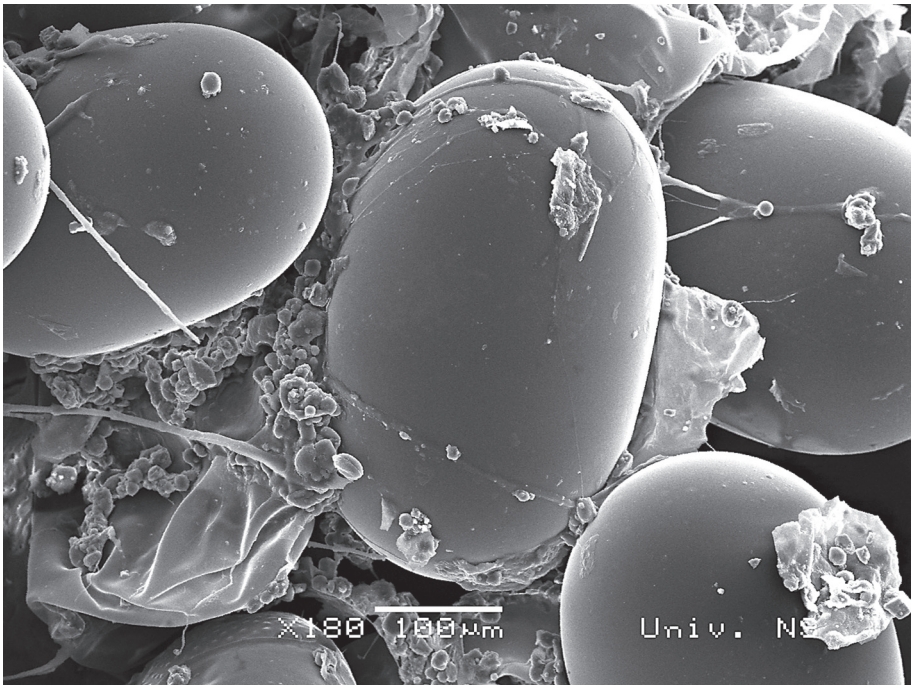


Figure 1. *D. gallinae* eggs recorded by electron microscope

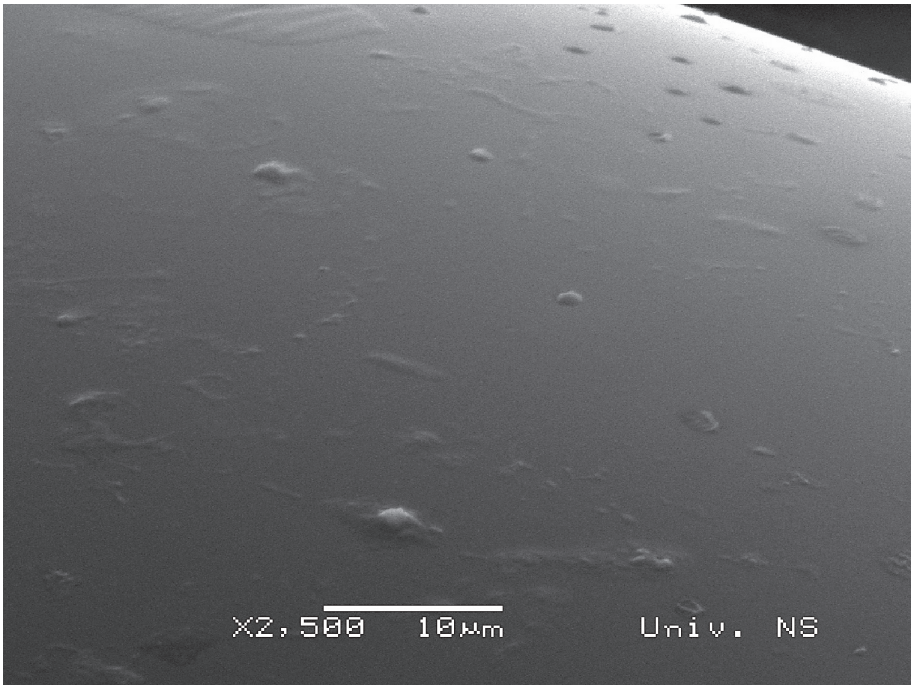


Figure 2. A detail from the surface of *D. gallinae* egg, recorded by electron microscope

After one to three days, at the temperature of 20-45 °C, larvae are laid from the eggs (Maurer and Baumgartner, 1992; Nordenfors et al.1999). Larvae do not feed. They have 3 pairs of legs. Their colour is seashell white. In the next 1-2 days they change into the protonymph. Protonymphs and deutonymphs have 4 pairs of legs, and in order to transform into the next form, they need to consume blood. In optimal environmental conditions, the development cycle from an egg to an adult can be finished in seven days (Nordenfors, 2000). In practical conditions, this cycle takes longer. For example, at 25 °C, the development of one generation of *D. gallinae* finishes in 16.8 days (Maurer and Baumgartner, 1992). The tests conducted in Sao Paolo, Brazil, showed that 15 to 42 generations of *D. gallinae* can develop in one year (Tucci et al., 2008).

The control of different development stages can have different impact depending on the species of arthropods.

The aim of our examination was to determine the efficacy of P 547/17 formulation (Pulcap) on the eggs and larvae, and the relevance of the findings for *D. gallinae* control.

MATERIAL AND METHODS

Female *D. gallinae* fed in isolation chambers in the laboratory, were moved to plastic Petri dishes, in order to lay eggs. The lids of Petri dishes were paired up according to the width and sticked with a duct tape in order to prevent mites from coming out. After the third day, adult mites were removed from the Petri dishes, and the laid eggs were counted and included in the testing.

The first testing group was exposed for 1 minute to the 20% water emulsion of P 547/17 formulation (hereinafter w.e.f.). This was done by first covering the eggs with w.e.f. P 547/17, and after 1 minute, the emulsion was poured out and drained for the next hour. Despite the draining, the Petri dishes still contained a certain amount of leftover water emulsion, i.e. the tested formulation. The testing groups were constantly exposed to 10%, 20%, 50% water emulsion, and to the concentrated P 547/17 (100%) formulation. The testing procedure was also conducted with two control groups: 1. water, with constant exposure and 2. negative control without exposure. The development of eggs, larvae and protonymphs was monitored until the 10th day of exposure (0-10). The temperature in the environment ranged from 19 to 23 ^{o}C, and humidity from 49 to 61%.

The results obtained by laboratory testing were interpreted by comparison with the clinical trials of the efficacy of P 547/17 water emulsion.

RESULTS

Our results are presented in tables 1 to 8, and figures 2 and 3.

Table 1. Testing 10% w.e.f. P 547/17, constant exposure.

10% w.e.f. P 547/17	Initial number of eggs	Development stage	After 10 days	
			Number	%
Constant exposure	116	Eggs	0	0
		Larvae	55	47.41
		Protonymphs	61	52.59
		Overall	116	100

Table 2. Testing 20% w.e.f. P 547/17, 1 minute exposure.

20% w.e.f. P 547/17	Initial number of eggs	Development stage	After 10 days	
			Number	%
1 min exposure	38	Eggs	2	5.3
		Larvae	4	10.5
		Protonymphs	32	84.2
		Overall	38	100

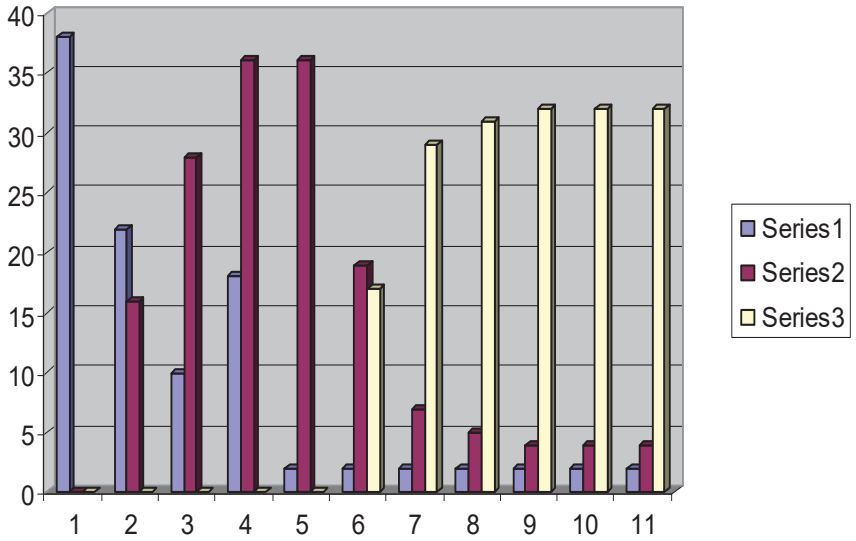


Figure 3. Testing 20% w.e.f. P 547/17, 1 minute exposure, development of eggs, larvae and protonymphs of *D. gallinae* presented by days of testing: 1. purple – eggs; 2. maroon - larvae; 3. yellow - protonymphs.

Table 3. Testing 20% w.e.f. P 547/17, constant exposure.

20% w.e.f. P 547/17	Initial number of eggs	Development stage	After 10 days	
			Number	%
Constant exposure	108	Eggs	4	3.70
		Larvae	32	29.63
		Protonymphs	72	66.67
		Overall	108	100

Table 4. Testing 50% w.e.f. P 547/17, constant exposure.

50% w.e.f. P 547/17	Initial number of eggs	Development stage	After 10 days	
			Number	%
Constant exposure	97	Eggs	10	10.31
		Larvae	80	82.47
		Proto-nymphs	7	7.22
		Overall	97	100

Table 5. Testing 100% w.e.f. P 547/17, constant exposure.

100% w.e.f. P 547/17	Initial number of eggs	Development stage	After 10 days	
			Number	%
Constant exposure	42	Eggs	4	9.52
		Larvae	18	42.86
		Proto-nymphs	20	47.62
		Overall	42	100

Table 6. Testing in water, constant exposure.

Well water	Initial number of eggs	Development stage	After 10 days	
			Number	%
Constant exposure	65	Eggs	7	10.77
		Larvae	19	29.23
		Proto-nymphs	39	60
		Overall	65	100

Table 7. Control group

Control group	Initial number of eggs	Development stage	After 10 days	
			Number	%
No exposure	24	Eggs	0	0
		Larvae	0	0
		Proto-nymphs	24	100
		Overall	24	100

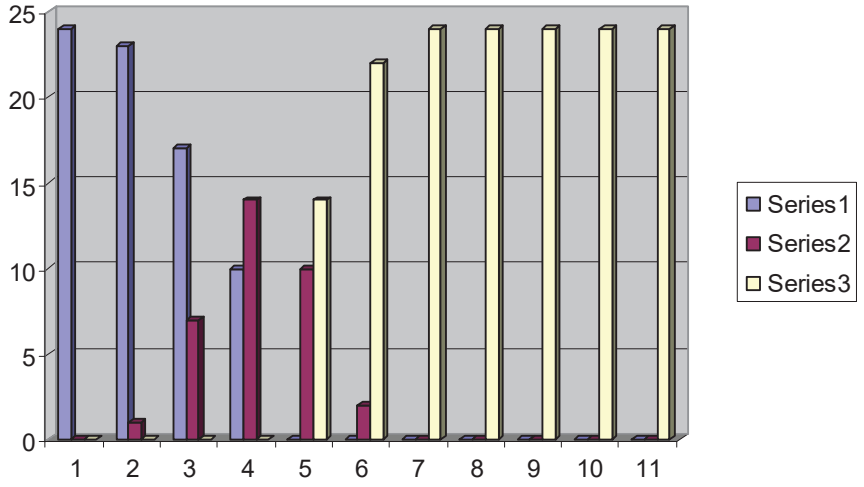


Figure 4. Testing control group, development of eggs, larvae and protonymphs of *D. gallinae* presented by days of testing: 1. purple – eggs; 2. maroon - larvae; 3. yellow - protonymphs.

Table 8. Comparative overview of values, percentage of laid eggs and transformed larvae of *D. gallinae*

Percentage (%) w.e.f. P 547/17 and others	**10**	**20**	**20**	**50**	**100**	**Water**	**Control**
Exposure	Constant	1 min	Constant	Constant	Constant	Constant	-
Percentage of laid eggs (%)	100	94.7	96.3	89.69	90.48	89.23	100
Percentage of changed larvae (%)	52.59	88.89	69.23	8.05	52.63	67.24	100

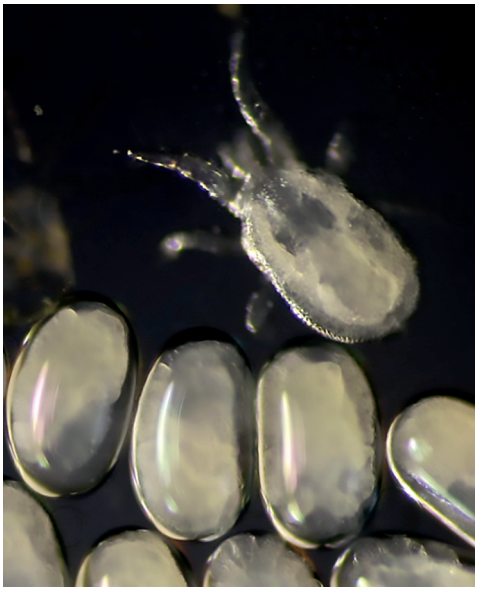


Figure 5. Eggs (with visible embryonic development) and larvae of *D. gallinae,* in w.e.f. P 547/17, recorded by optical microscope

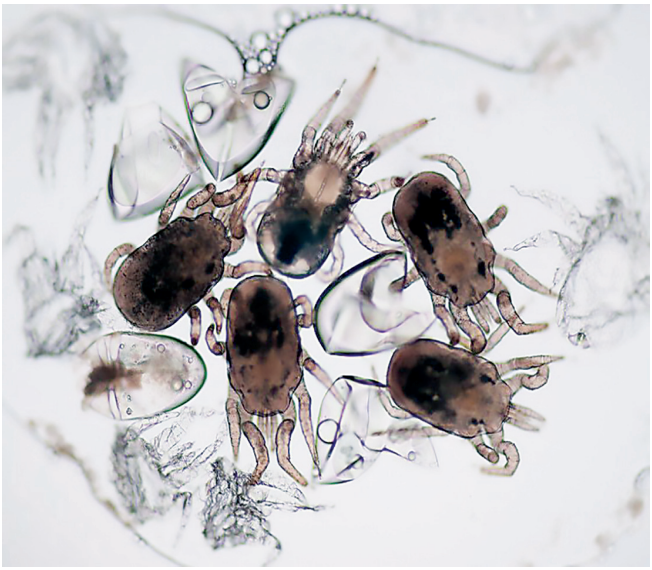


Figure 6. *D. gallinae* protonymph in w.e.f. P 547/17, recorded by optical microscope

DISCUSSION

In untreated, control group, all the eggs transformed into larvae and subsequently into protonymphs. According to the research by Nordenfors et al. (1999), 99% of eggs developed into larvae at 25 °C, and 92% of larvae further transformed into protonymphs.

Mineral oils have been used for the control of harmful insects and mites for more than a century (Agnello, 2002). There have been attempts to use various types of oils for *D. gallinae* control (Guimaraes and Tucci, 1992; Van Emous, 2005; Maurer et al., 2009; Camarda et al., 2018).

Pulcap is the first specialised formulation (P 547/17) and technology (project ID 1115) which uses water emulsion of inert oils for *D. gallinae* control. The aim is for the biological efficacy of P 547/17 to be thoroughly examined and documented. Preliminary observations from previous tests (Pavlićević et al., 2018) showed that P 547/17 water emulsion in working solutions has no significant effect on *D. gallinae* eggs.

There is general knowledge about the effect of inert oils. Unlike acaricides and insecticides (in the narrow sense of the word synthetic neurotoxic chemical compounds), oils do not act through specific receptors, but rather in multiple ways. Their effect depends on interaction between physical, chemical, anatomic, developmental and physiological characteristics and behaviour of the targeted mites or insects. The following have been described: affinity to epicuticle, immobilisation (in active stages), preventing respiration, disturbing water balance, penetrating the organism, disturbing cellular functions, etc. (Buteler and Stadler, 2011).

Certain oils are known to have effect on some arthropods' eggs. For example, in insects, Al Dabel et al. (2008) found high efficacy of horticultural oil on *Ostrinia nubilalis* eggs applied in 3-10% concentration. Buteler and Stadler (2011) determined that 2% horticultural mineral oil causes almost total mortality of *Choristoneura rosaceana* eggs.

Large numbers, short development cycle, i.e. great reproductive capacity of *D. gallinae* require high efficacy of a formulation used for their control. For direct 1 minute exposure, biological efficacy of formulations for *D. gallinae* control should be 95-100%. If constant and direct exposures are combined, efficient formulations should achieve 100% efficacy in laborato (ideal) conditions.

The tests were designed to maximise the contact of eggs and larvae with w.e.f. P 547/17 by direct and constant exposure. In spite of this, most eggs underwent their embryonic development smoothly. The degree of egg exposure ranged from 89 to 100%. Larvae existed in the tested fluids in high percentage and changed into protonymphs in 8-89 % of cases. 50% w.e.f. P 547/17 showed the highest

degree of efficacy on larval stage where 8% changed into protonymphs.

There is a significant difference in sensitivity between the development stages of eggs and larvae compared to adult *D. gallinae*. For adult *D. gallinae*, 1 minute direct exposure to 10% w.e.f. P 547/17 would be lethal (Pavlićević et al., 2017). Eggs and larvae were able to withstand, to a high degree, a constant direct exposure to water emulsion (10%, 20%, 50%), even concentrated (100%) P 547/17 formulation.

The results of conducted research indicate that eggs and larvae of *D. gallinae* can stand anoxia. We are not aware of any tests on the anoxia of *D. gallinae* eggs and larvae. However, hypoxia and anoxia have been indirectly tested in adult *D. gallinae*. Mites were put in Petri dishes, directly exposed to CO2 (2,500 ppm), by spraying for 10 seconds. After 120 hours, the effect of anoxia was 100% mortality of adult *D. gallinae* (Kang et al., 2020). Furthermore, we can assume that additional research will find that eggs and larvae of *D. gallinae* are, to a great extent, not sensitive to controlled CO2 management.

In comparison, controlled CO2 management has been used since the 1980s in plant protection in order to control harmful mites (Aharoni et al., 1981). Exposure to 60% CO^2 at 30 °C results in up to 100% mortality of adult two-spotted spider mite *Tetranychus urticae* (Oyamada and Murai 2013). Suzuki et al. (2015) determined that the exposure of eggs and adult females of *T. urticae* and *T. kanzawai* to anoxia caused by deoxidant over the period of 12 hours at 25 °C results in up to 100% mortality.

Laboratory tests were done in the conditions which enable the maximum effect of w.e.f. P 547/17. However, in practical conditions, it is not possible to expose all eggs and larvae of *D.gallinae* directly, sufficiently and constantly. The reasons for this are mites' way of life on hidden places, limited distribution (overlapping and covered places) of water emulsion in external application by spraying, as well as the very conditions in the poultry industry (absorbent surfaces or presence of dirt). Therefore, in practical conditions, we can expect worse results compared to those found in laboratory tests. Even in ideal laboratory conditions, even with high concentrations, the effect of P 547/17 formulation on *D. gallinae* eggs and larvae did not fulfil the conditions of total efficacy, and should be taken into account. The observed flaw of w.e.f. P 547/17 needs to be overcome by planning the method of application. The choice of method of w.e.f. P 547/17 application starts with the choice of the moment of application. Although w.e.f. P 547/17 can be used in both empty and populated poultry houses, the specific conditions and effects of control will vary significantly.

The development structure of *D. gallinae* invasion in hosts is reported as 35% eggs, 10% larvae, 33% nymphs and 22% adults (Van Emous, 2005). In a populated house, w.e.f. P 547/17 is used when necessary. The conditions in a

populated poultry house are characterised by lack of hygiene, worse distribution of the emulsion due to the presence of poultry (it covers the surfaces) and uninterrupted reproduction of *D. gallinae* (with all development stages present). The efficacy depends on hygienic situation (limited contact and reduced residual effect on surfaces), complexity of environmental conditions, quality of application and intensity and extensity of *D. gallinae* infestation. In the case of general low intensity infestation, one treatment is implemented, while in the case of general high intensity infestation, there are two treatments. The effects of suppression with w.e.f. P 547/17 in a populated house usually last for 4-5 months.

Formulation P 547/17 is primarily designed for prevention, since this is the primary and essential task of human and veterinary medicine. Treating an empty poultry house with w.e.f. P 547/17 prevents the infestation of a new, young flock of laying hens with *D. gallinae* invasion (present in the facility).

In an empty facility there is no host (poultry) and therefore no blood which is necessary for laying *D. gallinae* eggs. Adults will keep the ability to lay eggs for several days after emptying the poultry house, based on the previously consumed blood. At the temperature of 27 °C and humidity of 80%, egg laying will last for up to 11 days (Tucci et al., 2009). Then it will stop. The development of eggs and protonymphs will be limited to the phase of deutonymph, because from this moment, further development will depend on blood consumption. The absence of blood, i.e. the host (poultry) will prevent further development of protonymphs and deutonymphs. *D. gallinae* adults remain in an empty poultry house. In some cases, they can endure starvation for more than a year (Pavlićević et al., 2007). Therefore, the success of controlling adults is crucial for the effects which will be achieved in *D. gallinae* control.

The tests performed in laboratory conditions (Maurer and Baumgartner, 1992) indicate that development of eggs into larvae of *D. gallinae* at 10 °C lasts for 12 days, while at the temperature of 5 °C it lasts for up to 50 days or 28.2 days (Nordenfors et al. 1999). In practical conditions, we have not yet found a problem which could arise from a slower development of *D. gallinae* eggs and larvae at lower temperatures.

Poultry house preparation includes thorough cleaning, washing, deratisation, disinfection and drying. After that, w.e.f. P 547/17 is applied. On unabsorbent surfaces, it has long-lasting, highly efficient prolonged effect even on subsequently exposed mites (Pavlićević et al., 2017). The prolonged effect is especially noticeable in empty facilities, since residual layer is not exposed to dirt or removal.

Favourable hygienic situation, good conditions for the distribution of the water emulsion and sufficiently sensitive development stages of mites, housing down-time (enough time at the temperature conditions in which *D. gallinae* is

active), and correct application optimise the efficacy of w.e.f. P 547/17. When these conditions are fulfilled in an empty poultry house, P 547/17 results in *D. gallinae* eradication from production facilities, i.e. farms. Eradication has so far been proven in floor housing of parent flocks, cage housing system, conventional and enriched cages for layer housing (Pavlićević et al., 2018; 2019a,b, 2020) in the process of preparation). In aviaries and free range housing, the eradication has not yet been tested. *D. gallinae* eradication in a poultry house is considered successful if during one year of systematic monitoring, not a single mite is detected. If the conditions are only partially met, P 547/17 results in highly efficient *D. gallinae* suppression. If no conditions are met, *D. gallinae* suppression might not be effective (Pavlićević et al., 2019a).

After comparing the results obtained from laboratory (unsatisfactory effects) and clinical tests (highly efficient suppression and eradication), we believe that the development stages of eggs and larvae are not crucial for *D. gallinae* control. Despite their resistance, the need for feeding and short development period makes them inconsequential for the control procedure. Adult stage is crucial for the control.

In the appendix about the assessment of significance of *D. gallinae* eggs and larvae for the control, there is the research on the efficacy of the formulation of active substance fluralaner (isoxazoline). Fluralaner is administered to poultry *per os*, in drinking water and it works only on adults and development phases that feed on blood (protonymphs and deutonymphs). Therefore, fluralaner has no effect on eggs and larvae. Nevertheless, the effects of *D. gallinae* control can last for up to 238 days (Thomas et al., 2017).The development of *D. gallinae* eggs and larvae continues in aquatic environment as well. When washing the poultry house, the water itself (especially on floors) will not significantly affect the vitality of eggs and larvae (apart from mechanical removal). Therefore, they are expected to complete their development after the surfaces dry off (Thomas et al. 2017). However, a detailed washing is a prerequisite for creating the hygienic conditions which enable a quality contact with the water emulsion and prolonged effect on unabsorbent surfaces.

CONCLUSION

P 547/17 formulation does not have a significant impact on *D. gallinae* eggs and larvae. Although they have a higher resistance level, eggs and larvae are not very important for *D. gallinae* control. Adult control is crucial. Intensive and long-lasting prolonged effect of w.e.f. P 547/17 successfully eliminates adults, thereby including all developmental stages. Therefore, P 547/17 achieves a highly effective suppression, and with an optimal application in an

empty poultry house, it is even able to eradicate *D. gallinae* from facilities, i.e. poultry farms.

Laboratory and clinical tests, as well as the formulation and application technology make a unique method for *D. gallinae* control.

Author's contributions

AP provide the basic idea, coordinated the research and experimental study and processed the results, and IP additionally processed the results of the study and prepared the manuscript

Competing Interests

The authors declare that they have no competing interests.

REFERENCES

1. Agnello, A.M. 2002. Petroleum – Derived spray oils: chemistry, history, refining and formulation. In: Spray Oils Beyond 2000: Sustainable Pest and Disease Management. Eds. G.A.C. Beatie, D.M.Watson, M.L. Stevens, D.J. Rae and R.N. Spooner-Hart, University of Western Sydney Press, Australia, 2-18.
2. Aharoni Y., Stewart J.K., Guadagni D.G. 1981. Modified atmospheres to control western flower thrips on harvested strawberries. Journal of Economic Entomology, 74, 3, 338–340. doi: 10.1093/jee/74.3.338.
3. Al Dabel F., Mensah R., Frerot B. 2008. Effects of nC24 and nC27 petroleum spray oils on oviposition and egg survival of Ostrinia nubilalis Hübner (Lepidoptera, Pyralidae) and Trichogramma brassicae Bezdenko (Hymenoptera, Trichogrammatidae) adults on maize plants. International Journal of Pest Management, 54, 1, 5-11. doi:10.1080/09670870701549632.
4. Buteler M., Stadler T. 2011. A Review on the Mode of Action and Current Use of Petroleum Distilled Spray Oils. In. Pesticides in the Modern World – Pesticides Use and Management. Eds. M. Stoytcheva, InTech pub. Rijeka, Croatia, 119-136. doi: 10.5772/20394.
5. Camarda A., Pugliese N., Bevilacqua A., Circella E., Gradoni L., George D., Sparagano O., Giangaspero A. 2018. Efficacy of a novel neem oil formulation (RP03™) to control the poultry red mite Dermanyssus gallinae. Medical and Veterinary Entomology, 32, 3, 290-297. doi: 10.1111/mve.12296.

6. Guimaraes J.H., Tucci E.C. 1992. Evaluation of mineral oil in the control of Dermanyssus gallinae (De Geer, 1778) (Acari, Dermanyssidae) in field and laboratory conditions. Revista Brasileira de Entomologia, 36,859–862.
7. Kang, J., Hossain, M. A., Jeong, J., Park, H., Kim, J. H., Kang, M. S., Kwon, Y. K., Kim, Y. S., Park, S. W. 2020. Application of carbon dioxide as a novel approach to eradicate poultry red mites. Journal of Veterinary Science, 21, 2, e37. doi:10.4142/jvs.2020.21.e37.
8. Maurer V., Baumgartner J. 1992. Temperature influence on life table statistics of the chicken mite Dermanyssus gallinae (Acari: Dermanyssidae). Experimental and Apply Acarology, 15, 1, 27-40.
9. Maurer V., Perler E., Heckendorn F. 2009. In vitro efficacies of oils, silicas and plant preparations against the poultry red mite Dermanyssus gallinae. Experimental and Apply Acarology, 48, 1-2, 31–41. doi:10.1007/s10493-009-9254-2.
10. Nordenfors H, Höglund J., Uggla A. 1999. Effects of temperature and humidity on oviposition, molting and longevity of Dermanyssus gallinae (Acari: Dermanyssidae). Journal of Medical Entomology, 36, 1, 68-72. doi: 10.1093/jmedent/36.1.68.
11. Nordenfors H. 2000. Epidemiology and Control of the Poultry Red Mite, Dermanyssus gallinae. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala.
12. Oyamada K., Murai T. 2013. Effect of fumigation of high concentration carbon dioxide on two spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) and strawberry runner plant. Japanese Journal of Applied Entomology and Zoology, 57, 4, 249–256. doi: 10.1303/jjaez.2013.249.
13. Pavlićević A., Pavlović I., Dotlić M. 2007. A contribution to information on starvation survival capacity of poultry red mite *Dermanyssus gallinae*. Lucrari Stiintifice Medicina Veterinara 50, 9, 485-491.
14. Pavlićević A., Ratajac R., Dotlić M., Stojanov I., Pavlović I. 2017. An innovative formulation of paraffin and silicone oils for the control of the red poultry mite (Dermanyssus gallinae) –examination of the efficiency under laboratory conditions. Archives of Veterinary Medicine, 10, 2, 63-79. doi: 10.46784/e-avm.v10i2.74.
15. Pavlićević A., Ratajac R., Horvatek Tomić D., Stojanov I., Pavlović I. 2018. Dermanyssus gallinae eradication approach – application of inert compounds and integral animal health protection. Arhiv veterinarske medicine, 11, 1, 3 - 15. doi: 10.46784/e-avm.v11i1.12.
16. Pavlicevic A, Pavlovic I., Davidovic B., Ratajac R. 2019a. Clinical Examination of the Control Red Chicken Mites *Dermanyssus gallinae*. Scientific Papers: Animal Science and Biotechnologies, 52, 1, 105-115.

17. Pavlićević A., Ratajac R., Stojanov I., Pavlović I. 2019b. An example of rational *Dermanyssus gallinae* control – Pulcap. Archives of Veterinary Medicine, 12, 1, 71 – 85. doi: 10.46784/e-avm.v12i1.39.
18. Pavlicevic A., Pavlovic I. 2020. Pulcap in the control of Dermanyssus gallinae, current clinical findings. In: Proceedings of XX International Conference Poultry Production in Russia and in the World. Present State, Dynamics of Development, Prospective Inovations. Sergiyev Posad, Moscow Region, Russia, 14-16.10.2020, 663-664.
19. Pritchard J., Kuster T., Sparagano O., Tomley F. 2015. Understanding the biology and control of the poultry red mite Dermanyssus gallinae: a review. Avian Patology, 44, 3, 143-153. doi: 10.1080/03079457.2015.1030589.
20. Suzuki, T., Wang C.-H., Gotoh T., Amano H., Ohyama K. 2015. Deoxidant-induced anoxia as a physical measure for controlling spider mites (Acari: Tetranychidae). Experimental and Applied *Acarology,* 65, 3, 293- 305. doi: 10.1007/s10493-015-9881-8.
21. Thomas E, Chiquet M, Sander B, Zschiesche E, Flochlay AS. 2017. Field efficacy and safety of fluralaner solution for administration in drinking water for the treatment of poultry red mite (Dermanyssus gallinae) infestations in commercial flocks in Europe. Parasites and Vectors, 10, 1, 457. doi: 10.1186/s13071-017-2390-3.
22. Tucci E.C., Prado A.P., Araúlo R.P. 2008. Thermal requirements of *Dermanyssus gallinae* (De Geer, 1778) (Acari: Dermanyssidae). Revista Brasiliera de Parasitologia Veterinary, 17, 2, 67-72. doi: 10.1590/S1984-29612008000200002.
23. Tucci E.C., Prado A.P., Araúlo R.P. 2009. Influência do jejum sobre a fecundidade de *Dermanyssus gallinae* (De Geer, 1778) (Acari, Dermanyssidae). Arquivos Instituto Biológico São Paulo, 76, 1, 23-26. doi: 10.1590/1808-1657v76p0232009.
24. Van Emous R. 2005. Wage war against the red mite. Poultry International, 44, 26–33.
25. Wood, H.P. 1917. The chicken mite: its life history and habits. Bulletin of the United States Department of Agriculture, 553.

Received: 16.05.2021.
Accepted: 21.06.2021.

Original research article

UDC: 616.993(497.113)
636.09:616.993(497.113)
https://doi.org/10.46784/eavm.v14i1.267

EPIDEMIOLOGICAL CHARACTERISTICS OF LEPTOSPIROSIS IN VOJVODINA, SERBIA, 2009-2018, FROM THE ASPECT OF ONE HEALTH

Tatjana Pustahija[1*], Vladimir Vuković[1], Mioljub Ristić[1,2], Sara Savić[3], Živoslav Grgić[3], Smiljana Rajčević[1,2], Mirjana Štrbac[1], Tanja Tomašević[4,5], Snežana Medić[1,2]

[1]Institute of Public Health of Vojvodina, Centre for Disease Control and Prevention, Novi Sad, Republic of Serbia
[2]University of Novi Sad, Faculty of Medicine, Department of Epidemiology, Novi Sad, Republic of Serbia
[3]Scientific Veterinary Institute "Novi Sad", Department for Serology, Immunology and Biochemistry, Novi Sad, Republic of Serbia
[4]Institute of Public Health of Vojvodina, Centre for Analysis, Planning and Organization of Health Care, Novi Sad, Republic of Serbia
[5]University of Novi Sad, Faculty of Medicine, Department of Social Medicine and Health Statistics with Informatics, Novi Sad, Republic of Serbia

Abstract

This study summarizes demographical and chronological characteristics of human leptospirosis and reveals the spatial distribution of this disease in the Autonomous Province of Vojvodina during the period 2009-2018, as well as examines possible relationships between the occurrence of the disease and climatic factors. Additionally, it describes the seroprevalence of the disease among domestic animals in the same area and the same period. Pearson's correlation was used to explore correlations between different meteorological factors and trends in time-series of human cases. Overall 87 human cases of leptospirosis and five subsequent deaths (Case Fatality Rate - CFR: 5.75%) were recorded in the ten-year period. The average annual incidence rate was 0.45/100,000 (range: 0.16 - 1.50/100,000). The disease was more prevalent in males (M/F = 16.40 : 1), with the majority of cases reported in August (N = 23; 26.44%), September (N = 20; 23%) and October (N = 15; 17.24%). A statistically significant weak positive correlation was observed between the mean monthly air temperature and the

[1*] Corresponding Author: tatjana.pustahija@izjzv.org.rs

number of human cases of leptospirosis of the same month (r = 0.30, $p < 0.01$), and a statistically significant weak positive correlation was found between the number of human cases and the sum of precipitation in the previous month (r = 0.27, $p < 0.01$). The average annual seroprevalence among domestic animals in total was 1.13% (range: 0.23 - 3.65%). Seropositivity of tested samples of cats, dogs, cattle, donkeys, horses, sheep, pigs and goats was 25%, 9.50%, 8.55%, 6.25%, 1.59%, 0.25%, 0.18%, and 0.00, respectively. Human and animal leptospirosis occurs continuously in Vojvodina, which implies the need for constant and thorough monitoring of the epidemiological and epizootic situation of this disease. Further, more comprehensive parallel studies in humans and animals are needed as well as additional studies of living conditions of animals on farms with leptospirosis, and more extensive studies that will determine the influence of climatic/ environmental factors on the occurrence of leptospirosis in Vojvodina.

Key words: Human leptospirosis, animal leptospirosis, zoonosis, epidemiology, seroprevalence, one health

EPIDEMIOLOŠKE KARAKTERISTIKE LEPTOSPIROZE U VOJVODINI, SRBIJA, U PERIODU 2009-2018 – SA ASPEKTA JEDNOG ZDRAVLJA

Tatjana Pustahija[1], Vladimir Vuković[1], Miljub Ristić[1,2], Sara Savić[3], Živoslav Grgić[3], Smiljana Rajčević[1,2], Mirjana Štrbac[1], Tanja Tomašević[4,5], Snežana Medić[1,2]

[1]Institut za javno zdravlje Vojvodine, Centar za kontrolu i prevenciju bolesti, Novi Sad, Republika Srbija
[2]Univerzitet u Novom Sadu, Medicinski fakultet, Katedra za socijalnu medicinu i zdravstvenu statistiku sa informatikom, Novi Sad, Republika Srbija
[3]Naučni institut za veterinarstvo "Novi Sad", Odeljenje za imunološko-serološka i biohemijska ispitivanja, Novi Sad, Republika Srbija
[4]Institut za javno zdravlje Vojvodine, Centar za analizu, planiranje i organizaciju zdravstvene zaštite, Novi Sad, Republika Srbija
[5]Univerzitet u Novom Sadu, Medicinski fakultet, Katedra za socijalnu medicinu i zdravstvenu statistiku sa informatikom, Novi Sad, Republika Srbija

Kratak sadržaj

Ova studija je imala za cilj da prikaže demografske i hronološke karakteristike humane leptospiroze, kao i prostornu raspodelu ove bolesti

u Autonomnoj Pokrajini Vojvodini, Srbija, u periodu između 2009. i 2018. godine i da ispita moguće veze između pojave bolesti i klimatskih faktora. Pored toga opisuje seroprevalenciju leptospiroze među domaćim životinjama u istom periodu i na istom području. Pirsonov test korelacije je korišćen za određivanje korelacije između različitih meteoroloških faktora i trendova u vremenskim serijama humanih slučajeva leptospiroze. U posmatranom desetogodišnjem periodu ukupno je registrovano 87 slučajeva humane leptospiroze sa pet smrtnih ishoda (Letalitet: 5,75%). Prosečna godišnja stopa incidencije iznosila je 0,45/100.000 (opseg: 0,16 - 1,50/100.000). Bolest je češće registrovana kod osoba muškog pola (M/Ž = 16,40:1), sa najvećim brojem slučajeva prijavljenih u avgustu (N = 23; 26,44%), septembru (N = 20; 23,00%) i oktobru (N = 15; 17,24%). Zabeležena je statistički značajna slaba korelacija pozitivnog smera između srednje mesečne temperature vazduha i broja humanih slučajeva leptospiroze po mesecima (r = 0,30; $p < 0,01$) i statistički značajna slaba korelacija pozitivnog smera između mesečnog broja humanih slučajeva i sume padavina u prethodnom mesecu (r = 0,27; $p < 0,01$). Prosečna godišnja seroprevalencija leptospiroze među domaćim životinjama iznosila je 1,13% (opseg: 0,23% - 3,65%). Seroprevalenca kod mačaka, pasa, goveda, magaraca, konja, ovaca, svinja i koza iznosila je 25%, 9,50%, 8,55%, 6,25%, 1,59%, 0,25%, 0,18%, 0,00, redom. Leptospiroza ljudi i životinja se beleži kontinuirano, što ukazuje na potrebu stalnog i kvalitetnog praćenja epidemiološke i epizootiološke situacije ove bolesti. Potrebno je sprovođenje sveobuhvatnijih uporednih studija karakteristika humane i animalne leptospiroze, kao i dodatnih studija životnih uslova životinja na farmama sa leptospirozom i opsežnijih studija koje će utvrditi uticaj klimatskih i ekoloških faktora na pojavu leptospiroze u Vojvodini.

Ključne reči: Humana leptospiroza, leptospiroza životinja, zoonoze, epidemiologija, seroprevalencija, jedno zdravlje.

INTRODUCTION

Leptospirosis is globally widespread zoonosis with a major impact on public health in all continents except Antarctica (Adler and de la Peña Moctezuma, 2010). The causative agent of leptospirosis is spiral-shaped bacteria belonging to the genus *Leptospira,* family *Spirochetaceae* (WHO, 2003). The disease belongs to the group of natural-focal infectious diseases, with a large number of reservoirs among domestic and wild animals (WHO, 2003). Rodents, especially

rats are important asymptomatic *Leptospira* renal carriers (Levett, 2001). Animal-human transmission commonly occurs either by direct contact with the urine or tissues of infected animals or indirectly, through urine-contaminated environment, by penetrating through damaged skin or mucous membranes of eyes, mouth and nose (Antonijević, 2001; WHO, 2003). It is estimated that human leptospirosis can cause 1.03 million cases and 58,900 deaths each year, globally (Costa et al., 2015). The incidence of human leptospirosis ranges between 0.1 – 1 cases/100,000 inhabitants per year in temperate, non-endemic areas and between 10 – 100 cases/100,000 per year in humid, tropical, endemic areas (Sethi et al., 2010). For all these aspects, the "One Health" approach is of particular importance in solving problems related to zoonoses and achieving better prevention and control of these diseases because it includes multidisciplinary and multisectoral cooperation of different experts and connects humans, animals, plants and their common environment (Rabozzi et al., 2012).

The aim of this paper was to summarize the demographical and chronological characteristics of human leptospirosis and to determine the locations where the disease was identified, as well as to examine possible relationships between the occurrence of the disease and climatic factors in the Autonomous Province of Vojvodina (Vojvodina), Serbia, for the period between 2009 and 2018. We additionally described the seroprevalence of this disease among domestic animals in the same area and the same period.

MATERIAL AND METHODS

Data collection

Surveillance data for human cases were obtained from the Registry of Communicable Diseases, Centre for Disease Control and Prevention, Institute for Public Health of Vojvodina (IPHV), Novi Sad and analyzed retrospectively. The sources of the data were individual case reports, epidemiological surveys and annual reports of the IPHV for the observed ten-year period (2009-2018). For notification of leptospirosis we used a case definition of the European Centre for disease Prevention and control (ECDC, 2018). Veterinary data were obtained from the Scientific Veterinary Institute "Novi Sad" and Veterinary Specialized Institutes Sombor, Zrenjanin, Subotica and Pančevo. Meteorological data were obtained from annual reports of the Republic Hydrometeorological Service of Serbia (RHMS, 2019). These data include the mean annual air temperatures (in degrees Celsius), relative air humidity (in percent), the average annual precipitation (in mm) and numbers of rainy days, provided by

12 measuring stations located in the territory of Vojvodina. Demographic data was downloaded from the Statistical Office of the Republic of Serbia website (RZS, 2014).

Laboratory procedures

Laboratory diagnosis of human leptospirosis was confirmed by microscopic agglutination test (MAT) using the panels of *Leptospira* serovars or by polymerase chain reaction (PCR) at the Scientific Veterinary Institute "Novi Sad", specialized Veterinary Institutes in Subotica, Sombor and Zrenjanin, and by the enzyme-linked immunosorbent assay (ELISA) at the Institute of Public Health of Serbia (only human samples from South Banat district). For MAT, a four-fold increase in antibody titres in paired human serum samples or a single serum with titre ≥ 1:100 in a patient with clinical presentation of leptospirosis were considered as positive. Samples from pigs, cattle, sheep, horses, and goats were examined during the regular monitoring of animal health ordered by the Annual program of measures for each year on the territory of the Republic of Serbia (MAFWM). Dog and cat samples were tested only if animals had leptospirosis-like symptoms in case of travelling abroad to countries requesting such analysis. The detection of antibodies to *Leptospira* in animals was performed using MAT (cut-off 1:100).

Data analysis

Descriptive analysis was used to summarize demographic and chronological characteristics of human cases of leptospirosis, and by district of patient's residence. Annual incidence rate was calculated as the number of human cases per 100,000 individuals at risk for the observed period. Animal seroprevalence was calculated as the percentage of test-positive animals among the total number of tested animals. We used the Pearson's correlation to explore potential correlations between different meteorological factors and trends in time-series of leptospirosis cases. Analyses were performed using Stata, v16 (STATA StataCorp, College Station, TX, USA) and Quantum GIS (QGIS) version 3.4 was used for mapping. A $p < 0.05$ was considered statistically significant.

RESULTS

A total of 87 human cases of leptospirosis were recorded during the observed ten-year period in Vojvodina. The average annual incidence rate was

0.45/100,000 (range from 0.16/100,000 in 2015 and 2018, to 1.50/100,000 in 2014) (Figure 1). All registered cases occurred sporadically. Only one case of human leptospirosis was imported from neighbouring Croatia, while all others were autochthonous.

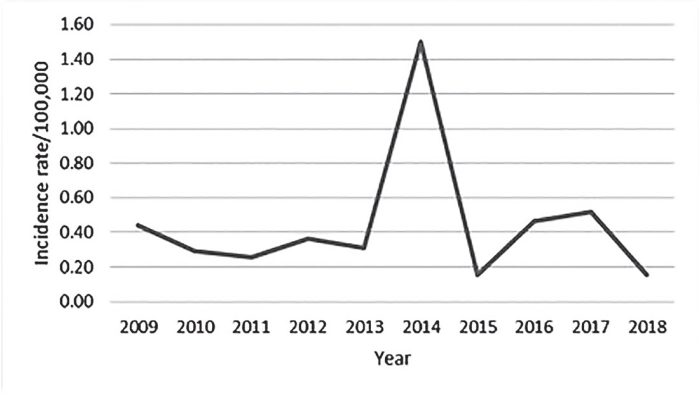


Figure 1. Incidence rate of human leptospirosis in Vojvodina, Serbia, in the period 2009-2018

The disease was more prevalent in males (M/F = 16.40:1). The age distribution of human cases showed an age-specific incidence peak in the age group 40 - 49, followed by age groups ≥ 60 and 50 - 59 years (1.74/100,000; 1.59/100,000 and 1.01/100,000, respectively) (Figure 2). Among males, the highest incidence rate occurred in the age group 40 – 49 (1.74/100,000), while in females the incidence peaked in the 50 – 59 age group (0.12/100,000). There were five deaths related to leptospirosis, giving the overall case fatality rate (CFR) of 5.75%. Deaths occurred more often in males (M/F = 4:1) and in the age group over 60 years (N = 3; 60%).

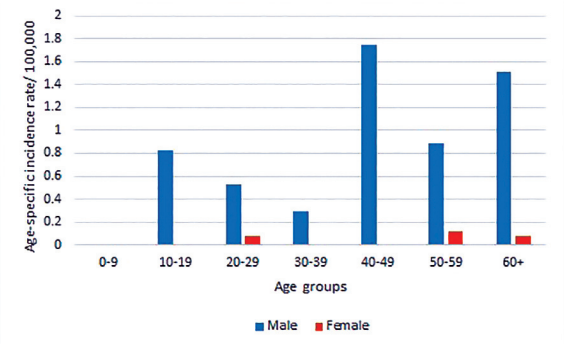


Figure 2. Distribution of human leptospirosis in Vojvodina, Serbia, in the period 2009-2018 by age and gender

Figure 3 reveals the incidence rate of leptospirosis per 100,000 inhabitants across the districts of Vojvodina. Human leptospirosis was registered in all seven Vojvodina districts, with the highest incidence rate in South Bačka (0.88/100,000) and West Bačka (0.74/100,000).

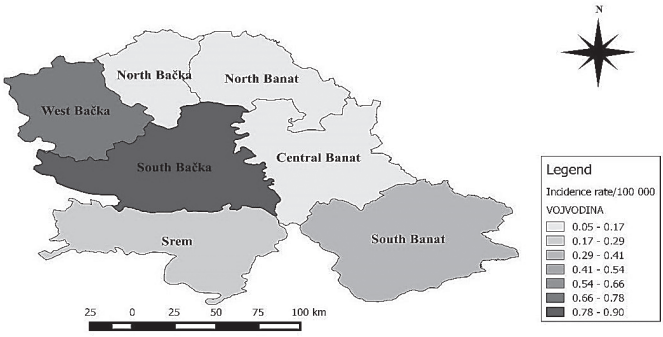


Figure 3. Distribution of human leptospirosis in Vojvodina, Serbia, in the period 2009-2018, by district of residence

The human leptospirosis revealed an extremely seasonal character with majority of cases reported in August (N = 23; 26.44%), September (N = 20; 23%) and October (N = 15; 17.24%). There were no human cases registered in January and February (Figure 4).

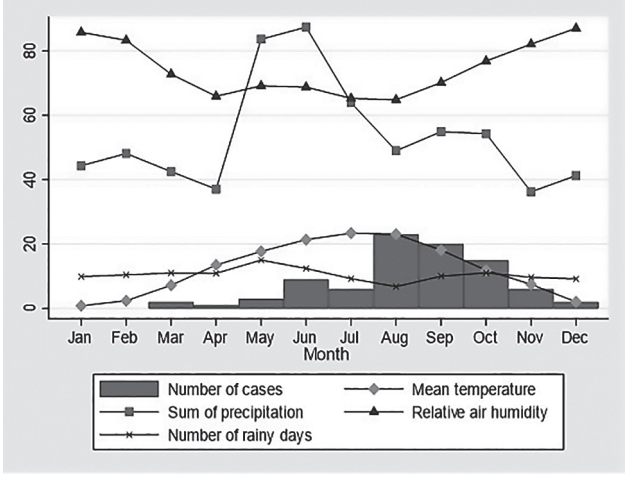


Figure 4. Seasonal distribution of human leptospirosis and the mean air temperature, the relative humidity, the sum of precipitation and the number of rainy days per month in Vojvodina, Serbia, in the period 2009-2018

The correlation of the explored meteorological factors and the seasonality of human leptospirosis is shown in Figures 4 and 5. A statistically significant weak positive correlation was observed between the mean monthly air temperature and the number of human cases of leptospirosis of the same month (r = 0.30, $p < 0.01$). However, no significant correlation was found between the monthly number of human cases of leptospirosis and the sum of precipitation and the relative humidity by the same month (r = 0.11, $p = 0.25$; r = -0.13, $p = 0.17$, respectively). In order to account for a potential lag effect of these meteorological factors, we considered one-month delay and found a statistically significant weak positive correlation between the number of human cases and the sum of precipitation in the previous month (r = 0.27, $p < 0.01$) and a significant weak negative correlation of human cases with relative humidity in the previous month (r = -0.20, $p = 0.02$).

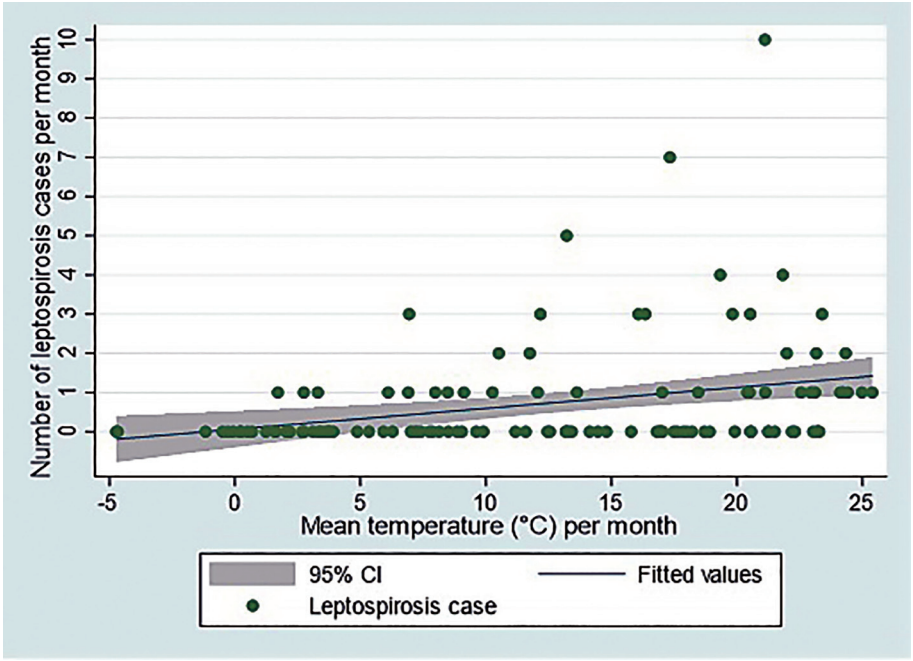


Figure 5. Correlation between the mean monthly temperature and the human cases of leptospirosis by month in Vojvodina, Serbia, for the period 2009-2018

During the observed ten-year period, a total of 220,875 domestic animals were examined. Seroprevalence of leptospirosis varied considerably (Table 1). The average annual seroprevalence was 1.13%, ranging from 0.23% (in 2017) to 3.65% (in 2015). There were no seropositive goats, and the seroprevalence varied from the lowest (0.18%) in pigs to the highest (25%) in cats.

Table 1. Seroprevalence of leptospirosis in domestic animals in Vojvodina, Serbia, in the period 2009-2018

Animal 2009		Years										
		2010	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**Total**	
Pigs	**E**	28,130	23,112	25,189	20,368	15,767	18,675	16,350	14,809	14,599	15,867	192,866
	P	29	32	75	35	11	10	133	9	1	14	349
	%	**0.10**	**0.14**	**0.30**	**0.17**	**0.07**	**0.05**	**0.81**	**0.06**	**0.01**	**0.09**	**0.18**
Cattle	**E**	4,231	2,957	7,251	3,695	749	1,394	1,787	1,238	751	642	24,695
	P	193	675	152	57	47	291	530	92	26	49	2,112
	%	**4.56**	**22.83**	**2.10**	**1.54**	**6.28**	**20.88**	**29.66**	**7.43**	**3.46**	**7.63**	**8.55**
Sheep	**E**	141	10	76	797	99	5	23	6	1	27	1185
	P	1	0	0	0	0	0	0	0	0	2	3
	%	**0.71**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**7.41**	**0.25**
Horses	**E**	183	177	184	86	152	61	52	83	66	86	1130
	P	0	0	2	1	1	0	1	10	0	3	18
	%	**0.00**	**0.00**	**1.09**	**1.16**	**0.66**	**0.00**	**1.92**	**12.05**	**0.00**	**3.49**	**1.59**
Goats	**E**	1	3	3	0	0	408	30	0	1	315	761
	P	0	0	0	0	0	0	0	0	0	0	0
	%	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Dogs	**E**	15	20	22	6	76	9	8	9	25	10	200
	P	2	0	0	1	0	4	1	1	9	1	19
	%	**13.33**	**0.00**	**0.00**	**16.67**	**0.00**	**44.44**	**12.50**	**11.11**	**36.00**	**10.00**	**9.50**
Donkeys	**E**	0	0	0	0	0	6	7	0	3	0	16
	P	0	0	0	0	0	0	1	0	0	0	1
	%	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**14.29**	**0.00**	**0.00**	**0.00**	**6.25**
Cats	**E**	0	0	0	0	0	0	0	1	1	2	4
	P	0	0	0	0	0	0	0	0	0	1	1
	%	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**50.00**	**25.00**
Total	**E**	32,701	26,279	32,725	24,952	16,843	20,558	18,257	16,146	15,447	16,949	22,0857
	P	225	707	229	94	59	305	666	112	36	70	2,503
	%	**0.69**	**2.69**	**0.70**	**0.38**	**0.35**	**1.48**	**3.65**	**0.69**	**0.23**	**0.41**	**1.13**

DISCUSSION

This study analysed the most important epidemiological and epizootiological characteristics of leptospirosis in Vojvodina over a ten-year period. As leptospirosis is an important public health problem, the analysis by "One health"

approach contributes to a better understanding of the disease dynamics. Thus, these findings might be relevant for timely planning and improving prevention and control measures against leptospirosis. To the best of our knowledge, this is the first study that shows the frequency of leptospirosis in humans in parallel to animals at the level of the entire Province, and the first one that analysed the seroprevalence in domestic animals including data from all epizootic areas of Vojvodina.

The average annual incidence of human leptospirosis was 1.6 times lower than that recorded during the previous 10-year period (2000 - 2009) in Vojvodina (Ristić et al., 2010), and 1.7 times lower than the one registered in Serbia in the period 2014 - 2018 (IPHS, 2018). On the other hand, the incidence of human leptospirosis was three times higher compared to incidence registered in the countries of the European Union (0.21/100,000 inhabitants) in the same period (ECDC, 2019). The highest incidence rate of leptospirosis was registered in 2014, when floods hit Serbia and the neighbouring countries. Some studies have already pointed to the importance of heavy seasonal rainfall and floods in the triggering of leptospirosis outbreaks, which is primarily related to sewage spills, which serve as a source of food for rats (Reis et al., 2008; Lau et al., 2010; Allan, 2016). Also, wet soil after floods allows *Leptospira* to survive longer (Desvars et al., 2011).

Similar to the results of several previously published studies (Holk et al., 2000; Christova et al., 2003; Goris et al., 2013) we found that leptospirosis occurs more frequently among males than in females. This could be explained by the fact that males are in higher risk of the disease due to occupational exposure to the source of infection and men are more likely to participate in activities such as fishing and hunting than females. We further found that as many as 80% of all cases of leptospirosis in Vojvodina were registered in age groups over 40, which is similar to the demographic distribution reported in Bulgaria, Croatia and Germany (Christova et al., 2003; Jansen et al., 2005 Topic et al., 2010). Overall CFR was slightly lower than that recorded in the Netherlands and Bulgaria (Christova et al., 2003; Goris et al., 2013), but even four times higher than in neighbouring Croatia (Habus et al., 2017). High CFR is a consequence of underestimation of leptospirosis and recognition of mainly severe forms of the disease (hospitalized patients), in which CFR may exceed 50% (Costa et al., 2015). The distribution of leptospirosis across Vojvodina districts reveals the highest incidence rate in the Districts of South Bačka and West Bačka. A possible explanation for that is the rich hydrographic network of these parts of Vojvodina, which are suitable places for the survival of *Leptospira* (Svircev et al., 2009). Seasonal characteristics of leptospirosis are influenced by the intensity of contact with reservoirs, as well as with contaminated

surface waters and soil (IPHV, 2018). Our results showed a seasonal peak of the disease in the summer and early autumn months (two thirds of cases were registered in August, September and October). A similar pattern has been found in Italy, Bulgaria, Croatia and other countries of the temperate climate zone (Ciceroni et al., 2000; Christova et al., 2003, Habus et al., 2017). We further found a positive correlation between the mean monthly air temperature and the monthly number of leptospirosis cases in Vojvodina. This correlation is mostly driven by frequent human outdoor activities, which include recreational activities (swimming and water sport activities), farming and garden activities during warm months. These activities increase the risk of human contact with contaminated water and reservoirs of infection, primarily rodents. Similar to our results, the positive correlation between human leptospirosis and rainfall in the previous month was established in the study in Martinique (Lhomme et al., 1996). This can be explained by the length of incubation period of the disease and the survival of the *Leptospira* for 1 - 2 months in the moist soil after rainfall.

The average annual seroprevalence of animal leptospirosis in Vojvodina was 1.5 times higher than in the study conducted in South Bačka district (1997 - 2001) (Grgić et al., 2003) and nine times lower than that observed in Croatia in the period 2009-2014 (Habus et al., 2017). Our results revealed a high percentage of seropositivity in cattle, that is, seven to nine times higher than in previous studies conducted in the South Bačka District (Grgić et al., 2005) and in the city of Belgrade (Vojinović et al., 2014a). This could be due to the high population of rodents on cattle farms, but also to the extended Program of animal health protection measures, which also included the analysis of abortions as a new criterion from 2016. Furthermore, the percentage of seropositive horses was noticeably lower than previously demonstrated (Vojinović et al., 2009; Turk et al., 2013; Vera et al., 2020). Although overall 0.18% of pigs tested positive for leptospiral infection, the seropositivity was consistently maintained. This indicates the constant presence of the disease among these animals, as previously reported (Grgić et al. 2002, Vojinović et al., 2014b). There were no goats tested positive for leptospirosis, and seropositivity among sheep was low and registered discontinuously. This result is probably due to the small number of samples compared to cattle and pigs, but also to the fact that sheep and goats are more commonly kept in the open space then objects indoor over the year.

This study has some limitations that should be taken into account when interpreting our results. Firstly, the reported incidence rates of human cases are underestimated due to the use of passive surveillance data. Insufficient recognition and diagnosis of the disease leads to the registration of mainly hos-

pitalized cases. Secondly, the overall number of examined pets (cats and dogs together) is still not high enough to draw out the exact conclusion. There is no program or obligation for testing these animals except if they are travelling abroad to the countries that require such type of analysis. Thirdly, the number of sheep, goat and donkey samples is pretty low compared to that of cattle and pigs, thus, the determined seroprevalence values should be considered with caution. Actually, it can be stated that the higher number of samples analyzed, the more credible information on seroprevalence.

Conclusion

Human and animal leptospirosis continuously occurs in Vojvodina, which implies the need for constant and thorough monitoring of the epidemiological and epizootic situation of this disease.

As the disease is often neglected and underestimated, that is, diagnosed mainly in hospitalized patients, education of physicians at all levels of health care system should be carried out in order to recognize and detect the disease in a timely manner. The presence of leptospirosis on cattle and pig farms should be considered a significant public health concern. Detailed studies of living conditions of animals on farms with leptospirosis have to be done, to establish whether there are other elements, besides rodents, which contribute to the presence of the disease on our farms. In order to develop the best prevention and control strategies against this disease, better surveillance of human leptospirosis and better animal monitoring are essential. Further, more comprehensive parallel studies in humans and animals are needed, as well as additional research to determine the influence of climatic/ environmental factors on the occurrence of leptospirosis in Vojvodina.

ACKNOWLEDGEMENT

We thank all colleagues from veterinary and human medicine who participated in the surveillance and monitoring of human and animal leptospirosis in Vojvodina, Serbia, during last ten years. Our special thanks to Tibor Molnar (Veterinary Specialized Institute Subotica, Serbia), Bratislav Kisin (Veterinary Specialized Institute Sombor, Serbia), Aleksandar Živulj (Veterinary Specialized Institute Pančevo, Serbia) and Vladimir Bursać (Veterinary Specialized Institute Zrenjanin, Serbia) for their invaluable contributions to this study.

Author's Contribution:

PT and VV made contributions to conception and design of the article; PT and VV wrote the manuscript; RM and MS coordinated the work and revised the manuscript; SS and GŽ were involved in the data collection and revised the manuscript. PT and VV prepared the final draft; VV and TT performed statistical analysis, PT, RS and ŠM contributed in results analysis and the way of results presenting.

Competing interest

The authors declare that they have no competing interest.

REFERENCES

1. Adler B., de la Peña Moctezuma A. 2010. Leptospira and leptospirosis. Veterinary Microbiology, 140, 3/4, 287-296. doi: 10.1016/j.vetmic.2009.03.012.
2. Allan KJ. 2016. Leptospirosis in northern Tanzania: investigating the role of rodents and ruminant livestock in a neglected public health problem. Doctoral Thesis, University of Glasgow, Glasgow.
3. Antonijević B. 2001. Zoonoze, Eds. Đerić B.J., Pijanović P., Zavod za udžbenike i nastavna sredstva, Beograd, Serbia, 1st edition, ISBN 978-86-1736-2971.
4. Christova I., Tasseva E., Manev H. 2003. Human leptospirosis in Bulgaria, 1989-2001: epidemiological, clinical, and serological features. Scandinavian Journal of Infectious Disease, 35, 11/12, 869-872. doi: 10.1080/00365540310016709.
5. Ciceroni L., Stepan E., Pinto A., Pizzocaro P., Dettori G., Franzin L., Lupidi R., Mansueto S., Manera A., Ioli A., Marcuccio L., Grillo R., Ciarrocchi S., Cinco M. 2000. Epidemiological trend of human leptospirosis in Italy between 1994 and 1996. European Journal of Epidemiology, 16, 1, 79-86. doi: 10.1023/a:1007658607963.
6. Costa F., Hagan J.E., Calcagno J., Kane M., Torgerson P., Martinez-Silveira M.S., Stein C., Abela-Ridder B., Ko A.I. 2015. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Neglected Tropical Diseases, 9, 9, e0003898. doi: 10.1371/journal.pntd.0003898.
7. Desvars A., Jégo S., Chiroleu F., Bourhy P., Cardinale E., Michault A. 2011. Seasonality of human leptospirosis in Reunion Island (Indian Ocean) and its association with meteorological data. PLoS One, 6, 5, e20377. doi: 10.1371/journal.pone.0020377.

8. European Centre for Disease Prevention and Control (ECDC). 2018. EU Case definition. Available at: https://www.ecdc.europa.eu/en/surveillance-and-disease-data/eu-case-definitions Accessed 09.05.2021.
9. European Centre for Disease Prevention and Control (ECDC). 2019. Surveillance Atlas of Infectious Diseases. Available at: https://atlas.ecdc.europa.eu/public/index.aspx Accessed 05.05.2021.
10. Goris M.G., Boer K.R., Duarte T.A., Kliffen S.J., Hartskeerl R.A. 2013. Human leptospirosis trends, the Netherlands, 1925-2008. Emerging Infectious Diseases, 19, 3, 371-378. doi: 10.3201/eid1903.111260.
11. Grgić Ž., Vidić B., Đuričić B., Savić-Jevđenić S., Stojanov I. 2005. Findings of specific antibodies against Leptospira interrogans in cattle blood sera. Veterinarski Glasnik, 59, 5/6, 611-618. doi: 10.2298/VETGL0506611G.
12. Grgić Ž., Vidić B., Savić-Jevđenić S., Stojanov I. 2002. Swine leptospirosis in Southern Bačka district from 1997 to 2001. Veterinarski glasnik, 56, 3/4, 195-202. doi:10.2298/VETGL0204195G.
13. Grgić Ž., Vidić B., Savić-Jevđenić S., Stojanov I. 2003. Leptospirosis in domestic animals. Savremena Poljoprivreda, 52, 3/4, 459-464.
14. Habus J., Persic Z., Spicic S., Vince S., Stritof Z., Milas Z., Cvetnic Z., Perharic M., Turk N. 2017. New trends in human and animal leptospirosis in Croatia, 2009-2014. Acta Tropica, 168, 1-8. doi:10.1016/j.actatropica.2017.01.002.
15. Holk K., Nielsen S.V., Rønne T. 2000. Human leptospirosis in Denmark 1970-1996: an epidemiological and clinical study. Scandinavian Journal of Infectious Diseases, 32, 5, 533-538. doi: 10.1080/003655400458839.
16. Institute of Public Health of Serbia (IPHS). 2018. Infectious Disease Report in the Republic of Serbia for 2018. Available at: http://www.batut.org.rs/download/izvestaji/GodisnjiIzvestajOZaraznimBolestima2018.pdf Accessed 08.05.2021.
17. Institute of Public Health of Vojvodina (IPHV). 2018. Communicable diseases in AP Vojvodina in 2018-Annual report. Available at: http://izjzv.org.rs/publikacije/ZarazneBolesti/ZB_2018.pdf Accessed 10.05.2021.
18. Jansen A., Schöneberg I., Frank C., Alpers K., Schneider T., Stark K. 2005. Leptospirosis in Germany, 1962-2003. Emerging Infectious Diseases, 11, 7, 1048-1054. doi: 10.3201/eid1107.041172.
19. Lau C.L., Smythe L.D., Craig S.B., Weinstein P. 2010. Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? Transactions of The Royal Society of Tropical Medicine and Hygiene, 104, 10, 631-638. doi: 10.1016/j.trstmh.2010.07.002.
20. Levett PN. 2001. Leptospirosis. Clinical Microbiology Review, 14, 2, 296-326. doi:10.1128/CMR.14.2.296-326.2001.

21. Lhomme V., Grolier-Bois L., Jouannelle J., Elisabeth L. 1996. Leptospirosis in Martinique from 1987 to 1992: results of an epidemiological, clinical and biological study. Médecine et Maladies Infectieuses, 26, 2, 94–98. doi:10.1016/S0399-077X(96)80161-2.
22. Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (MAFWM). Rulebook on determining the Program of animal health protection measures. Available at: http://www.minpolj.gov.rs/dokumenti/pravilnici/ Accessed 15.05.2021.
23. Rabozzi G., Bonizzi L., Crespi E., Somaruga C., Sokooti M., Tabibi R., Vellere F., Brambilla .G., Colosio C. 2012. Emerging zoonoses: the "one health approach". Safety and Health at Work, 3, 1, 77-83. doi: 10.5491/SHAW.2012.3.1.77.
24. Reis R.B., Ribeiro G.S., Felzemburgh R.D., Santana F.S., Mohr S., Melendez A.X., Queiroz A., Santos A.C., Ravines R.R., Tassinari W.S., Carvalho M.S., Reis M.G., Ko A.I. 2008. Impact of environment and social gradient on Leptospira Infection in urban slums. PLoS Neglected Troical Disease, 2, 4, e228. doi: 10.1371/journal.pntd.0000228.
25. Republic Hydrometeorological Service of Serbia (RHMS). 2019. Available at: http://www.hidmet.gov.rs/eng/meteorologija/klimatologija.php Accessed 03.05.2021.
26. Ristić M., Šeguljev Z., Vidić B., Petrović V., Ilić S. 2010. Structure and distribution of leading zoonoses in Vojvodina in 2000-2009 period. Archive of Veterinary Medicine, 3, 1, 63-72. doi:10.46784/e-avm.v3i1.193.
27. Sethi S., Sharma N., Kakkar N., Taneja J., Chatterjee S.S., Banga S.S., Sharma M. 2010. Increasing trends of leptospirosis in northern India: a clinico-epidemiological study. PLoS Neglected Tropical Diseases, 4, 1, e579. doi: 10.1371/journal.pntd.0000579.
28. Statistical Office of Republic of Serbia (RZS). 2014. Census Atlas, 2011. Available at: https://publikacije.stat.gov.rs/G2014/PdfE/G20144012.pdf Accessed 03.05.2021.
29. Svircev Z., Marković S.B., Vukadinov J., Stefan-Mikić S., Ruzić M., Doder R., Fabri M., Canak G., Turkulov V., Stojanović D.B., Draganić M. 2009. Leptospirosis distribution related to freshwater habitats in the Vojvodina region (Republic of Serbia). Science China Life Sciences, 52, 10, 965-971. doi: 10.1007/s11427-009-0124-2.
30. Topic M.B., Habus J., Milas Z., Tosev E.C., Stritof Z., Turk N. 2010. Human leptospirosis in Croatia: current status of epidemiology and clinical characteristics. Transactions of The Royal Society of Tropical Medicine and Hygiene, 104, 3, 202-206. doi: 10.1016/j.trstmh.2009.05.018.

31. Turk N., Milas Z., Habuš J., Štritof Majetić Z., Mojčec Perko V., Barbić Lj., Stevanović V., Perharić M., Starešina, V. 2013. Equine leptospirosis in Croatia - occurrence of subclinical infections and abortions. Veterinarski arhiv, 83, 3, 253-262.
32. Vera E., Taddei S., Cavirani S., Schiavi J., Angelone M., Cabassi C.S., Schiano E., Quintavalla F. 2020. *Leptospira* Seroprevalence in Bardigiano Horses in Northern Italy. Animals, 10, 1, 23. doi:10.3390/ani10010023.
33. Vojinović D., Žutić J., Stanojević S. 2009. Seroprevalence of leptospirosis in horses in the territory of Belgrade during the period from 1998 to 2008. Veterinarski Glasnik, 63, 3/4, 163-169. doi:10.2298/VETGL0904163V.
34. Vojinović D., Jovičić D., Đuričić B., Ilić Ž., Samokovlija A. 2014a. Leptospira infections in cattle at the territory of Belgrade in the period from 2000. to 2010. Veterinarski Glasnik, 68, 1/2, 11-22. doi:10.2298/VETGL1402011V.
35. Vojinović D., Vasić A., Jovičić D., Đuričić B., Ilić Ž. 2014b. Pigs leptospirosis at the territory of Belgrade. In Serbian. Veterinarski Glasnik, 68, 5/6, 331-338. doi:10.2298/VETGL1406331V.
36. World Health Organization. 2003. Human leptospirosis: guidance for diagnosis, surveillance and control. Available at: https://apps.who.int/iris/handle/10665/42667 Accessed 02.05.2021.

Received: 10.06.2021.
Accepted: 22.07.2021.

Original research article
UDK 595.42:[616.98:579.834
https://doi.org/10.46784/eavm.v14i1.269

DETECTION OF *BORRELIA* SPIROCHETES IN TICKS USING q16 REAL-TIME PCR

Marina Žekić Stošić[1*], Snežana Tomanović[2],
Ratko Sukara[2], Smiljana Milošević[3], Sara Savić[1]

[1] Scientific Veterinary Institute „Novi Sad“, Novi Sad, Republic of Serbia
[2] Institute for medical research, University of Belgrade, Belgrade, Republic of Serbia
[3] Veterinary Practice "Basna", Novi Sad Republic of Serbia

Abstract

Lyme disease is a tick-borne disease caused by *Borrelia burgdorferi* sensu lato spirochaetes. It is transmitted by several hard ticks of the *Ixodes* genus, mainly *Ixodes ricinus* in Europe. Higher temperatures caused by climate changes are linked to a heightened activity of ticks for most of the year. Therefore, the awareness of tick-borne diseases is increasing in the region. The aim of this study was to estimate the use of molecular method with Genesig q16 PCR in real time, (Primerdesign Ltd., United Kingdom), as a diagnostic tool for rapid identification of causative agent for Lyme disease in ticks. *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* are the most important causative agents of Lyme disease in this region. With this method a targeted gene is detected and it has been previously discovered that it is a good genetic marker for the three strains of *Borrelia*. A total of 90 ticks were collected after being removed from humans. Every tick collected was identified regarding its species. A total of 79 ticks belonging to the *Ixodes* genus were tested for the presence of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* using real-time PCR assay targeting the recA gene. In total, 8 of them tested positive. Representative samples were tested by conventional PCR and the obtained results were in accordance with the ones obtained by qPCR. This study showed that the Genesig q16 Real-Time PCR is an easy diagnostic test for fast detection of *Borrelia* spirochetes in ticks.

Key words: Lyme disease, tick-borne disease, real-time PCR, *Borrelia*.

[1*] Corresponding Author: marina@niv.ns.ac.rs

UPOTREBA q16 REAL-TIME PCR-a ZA UTVRĐIVANJE PRISUSTVA SPIROHETA *BORRELIA* U KRPELJIMA

Marina Žekić Stošić[1]*, Snežana Tomanović[2],
Ratko Sukara[2], Smiljana Milošević[3], Sara Savić[1]

[1]Naučni institut za veterinarstvo Srbije, Beograd, Republika Srbija
[2]Institut za medicinska istraživanja, Univerzitet u
Beogradu, Beograd, Republika Srbija
[3]Veterinarska ambulanta "Basna", Novi Sad, Republika Srbija

Kratak sadržaj

Lajmsku bolest uzrokuju spirohete *Borrelia burgdorferi s.l*, a prenose krpelji. Nosioci spiroheta su uglavnom tvrdi krpelji iz roda *Ixodes*, i to najčešće *Ixodes ricinus* u Evropi. Klimatske promene i porast spoljašnjih temperature uslovili su i povećanu aktivnost krpelja tokom većeg dela godine, pa se samim tim u regionu povećao značaj bolesti koje se prenose krpeljima. Cilj ove studije bio je da se proceni upotreba molekularne metode pomoću Genesig q16 PCR-a u realnom vremenu (Primerdesign Ltd., Velika Britanija), kao dijagnostičkog alata za brzo otkrivanje uzročnika lajmske bolesti kod krpelja u rutinskoj laboratorijskoj dijagnostici. *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* i *Borrelia afzelii* su najznačajniji uzročnici lajmske bolesti u ovom regionu. Ovom metodom se otkriva ciljni gen, za koji se prethodno pokazalo da je dobar genetski marker za ove tri vrste. Uklanjanjem sa ljudi, prikupljeno je ukupno 90 krpelja. Svaki od krpelja je identifikovan u odnosu na vrstu. Ukupno 79 krpelja, za koje je utvrđeno da pripadaju rodu *Ixodes*, su testirani na prisustvo *Borrelia burgdorferi* s.s, *Borrelia garinii* i *Borrelia afzelii* pomoću PCR testa u realnom vremenu, usmerenog na gen recA. Rezultati testa su pokazali 8 pozitivnih uzoraka. Analiza reprezentativnih uzoraka drugom metodologijom je dala usaglašene rezultate. Ova studija je pokazala da je upotreba Genesig q16 PCR u realnom vremenu jednostavan test za brzo otkrivanje spiroheta Borrelia kod krpelja.

Ključne reči: Lajmska bolest, bolesti prenosive krpeljima, PCR u realnom vremenu, *Borrelia*

INTRODUCTION

Lyme disease is a tick-borne zoonosis, caused by *Borrelia burgdorferi* sensu lato (s.l.) bacteria. Lyme disease mostly occurs in people and dogs, but it affects other animals as well (Potkonjak et al., 2016b). In humans, vector-borne diseases represent more than 17% of all known infectious diseases (WHO, 2017). Since the discovery of the cause of Lyme disease (Steere et al., 1977; Burgdorfer et al., 1982), tick-borne infections are the subject of intensive research all over the world. Lyme disease is the most prevalent arthropod-borne infection in the Northern Hemisphere (Stanek et al. 2012). Hard ticks, mostly belonging to *Ixodes* genus are the main vectors for *Borrelia* spirochetes. *Ixodes ricinus* is a predominant vector of Lyme disease in Europe. Ticks that carry *B. burgdorferi* s.l. in Serbia belong to *I. ricinus* species as well (Savić et al., 2010).

The change of vegetation and current climate changes (particularly mild winters) increase the activity of the tick population. Current climate changes affect regional vector introduction, vector shift to higher latitudes and altitudes and extended annual periods of vector activities (Leschnik, 2020). There is no longer seasonal occurrence of ticks. So far we know that Serbia is an endemic area for a large number of tick-borne infections (Potkonjak et al., 2016a).

Diagnosis of *B. burgdoferi* s.l. in ticks relies on the detection of bacteria in their abdominal gut. In live ticks, detection of spirochetes is possible by dark-field microscopy. Detection of bacteria in dead ticks is not possible when this method is used. Compared to the previous assay, molecular method, such as PCR assay, has higher sensitivity for the detection of causing agent and improves the diagnostics in the laboratory. It can be performed on both live and dead ticks, which is a considerable advantage. With the progress in molecular diagnostics, new species, strains, and genetic variants of microorganisms are detected in ticks worldwide, and the list of potential tick-borne pathogens is increasing (Dantas-Torres et al., 2015). In the past decade, most of the research conducted in Serbia widely used molecular methods for proving the presence of pathogens in ticks (Milutinović et al., 2008; Savić et al., 2010; Tomanović et al., 2010a; Tomanović et al., 2010b; Radulović et al., 2011; Tomanović et al., 2013; Potkonjak et al., 2016a; Potkonjak et al., 2016b; Potkonjak et al., 2017). The screening of DNA samples was performed mostly by conventional PCR assay, targeting specific bp fragment. Five *Borrelia* species are confirmed as pathogens in Europe (*B. burgdorferi* sensu stricto (s.s.), *B. afzeli*, *B. garinii*, *B. spielmani* and *B. bavariensis*). In general, the prevalence of *B. burgdorferi* s.l. in *I. ricinus* ticks from Serbia is 21.1% - 42.5% (Milutinović et al., 2008; Čekanac et al., 2010; Savić et al., 2010; Potkonjak et al., 2016a; Potkonjak et al., 2016b). So far, the following species from ticks have been registered in

Serbia: *B. lusitaniae*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. burgdorferi* s.s. and *B. bavariensis* (Ćakić et al., 2019); *B. burgdorferi* s.s, *B. garinii* and *B. afzelii* are the most significant causative agents of Lyme disease in this region (Milutinović et al. 2008; Savić et al, 2010; Potkonjak et al. 2016).

This study aims to evaluate the use of Genesig q16 Real-Time PCR (designed and launched by Primerdesign Ltd. UK) as a tool for detection of causative agents of Lyme disease in ticks in routine laboratory conditions.

MATERIAL AND METHODS

Sample collection

The ticks were collected from human patients who visited their general practitioner because of a tick bite. A total of 90 ticks were collected. The species of each tick was identified at the Scientific Veterinary Institute "Novi Sad". Seventy-nine ticks were identified as *I. ricinus*. These ticks were analysed for the presence of *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* using q16 real-time PCR method. The collected ticks were stored at -20 °C before the analysis. Before the extraction of DNA, a two-step wash protocol of ticks was performed (using 70% ethanol and distilled water). After that, the samples were processed in TissueLyser LT (Qiagen). Sample disruption with small beads and phosphate buffered saline (PBS) is a crucial step before extraction. This way the bacteria are released from hard tick and the isolation of potential DNA is then possible.

DNA extraction

DNA was isolated from whole tick tissues using the Genesig Easy extraction kit (Primedesign Ltd), by adding metal beads and using a magnetic separator. The DNA extraction is a six-step process that takes 45 min to complete. Sample lyses are stimulated by incubation with lysis buffer and proteinase K. Proteinase K given in the extraction kit is active at room temperature, and when using the incubation it is shorter compared to other protocols of DNA extraction. This step is followed by the addition of binding buffer and Genesig easy magnetic beads. After magnetic separation, the magnetic beads are washed through a three-step process with buffer 1, buffer 2 and 80% ethanol in order to remove contaminants and salts. In the last step, high purity DNA/RNA is eluted with a slightly saline elution buffer. Prepared in this way, the ticks DNA can be used directly in reactions as a sample.

The detection of the presence of a target DNA was done by real-time quantitative PCR (Genesig q16, Primedesign ltd), using Lyme disease genesig® Easy

kit. The kit is designed for the in vitro quantification of "Lyme disease genome". This kit detects a target gene (recA gene) which has previously been proved to be a good genetic marker for all three species (*B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.). The primers and probe sequences in this kit have 100% homology with over 95% of reference sequences in the NCBI databases based on comprehensive bioinformatics analysis.

There are 50 cycles in this qPCR protocol. Every cycle consists of: 2 min of enzyme activation at 95 °C, followed by 10 s of denaturation at 95 °C and 1 min of data collection at 60 °C.

Interpretation of the results

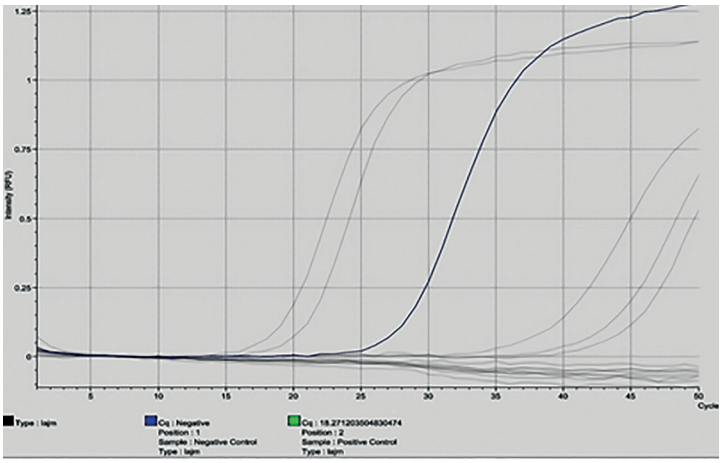
The interpretation of the results was done according to the instructions of the producer (PrimeDesign). Positive control template is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised. Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted. If the sample amplifies > 5 Cq earlier than the negative control, then the sample should be reinterpreted with the negative control verified as negative. If the sample amplifies < 5Cq earlier than the negative control, then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Detection of B. burgdorferi s.l. DNAs in tick samples by conventional PCR

Representative DNA samples prepared from tick tissue were subjected to additional method for detection of *B. burgdorferi* s.l. - *rrf-rrl* rDNA intergenic spacer PCR. Primers corresponding to the 3' end of 5S rDNA (*rrf*) (RIS1; 5'-CTG CGA GTT CGC GGG AGA-3' and RIS3; 5'-GGA GAG TAG GTT ATT GCC AGG-3') and the 5' end of 23S rDNA (*rrl*) (RIS2; 5'-TCC TAG GCA TTC ACC ATA-3' and RIS4; 5'-GAC TCT TAT TAC TTT GAC C-3') were used for first-step (RIS1 and RIS2) and nested-PCR (RIS3 and RIS4) under the previously described PCR conditions (Masuzawa et al., 1996). The quality of extracted DNA was evaluated by PCR targeted to the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes according to a previously described method (Radulović et al., 2010).

RESULTS

The ticks were analysed for the presence of three pathogens *B. burgdorferi* s.l. species (*B. garinii, B. afzelii* and *B. burgdorferi* s.s.). Pathogen *Borellia* DNA was amplified in 8 of 79 ticks (10%).



#	Test	Sample	Status	Copy Number	Cq (Test)	Cq (I.C.)
1	lajm	Negative Control	PASS	n/a	n/a	n/a
2	lajm	Positive Control	PASS	n/a	18.27	19.99
3	lajm	Sample 1	NEGATIVE	n/a	n/a	30.82
4	lajm	Sample 2	NEGATIVE	n/a	n/a	25.60
5	lajm	Sample 3	NEGATIVE	n/a	n/a	25.21
6	lajm	Sample 4	POSITIVE	1	44.68	26.40
7	lajm	Sample 5	NEGATIVE	n/a	n/a	25.51
8	lajm	Sample 6	NEGATIVE	n/a	n/a	25.38
9	lajm	Sample 7	NEGATIVE	n/a	n/a	25.40
10	lajm	Sample 8	NEGATIVE	n/a	n/a	25.92
11	lajm	Sample 9	NEGATIVE	n/a	n/a	25.99
12	lajm	Sample 1B garini	POSITIVE	1	43.12	25.59
13	lajm	Sample 2B ss	POSITIVE	1	39.29	25.99
14	lajm	Sample 3B afzelii	POSITIVE	1,795	27.39	26.05
15	lajm	Sample 4B afzelii	POSITIVE	293,141	20.04	26.20

Figure 1. The results obtained from a run of 14 samples and positive and negative controls with the status of the samples at the end of the run with Cq values.

Five representative samples were subjected to the additional method for *Borrelia* detection and evaluation of the quality of extracted DNA at the Institute for Medical Research, Belgrade University, Serbia. The quality of extracted DNA was evaluated by PCR targeted to the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes (the upper part of figure 2). After that, two positive and three negative samples (representative samples) were tested with conventional nested PCR for the presence of *B burgdorferi* s.l. Both positive and negative results were in accordance with the results of qPCR, which is shown in the lower section of Figure 2.

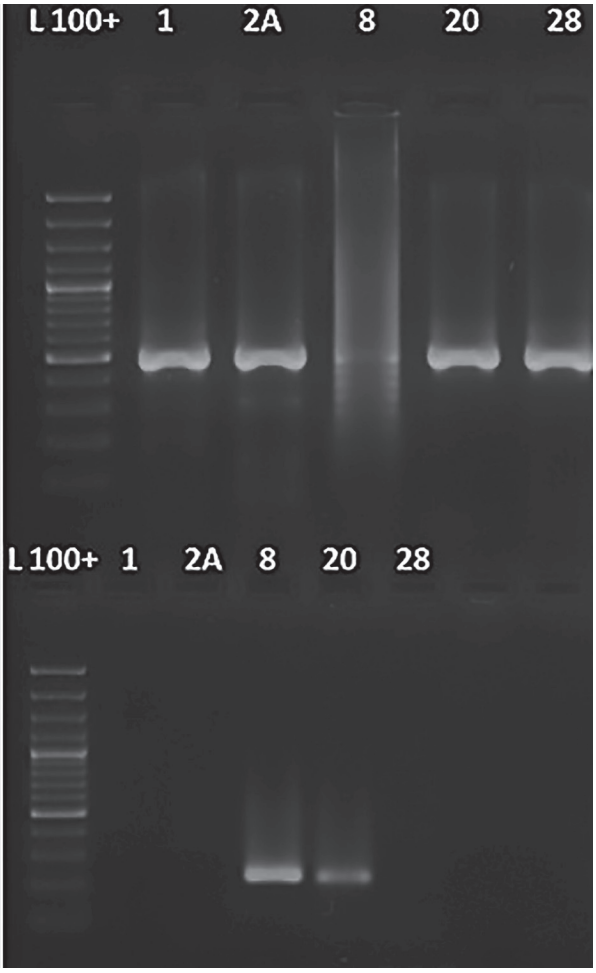


Figure 2. Conventional PCR results

DISCUSSION

Polymerase chain reaction (PCR) technique provides more precise detection of causative agents of many infectious diseases. It is a method with high level of specificity and sensitivity, but it also has some limitations that need to be considered during the diagnostic procedure. It is very important to know what exactly is detected, in which sample and at which stage of infection. For detection of pathogen DNA, PCR method requires much less pathogen to be present in the sample than other detection methods which usually means more positive results than in the cases when other detection methods are used. Even though the sensitivity of PCR method is rather high, cross-contamination, unspecific amplification and false-negative signals are possible to occur. Another flaw of this method is that the enzymes involved in the reaction can be sensitive to inhibitors found in blood, in by-products of blood-derived DNA isolations, and in other body fluids (Zarlenga and Higgins, 2001). In the samples like engorged ticks that have taken a blood meal from a human or other animal host, determination of *B. burgdorferi* infection can be potentially inhibited (Shwartz et al., 1997) and therefore challenging. Before the extraction of DNA, removing all impurities from ticks is a very important step, as well as the proper amount of PBS for the next step /maceration: if the tick is more engorged, then the bigger amount of PBS for preparation of sample is needed. In this study, the quality of extracted DNA was estimated by internal control of Lyme disease genesig® Easy kit, and in representative samples double-checked with conventional PCR targeted to the internal transcribed spacer 2 (ITS2) region of tick-ribosomal DNA genes following the previously described method (Radulović et al., 2010). The extracted DNA in our study was sufficient for reliable detection of Borrelial DNA.

When it comes to the detection of causative agents of Lyme borreliosis by PCR, attention also needs to be paid to test specificity (Aguero-Rosenfeld et al., 2005). The selection of gene targets for amplification has varied. PCR protocols amplified different Borrelial genes, chromosomal such as flagellin gene (Pahl et al., 1999; Ramamoorthi et al., 2005), 23S rRNA (Schwartz et al., 1992), p66 gene (Park et al., 1993), recA gene (Chan et al., 2013), and plasmids-encoded osp genes (Moter et al.,1994; Hovius et al., 2007). The most frequently cited target is the plasmid-encoded ospA, which occurs in multiple copies in each *B. burgdorferi* cell (Dumler, 2001).

Casati and coauthors (2014) successfully analysed 874 *I. ricinus* ticks, by RT-PCR method targeting recA gene. The DNA sequence analysis performed in this study enabled the characterization and identification of *B. burgdorferi*

s.l. strains. Five genospecies were detected: *B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, *B. valaisiana*, and *B. lusitaniae*. RecA gene was reliable and fast for real time-PCR detection of *B. burgdorferi* s.l. species and differentiation of three species of *Borrelia* commonly associated with Lyme disease (*B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*) in one different study as well (Mommert et al., 2001). The variability of this fragment is relatively high (23.5%) compared to other genetic markers. Only the flaA gene has shown a slightly higher variability (26.3%) than recA, but with a longer fragment sequenced (580 bp) (Casati et al., 2004).

All the other quality control criteria also were checked in this study, while performing diagnostic procedure with q16 PCR. According to the manufacturer of the kit, the quality control criteria of reaction were satisfied, which indicates that the analysis was not compromised in any way. Positive control template is expected to be amplified between Cq 16 and 23 and negative control passed as well. There were no inconclusive results. As shown in the Figure 1, internal controls of the samples and positive control were ranging between 25.21 and 26.40. The normal range of internal control according to the manufacture protocol is 25 ± 3 Cq. Anything higher than that indicates some kind of inhibition, which we did not have in our study. One sample did show internal control Cq of 30.82, but after diluting it at 1:10, the sample was re-run. After that, internal control was good, meaning that there was no inhibition.

In our study recA from *B. burgdoferi* s.l. was successfully determined. The sensitivity of PCR can be increased using a nested- PCR procedure, were two rounds of amplifications are performed (Schmidt, 1997). Representative samples in our study were re-run by nested PCR in another laboratory and the obtained results were the same as the ones gained by q16 PCR methodology.

Conclusion

This research showed that Genesig q16 PCR real-time method is a fast and the procedure for detection of *B. burgdorferi* s.l. species with proven pathogenicity in ticks is easy to handle. Since the kit is already labelled for detection of *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s, positive results gained using this kit mean that one of these pathogen of *Borrelia* strain is present. Machine and software are easy to use. It takes a few hours to get the results (once the extraction of DNA starts). Even though it is not possible to distinguish between the *B. burgdorferi* s.l., positive results mean that there is at least one of *B. burgdorferi* s.l. pathogen strain (*B. afzelii*, *B. garinii* and *B. burgdorferi* s.s.) present in the tick.

ACKNOWLEDGEMENT

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia by the Contract of implementation and financing of scientific research work of NIV-NS in 2021, Contract No: 451-03-9/2021-14/200031 from 05/02/2021, and IMI-BG, Contract No: 451-03-9/2021-14/200015.

Author's Contribution:

SS and MŽS made contributions to the idea of the publication, organisation of work and writing the manuscript; MŽS, ST and RS did the laboratory analysis, MS participated in the writing of the manuscript and ST, RS and SS reviewed the manuscript and participated in the final draft of the manuscript.

Competing interest

The authors declare that they have no competing interest.

REFERENCES

1. Aguero-Rosenfeld M.E., Wang G., Schwartz I., Wormser G.P. 2005. Diagnosis of lyme borreliosis. Clinical microbiology reviews, 18, 3, 484–509. doi: 10.1128/CMR.18.3.484-509.2005.
2. Burgdorfer W., Barbour A.G., Hayes S.F., Benach J.L., Grunwaldt E., Davis, J.P. 1982. Lyme disease-a tick-borne spirochetosis? Science, 216, 4552, 1317-1319. doi: 10.1126/science.7043737.
3. Ćakić S., Veinović G., Cerar T., Mihaljica D., Sukara R., Ružić-Sabljić E., Tomanović S. 2019. Diversity of Lyme borreliosis spirochetes isolated from ticks in Serbia. Medical and veterinary entomology, 33, 4, 512-520. doi: 10.1111/mve.12392.
4. Casati S., Bernasconi M.V., Gern L., Piffaretti J.C. 2004. Diversity within *Borrelia burgdorferi* sensu lato genospecies in Switzerland by recA gene sequence. FEMS microbiology letters, 238, 1, 115-123. doi: 10.1111/j.1574-6968.2004.tb09745.x.
5. Čekanac R., Pavlovic N., Gledovic Z., Grgurevic A., Stajkovic N., Lepsanovic Z., Ristanovic E. 2010. Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks in Belgrade area. Vector borne and zoonotic diseases,10, 5, 447-452. doi: 10.1089/vbz.2009.0139.

6. Chan K., Marras S.A.E., Parveen N. 2013. Sensitive multiplex PCR assay to differentiate Lyme spirochetes and emerging pathogens *Anaplasma phagocytophilum* and *Babesia microti*. BMC Microbiology, 13, 295-295. doi: 10.1186/1471-2180-13-295.
7. Dantas-Torres F. 2015. Climate change, biodiversity, ticks and tick-borne diseases: The butterfly effect. International journal for parasitology. Parasites and wildlife, 4, 3, 452–461. doi: 10.1016/j.ijppaw.2015.07.001.
8. Dumler J.S. 2001. Molecular diagnosis of Lyme disease: review and meta-analysis. Molecular diagnosis: a journal devoted to the understanding of human disease through the clinical application of molecular biology, 6, 1, 1–11. doi: 10.1054/modi.2001.21898.
9. Hovius J.W., Li X., Ramamoorthi N., van Dam A.P., Barthold S.W., van der Poll T., Speelman P., Fikrig E. 2007. Coinfection with *Borrelia burgdorferi* sensu stricto and *Borrelia garinii* alters the course of murine Lyme borreliosis. FEMS immunology and medical microbiology, 49, 2, 224–234. doi: 10.1111/j.1574-695X.2006.00177.x.
10. Leschnik M. 2020. Focus on common small animal vector-borne diseases in central and southeastern Europe. Acta Veterinaria-Beograd, 70, 2, 147-169. doi: 10.2478/acve-2020-0011.
11. Masuzawa T., Komikado T., Iwaki A., Suzuki H., Kaneda K., Yanagihara Y. 1996. Characterization of Borrelia sp. isolated from *Ixodes tanuki*, *I. turdus*, and *I. columnae* in Japan by restriction fragment length polymorphism of rrf (5S) – rrl (23S) intergenic spacer amplicons. FEMS Microbiology Letter, 142, 1, 77-83. doi: 10.1111/j.1574-6968.1996.tb08411.x.
12. Milutinović M., Masuzawa T., Tomanović S., Radulović Z., Fukui T., Okamoto Y. 2008. Borrelia burgdorferi sensu lato, *Anaplasma phagocytophilum, Francisella tularensis* and their co-infections in host-seeking *Ixodes ricinus* ticks collected in Serbia. Experimental and Applied Acarology, 45, 3-4, 171-183. doi: 10.1007/s10493-008-9166-6.
13. Moter S.E., Hofmann H., Wallich R., Simon M.M., Kramer M.D. 1994. Detection of *Borrelia burgdorferi* sensu lato in lesional skin of patients with erythema migrans and acrodermatitis chronica atrophicans by ospA-specific PCR. Journal of Clinical Microbiology, 32, 12, 2980-2988. doi:10.1128/jcm.32.12.2980-2988.
14. Pahl A., Kühlbrandt U., Brune K., Röllinghoff M., Gessner A. 1999. Quantitative Detection of *Borrelia burgdorferi* by Real-Time PCR. Journal of Clinical Microbiology, 37, 6, 1958-1963. doi:10.1128/JCM.37.6.1958-1963.1999.
15. Park K.H., Chang W.H., Schwan T.G. 1993. Identification and characterization of Lyme disease spirochetes, *Borrelia burgdorferi* sensu lato, isolated

in Korea. Journal of Clinical Microbiology, 31, 7, 1831-1837. doi: 10.1128/jcm.31.7.1831-1837.1993.
16. Potkonjak A., Gutiérrez R., Savić S., Vračar V., Nachum-Biala Y., Jurišić A., Kleinerman G., Rojas A., Petrović A., Baneth G., Harrus S. 2016a. Molecular detection of emerging tick-borne pathogens in Vojvodina, Serbia. Ticks and tick-borne diseases, 7, 1, 199–203. doi: 10.1016/j.ttbdis.2015.10.007.
17. Potkonjak A., Kleinerman G., Gutiérrez R., Savić S., Vračar V., Nachum-Biala Y., Jurišić A., Rojas A., Petrović A., Ivanović I., Harrus S., Baneth G. 2016b. Occurrence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks with first identification of *Borrelia miyamotoi* in Vojvodina, Serbia. Vector Borne Zoonotic Diseases, 16,10, 631-635. doi: 10.1089/vbz.2016.2008.
18. Potkonjak A., Petrović T., Ristanović E., Lalić I., Vračar V., Savić S., Turkulov V., Čanak G., Milošević V., Vidanović D., Jurišić A., Petrović A., Petrović V. 2017. Molecular Detection and Serological Evidence of Tick-Borne Encephalitis Virus in Serbia. Vector borne and zoonotic diseases, 17, 12, 813–820. doi: 10.1089/vbz.2017.2167.
19. Radulović Ž., Milutinović M., Tomanović S., Mulenga A. 2010. Exon variability of gene encoding glycerol-3-phosphate dehydrogenase of Ixodes ricinus ticks. Parasite, 17, 4, 363-368. doi: 10.1051/parasite/2010174363.
20. Radulović Ž., Chochlakis D., Tomanović S., Milutinović M., Tselentis Y., Psaroulaki A. 2011. First detection of spotted fever group Rickettsiae in ticks in Serbia. Vector borne and zoonotic diseases (Larchmont, N.Y.), 11(2), 111–115. doi: 10.1089/vbz.2009.0254.
21. Ramamoorthi N., Narasimhan S., Pal U., Bao F., Yang X.F., Fish D., Anguita J., Norgard M.V., Kantor F.S., Anderson J., Koski R.A., Fikrig E. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature, 436, 7050, 573–577. doi: 10.1038/nature03812.
22. Savić S., Vidić B., Lazić S., Lako B., Potkonjak A., Lepsanović Z. 2010. *Borrelia burgdorferi* in ticks and dogs in the province of Vojvodina, Serbia. Parasite, 17, 4, 357–361. doi: 10.1051/parasite/2010174357.
23. Schmidt B.L. 1997. PCR in laboratory diagnosis of human *Borrelia burgdorferi* infections. Clinical microbiology reviews, 10, 1, 185–201. doi: 10.1128/CMR.10.1.185.
24. Schwartz I., Wormser G.P., Schwartz J.J., Cooper D., Weissensee P., Gazumyan A., Zimmermann E., Goldberg N.S., Bittker S., Campbell G.L., Pavia C.S. 1992. Diagnosis of early Lyme disease by polymerase chain reaction amplification and culture of skin biopsies from erythema migrans lesions. Journal of clinical microbiology, 30, 12, 3082–3088. doi: 10.1128/jcm.30.12.3082-3088.1992.

25. Stanek G., Wormser G.P., Gray J., Strle F. 2012. Lyme borreliosis. Lancet 379, 9814, 461– 473. doi: 10.1016/S0140-6736(11)60103-7.
26. Steere A.C., Malawista S.E., Snydman D.R., Shope R.E., Andiman W.A., Ross M.R., Steele F.M. 1977. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis and rheumatism, 20, 1, 7–17. doi: 10.1002/art.1780200102.
27. Tomanović S., Radulović Z., Masuzawa T., Milutinović M. 2010a. Coexistence of emerging bacterial pathogens in *Ixodes ricinus* ticks in Serbia. Parasite, 17, 3, 211–217. doi: 10.1051/parasite/2010173211.
28. Tomanović S., Radulović Z., Masuzawa T., Milutinović M., Stanisavljević L. 2010b. Potential infectivity of *Anaplasma phagocytophilum* strains in *Ixodes ricinus* ticks from Serbia. Acta veterinaria Hungarica, 58, 2, 231–242. doi: 10.1556/AVet.58.2010.2.9.
29. Tomanović S., Chochlakis D., Radulović Z., Milutinović M., Cakić S., Mihaljica D., Tselentis Y., Psaroulaki A. 2013. Analysis of pathogen co-occurrence in host-seeking adult hard ticks from Serbia. Experimental and applied acarology, 59, 3, 367–376. doi: 10.1007/s10493-012-9597-y.
30. WHO. 2017. Vector-borne Diseases. World Health Organization, Geneva. Available at: [https://www.who.int/en/news-room/fact-sheet/detail/vector-borne-diseases]. Accessed 10.05.2021.
31. Zarlenga D.S., Higgins J. 2001. PCR as a diagnostic and quantitative technique in veterinary parasitology. Veterinary Parasitology 101, 3-4, 215–230. doi: 10.1016/s0304-4017(01)00568-4.

Received: 22.07.2021.
Accepted: 23.08.2021.

Case Report

UDC: 636.8:616.24-008.4
https://doi.org/10.46784/eavm.v14i1.268

WHAT IS YOUR DIAGNOSIS? DYSPNOEIC CAT

Erdem Gülersoy[1*], Mehmet Burak Ateş[2], Mustafa Yalçin[3], Süleyman Serhat İyigün[4], Zeynep Çelik[2], Burcu Büşra Erol[4], Hasan Güzelbekteş[4]

[1]Harran University, Faculty of Veterinary Medicine, Department of Internal Medicine, Şanlıurfa, Turkey
[2]Selçuk University, Faculty of Veterinary Medicine, Department of Pathology, Konya, Turkey
[3]Selçuk University, Faculty of Veterinary Medicine, Department of Surgery, Konya, Turkey
[4]Selçuk University, Faculty of Veterinary Medicine, Department of Internal Medicine, Konya, Turkey

Abstract

In this report, a 4-year-old male cat which was brought to Selcuk University, Faculty of Veterinary Medicine, Department of Internal Medicine clinics because of increased dyspnoea, anorexia, weight loss, nasal discharge and excessive salivation over a few days is described. In haemotochemical analysis, leukocytosis, hyperkalaemia, low venous partial pressure and saturation of oxygen level along with azotemia and hypocholesterolemia were determined. For further diagnosis, imaging techniques such as ultrasonography, radiography and computed tomography were performed. While no abnormalities were observed in abdominal ultrasonography, severe left mediastinal shift was detected on radiography; heterogeneous, hypodense areas interpreted as necrotic and granulomatous areas on computed tomography were observed. A diffuse granulomatous pneumonia with severe mediastinal shift was diagnosed based on clinical and laboratory analyses. Humane euthanasia was performed at the request of the owner as cat's general condition started to worsen. The diagnosis was confirmed in the necropsy. In addition, fungal cell walls were observed around the necrotic areas and it was determined that the agent is *Cryptococcus*. As a result, the diagnostic differentiation and difficulties of dyspnoeic cats were stated through a case of a cat with respiratory distress due to diffuse granulomatous pneumonia

[1*] Corresponding Author: egulersoy@harran.edu.tr

accompanied by necrotic areas and *Cryptococcus*, and it was concluded that the management protocol which was performed in the present case would be beneficial for clinicians.

Key words: Dyspnea, granulomatous, necrosis, pneumonia, *Cryptococcus*, diagnostic challenge

KOJA JE VAŠA DIJAGNOZA? MAČKA SA DISPNEJOM

Erdem Gülersoy[1*], Mehmet Burak Ateş[2], Mustafa Yalçin[3], Süleyman Serhat İyigün[4], Zeynep Çelik[2], Burcu Büşra Erol[4], Hasan Güzelbekteş[4]

[1]Univerzitet Harran, Fakultet veterinarske medicine,
Departman za internu medicinu, Sanliurfa, Turska
[2]Univerzitet Selcuk, Fakultet veterinarske medicine,
Departman za patologiju, Konja, Turska
[3]Univerzitet Selcuk, Fakultet veterinarske medicine,
Departman za hirurgiju, Konja, Turska
[4]Univerzitet Selcuk, Fakultet veterinarske medicine,
Departman za internu medicinu, Konja, Turska

Kratak sadržaj

U ovom izveštaju opisan se slučaj četvorogodišnjeg mačka koji je donet na kliniku za internu medicinu Fakulteta Veterinarske Medicine Univerziteta Selcuk, zbog pojačane dispneje, gubitka na težini i preterane salivacije koja je trajala nekoliko dana. Hematološkom analizom utvrđena su leukocitoza, hiperkalemija, nizak venski parcijalni pritisak i zasićenost kiseonikom zajedno sa azotemijom i hipoholesterolemijom. Za dalju dijagnozu, sprovedene su tehnike snimanja poput ultrazvuka, radiografije i kompjuterske tomografije. Iako nisu uočene abnormalnosti u ultrazvuku abdomena, na radiografiji je otkriven ozbiljan pomak levog medijastinuma; primećena su heterogena, hipodenska područja interpretirana kao nekrotična i granulomatozna područja na kompjuterizovanoj tomografiji. Na osnovu kliničkih i laboratorijskih analiza dijagnostikovana je difuzna granulomatozna pneumonija sa teškim pomeranjem medijastinuma. Humana eutanazija izvršena je na zahtev vlasnika jer je opšte stanje mačke počelo da se pogoršava. Dijagnoza je potvrđena obdukcijom. Osim toga, uočeni su gljivični ćelijski zidovi

oko nekrotičnih područja i utvrđeno je da je uzročnik *Cryptococcus*. Kao rezultat toga, dijagnostička diferencijacija i problemi kod mačaka sa dispnejom prikazane su na slučaju mačke sa respiratornim poremećajem usled difuzne granulomatozne upale pluća praćene nekrotičnim područjima i kriptokokom, te se može zaključiti da bi protokol dijagnostike koji je izveden u ovom slučaju bio koristan kliničarima.

Ključne reči: dispneja, *granulomatozna*, nekroza, upala pluća, *Cryptococcus*, izazov u postavljaju dijagnoze

CASE PRESENTATION

Anamnestic data

A four-year-old male cat weighing 3.5 kg was brought with severe lethargy and increasing dyspnoea and respiratory distress, nasal discharge, excessive salivation, loss of appetite and weight loss that were persisting for a few days. It was an indoor cat with access to the outdoors and it was fed homemade diet.

In physical examination, severe laboured abdominal breathing, nasal discharge and excessive salivation were established. Mucous membranes were slightly hyperaemic, with neither jaundice nor cyanosis. There was no jugular distension. Gingival capillary refill time was determined to be 2 seconds. Body temperature was 39.2 °C. During palpation, mildly enlarged mandibular lymph nodes were observed and no abdominal mass was detected. On thoracic auscultation, bronchial crackles in the right lung lobe and dysphonia in the left lobe were noted. The cat was initially stabilised with oxygen therapy (10 liters /minute) by oxygen chamber. Venous blood (jugular venepuncture), urine (mid-stream free-flow) and faecal samples (rectal swab) were taken for laboratory analysis. Abdominal ultrasonography, thoracic and abdominal radiography, and thoracic computed tomography (CT) examinations were performed.

Laboratory Results

A complete blood count (using a MS4e®, Melet Schloesing Laboratoires, France), serum biochemistry (using a BT 3000® plus analyser, Biotecnica Instruments SpA, Rome, Italy), and acid/base, blood gas and electrolyte measurements (using an ABL90 Flex® blood gas analyser, Radiometer Medical ApS, Bronshoj, Denmark) were performed. The results are presented in Tables 1 - 3 respectively.

Table 1. Haematologic analysis results

Parameters	Units	Tested Sample	Reference values
White cell count	$x10^9/L$	**62.3**	5.0 - 19.0
Granulocytes*	$x10^9/L$	**24.9**	2.0 - 15.2
Lymphocytes	$x10^9/L$	**37.2**	0.2 - 5.7
Monocytes	$x10^9/L$	**2.2**	0.1 - 1.1
Haemoglobin	g/dL	**16.5**	9.5 - 15.0
Haematocrit	L/L	**0.64**	0.24 - 0.45
Red cell count	$x10^{12}/L$	**13.5**	4.0 - 9.0
Mean corpuscular volume	fl	48	35.5 - 55.0
Mean corpuscular hemoglobin	pg	12	16 – 24
Mean corpuscular hemoglobin concent.	g/dL	26	28 – 40
Red cell distribution width	%	**12.7**	8.0 - 12.0
Platelets	$x10^9/L$	**20**	180 – 500

* Includes neutrophils, eosinophils, basophils and mast cells

Table 2. Biochemistry analysis results

Parameters	Units	Tested sample	Reference values
Urea	mmol/L	**34.5**	5.0 - 12.9
Creatinine	mmol/L	**0.42**	0.05 - 0.21
Aspartate aminotransferase	U/L	**122**	10 - 100
Alanine aminotransferase	U/L	94	10 - 100
Alkaline phosphatase	U/L	**104**	6 - 102
Amylase	U/L	668	100 - 1200
Glucose	mmol/L	8.3	3.6 - 9.4
Magnesium	mmol/L	0.66	0.62 - 1.03
Lactate dehydrogenase	U/L	480	20 - 500
Total bilirubin	umol/L	**9**	2 - 7
Direct bilirubin	umol/L	3	0 - 7
Phosphorus	mmol/L	2.1	0.8 - 2.6

Parameters	Units	Tested sample	Reference values
Cholesterol	mmol/L	**1.7**	1.9 - 5.7
Calcium	mmol/L	2.20	2.05 - 2.69
Triglycerides	mmol/L	1.7	0.3 - 1.8
Gamma-glutamyl transferase	U/L	2	1 - 10
Total Protein	g/L	68	52 - 88
Albumin	g/L	27	25 - 39
Creatine kinase	U/L	**277**	10 - 200

Table 3. Acid/base, blood gas and electrolyte measurement results

Parameters	Units	Tested Sample	Reference values
pH		**7.22**	7.35 - 7.45
pCO2	mm Hg	**27**	40 - 45
pO2	mm Hg	34	30 - 42
O2 saturation	%	**55**	95 - 100
Bicarbonate	mmol/L	**12.7**	19 - 24
Base excess	mmol/L	**-14.8**	-4 - 4
Lactate	mmol/L	1.9	0 - 2
Sodium	mmol/L	164	150 - 165
Chloride	mmol/L	**135**	104 - 128
Potassium	mmol/L	**6.9**	3.4 - 5.6
Calcium	mmol/L	**1.05**	2.0 - 2.7

pH: Power of hydrogen; pCO2: Partial pressure of carbon dioxide; pO2: Partial pressure of oxygen; O2: Oxygen

Feline leukaemia virus (FeLV) antigen, feline immunodeficiency virus (FIV) antibody and feline coronavirus (FCoV) antibody tests (ASAN PHARM® Co. Ltd, South Korea) were performed according to the manufacturer's instructions and were all negative. Urinalysis using a refractometer revealed specific gravity of 1035. The urine was transparent and dark yellow in colour. Urine dipstick (URIT-31®, India) revealed a pH of 6.0, protein 1+, trace amount of glucose and negative bilirubin and ketones. Microscopic examination of urine

sediment revealed low numbers of large polygonal squamous cells, occasional erythrocytes and no casts, leukocytes or crystals. The faecal flotation test was negative for common feline endoparasites such as *Toxocara cati*, *Ancylostoma* spp., *Dipylidium* spp., *Isospora*, and *Giardia*.

Diagnostic Imaging

Abdominal ultrasonography revealed no abnormalities in the morphology and echogenicity of the abdominal organs and no effusion was detected. Ventrodorsal and lateral thoracic radiographs are shown in Figure 1. CT was performed using a Toshiba® Asteion machine (Asteion, Toshiba Medical Systems Corporation, Japan) in helical thoracic scanning mode, using 120 kV, 100 mA and 3 mm section thickness values. Prior to the CT, the cat was sedated with dexmedetomidine (Domitor®, Zoetis) at 40 mcg/kg dosage according to the patient's weight. Representative CT images are shown in Figure 2.

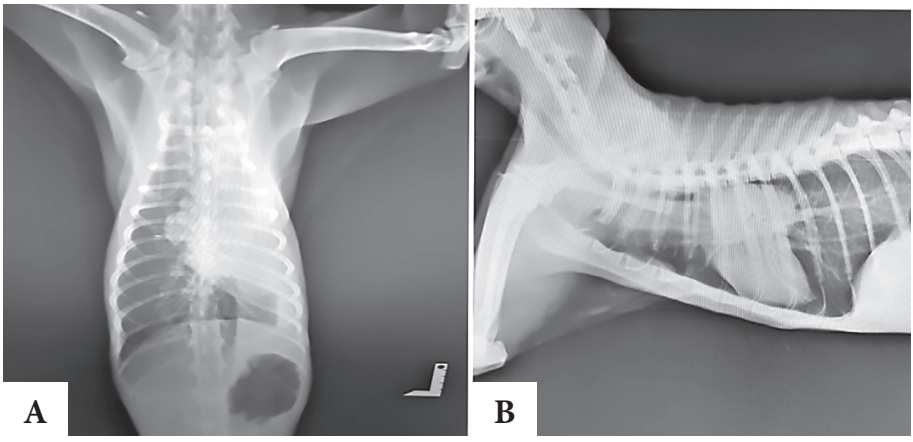


Figure 1. Ventrodorsal and lateral thoracic radiographs of the cat (A and B, respectively).

Dyspnoea is a common finding in cats and is often the only symptom of a thoracic disease. A wide variety of conditions such as viral and bacterial diseases, neoplasms, mycotic infections, tracheal diseases such as collapse and stenosis, cardiac diseases and tracheal diseases such as collapse, stenosis were reported to be causative (Blaxter, 1986; Sharp, 2013). After all the above-mentioned information and applied diagnostic tests and methods, the following questions arise: What is your interpretation of these laboratory tests and imag-

ing results as they relate to this case? What are your differential diagnoses? Are there any further tests that you would perform?

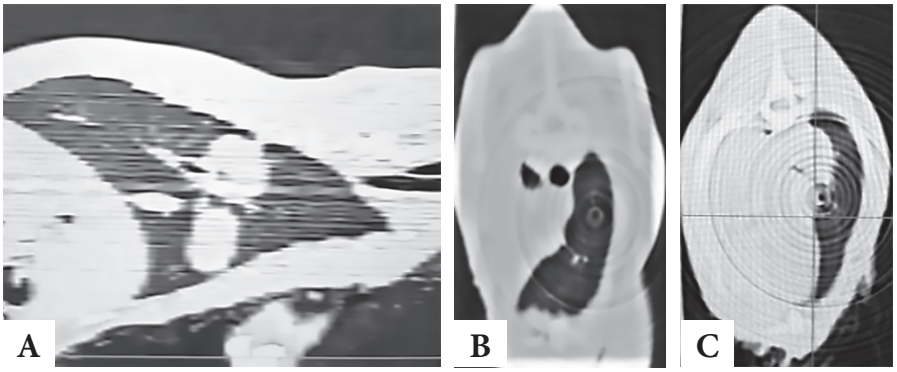


Figure 2. CT images of the cat (A, B and C, respectively)

Clinical Pathology

Polycythaemia with a moderate to severe prerenal azotaemia was noted. These combined findings support the presence of significant dehydration (Pavelski et al., 2018). In the leukogram, an increase in granulocytes, lymphocytes and monocytes were observed. Further interpretation was required such as assessment of a blood smear in order to determine the types of granulocytes, like whether there was a left-shift and to evaluate cell morphology, especially of the lymphocytes. An inflammatory leukogram was highly likely. Therefore, lymphocytes may reflect antigenic stimulation or lymphoid leukaemia (Wang et al., 2014). Thrombocytopenia in the presented case was the result of clumping of thrombocytes (pseudo thrombocytopenia) which is a common issue in cats and might be secondary to blood sampling technique and handling of peripheral blood (Sumner and Rozanski, 2013). The mild increase in creatine kinase and aspartate aminotransferase were likely the result of muscle ischaemia from dehydration and recumbency, while marginal hypocholesterolaemia was probably due to inanition. The importance of the marginal increases in alkaline phosphatase and total bilirubin were moot (Bart et al., 2000). Acid-base results revealed a marked metabolic acidosis (decreased pH and bicarbonate concentration and increased base deficit), with secondary respiratory alkalosis (decreased pCO2). The metabolic acidosis explains the hyperkalae-

mia. It was likely due to dehydration, which also explains the hyperchloraemia (Sumner and Rozanski, 2013). Apart from hypersthenuria, the urinalysis was unremarkable.

Radiographs and CT images

A severe left mediastinal shift, hyperinflation of the right lung lobe, diffuse infiltration of bronchial tissue and a stomach dilated with gas are shown in Figure 1A (ventrodorsal view). In Figure 1B (left lateral view), a radiopaque tumour-like granulomatous structure in the carina is shown. No cardiomegaly was apparent.

A solitary tumour-like structure (red arrow) with mineralized opacity in the left medial lung lobe and carina and heterogeneous hypodense areas (orange arrows) interpreted as necrotic and granulomatous lesions are shown in Figure 2A. Severe mediastinal shift is apparent in Figure 2B. Total consolidation of left lung lobe is shown in Figure 2C (left lung lobe and heart). No pleural, pericardial, or pulmonary effusions were evident.

Differential diagnoses

Based on the pulmonary imaging findings, differential diagnoses considered were focal pneumonia, granulomatous lesions, primary or metastatic lung tumors. The most common causes of pneumonia in cats are bacterial. However, fungal, parasitic and viral pneumonia should also be considered (Blaxter, 1986; Cohn, 2009). For further differential diagnosis, it was decided to take bronchoalveolar lavage fluid (BALF) and fine needle aspiration from the affected lung and to prepare the collected sample for culture. Due to the exacerbated respiratory distress in the patient during imaging examinations, the planned further differential diagnostic methods could not be performed and the treatment phase was initiated immediately.

Treatment and Outcome

The cat was hospitalized and intravenous (IV) fluid therapy (0.9% isotonic saline, with vitamin-amino acid supplements; Duphalyte®, Zoetis, 10 mL/kg), antibiotics (ceftriaxone, 25 mg/kg IV), corticosteroids (prednisolone, 1 mg/kg IV), nebulization (salbutamol, 100 mcg) and oxygen therapy (oxygen chamber, 10 L/min) were administered. Despite the treatment, the general condition of the cat did not improve. Humane euthanasia was performed at the re-

quest of owner after 2 days after admission to the hospital. Cat necropsy was performed.

Pathological Examination

During the necropsy, multiple grey-white granulomatous foci (approximately 1 cm, between 0.75 - 1.55 cm) on the lungs were observed. The bronchial lymph nodes in the middle of both lung lobes were markedly enlarged (approximately 4.5 cm). There was a white-coloured mucopurulent exudate in the trachea and on the left caudal lobe after sectioning. There were large whitish grey areas, which were more severe in the cranial lobe of the right lung, oval-round foci characteristics spreading over large areas in the caudal lobes, without exudate leakage on cross-sectioning. The enlarged lymph nodes were whitish in colour on sectioning with solid consistency. Cardiac examination revealed clotted blood in both ventricles (Figure 3). Although there were minimal and insignificant macroscopic changes in other organs, sampling for histopathology was considered to be useful.

The samples taken from the lung, lymph nodes, heart, brain, intestines, kidney, brain, pancreas and spleen were fixed in 10% formalin solution for histopathology. Hematoxylin-eosin (HE), periodic acid Schiff (PAS), Grocott-Gomori's methenamine silver (GMS) and Ziehl-Neelsen (ZN) stains were used (Luna, 1968). The samples of these tissues were sent for microbiology testing.

Diffuse necrosis in the lungs and typical granuloma structures consisting of a small number of lymphocytes, many plasmacytes and epithelioid histiocytes, fibrocytes, fibroblasts and collagen threads around these necrotic foci were determined during routine HE staining of histopathologic examination. In some parts of the lungs, alveolar structures were completely deficient, and were filled with neutrophil leukocytes, alveolar macrophages and shed epithelial cells. Similar findings were observed in the bronchi and bronchioles. In addition, degeneration of bronchial epithelium and severe hyperplasia and desquamation of glands were detected. Thrombus and severe haemorrhagic leaks were noted in the pulmonary vessels. In addition, pale eosinophilic structures with oval-spheroid structure (approximately 20 μm), especially surrounding the necrotic areas in the lung, were observed.

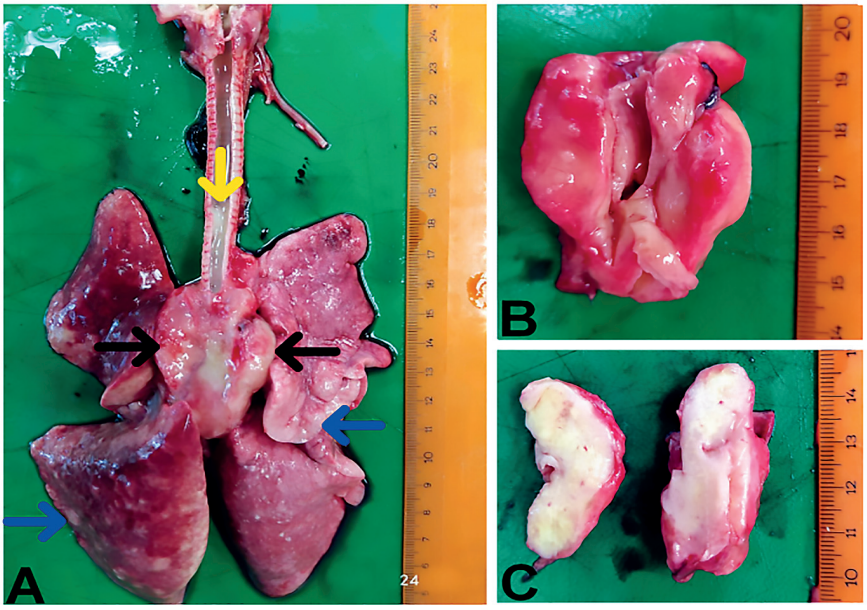


Figure 3. Macroscopic photographs of the lung and bronchial lymph nodes. **A:** General appearance of the lung, excessively enlarged lymph node between the cranial lobes (black arrow), diffuse gray-white foci in the lung (blue arrow), mucopurulent exudate in the trachea (yellow arrow); **B:** Extremely enlarged lymph node; **C:** Sectional face of the lymph nodule.

These structures were determined to be positive using PAS and GMS staining. No acid-resistance bacteria were found in the ZN staining. Granulomatous lesions were observed both in the affected lymph nodes and in the lungs. Degeneration and desquamation in the tubular epithelium of the kidneys, protein-rich fluid in the lumen, plasmahistiocytic cell infiltration in the interstitium and an increase in the fibrous tissue were detected. Congestion in the liver, atrophy of hepatocytes and compensation hypertrophy of the vena centralis were observed. In addition to the increase in the connective tissue of the pancreas, degeneration and desquamation were observed in the duct epithelium (Figure 4). It was reported that no bacterial agent could be isolated in microbiological analyses (48 hours, routine set of culture media was MacConkey agar as the tissue materials were mostly purulent).

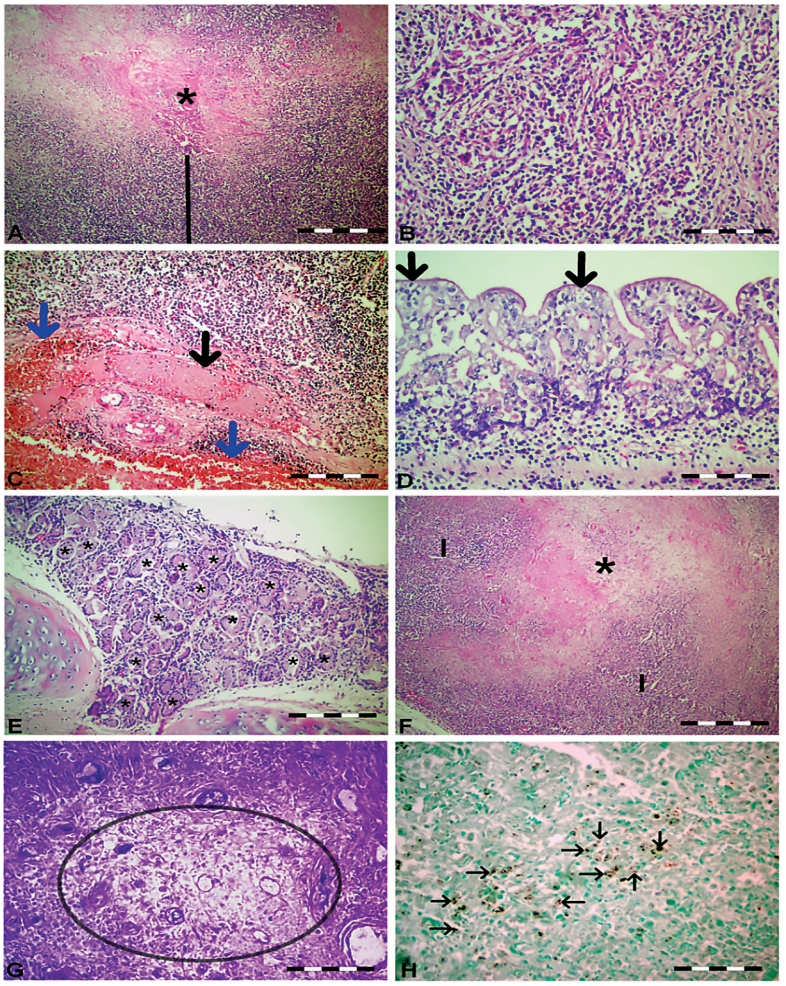


Figure 4. **A:** Granulomatous pneumonia, typical granuloma structure, necrosis (asterisk) in the middle and the surrounding inflammatory cell line (line), HE, Lung, Scale bar: 200 µm; **B:** Inflammatory and connective tissue cells forming the granuloma, HE, Lung, Scale bar: 50 µm; **C:** Thrombosis in vein (black arrow) and bleeding (blue arrow), HE, Lung, Scale bar: 100 µm; **D:** Degeneration in bronchial epithelium (black arrow), HE, Lung, Scale bar: 50 µm; **E:** Severe hyperplasia and desquamation in the bronchial glands (asterisk), HE, Lung, Scale bar: 100 µm, **F:** Granulomatous lymphadenitis, necrosis in the middle (asterisk) and the surrounding inflammatory cell line (line), HE, lymph node, Scale bar: 200 µm; **G:** Pale eosinophilic fungal (Cryptococcosis) structures with oval-spheroid structure around the necrosis areas in the lung, PAS, Lung, Scale bar: 100 µm; **H:** Many positively stained fungal agents, GMS stain, Lung, Scale bar: 100 µm.

DISCUSSION

In the present case, a definitive diagnosis of a diffuse granulomatous pneumonia with severe mediastinal shift caused by pulmonary *Cryptococcosis* was established based on clinical, laboratory and necropsy findings.

The first approach of dyspnoeic cats is to determine the localization of the lesion in the respiratory tract, lung lobe or pleural space and to make differential diagnosis on the basis of the patient's anamnesis, signalment, physical, laboratory and imaging findings. Since the mediastinum is in contact with the facial surfaces of the neck and retroperitoneal space, pneumomediastinum, diaphragmatic hernia, pleural effusion, atelectasis and masses can be detected in the presence of mediastinal shift (Blaxter, 1986; Cohn 2009). Although the exact etiology of this pathological condition is unclear in many cases, it is known to be highly correlated with viral infections such as feline leukaemia and feline immunodeficiency virus (Newman and Schaible, 2019). Studies have reported that fixed shift of mediastinum is associated with mass lesions such as scoliosis, pneumonectomy, atelectasis, pleural operations, effusion or granulomatous structures (Pavelski et al., 2018). In our case, histopathological examination confirmed that mediastinal shift detected in radiographic examination was associated with diffuse granulomatous lesions.

As stated by various researchers (Bart et al., 2000; Cohn, 2009), radiographic examination is one of the most useful diagnostic tools in cats with respiratory distress and should be done with caution in dyspnoeic cats. Lateral and dorsoventral or ventrodorsal imaging is essential for complete evaluation (Elsmo et al., 2018). Radiopaque areas were seen in the left thoracic region on ventrodorsal radiological examination. Radiopaque granulomatous appearance in the mediastinal and carina regions was noted on lateral radiography. Although the use of CT in feline thorax has been rarely described, feline CT anatomy has been reported by Samii et al. (1998). In the present case, unilateral lymph node enlargement and mediastinum invasion were detected in the CT examination. In addition, patterns with different opacity as a result of air loss in the lungs were detected. Hypodense areas refer to necrotic debris. With CT imaging of feline thorax, differential diagnosis of granulomatous lesions, pulmonary abscesses, primary lung tumours, metastatic lung tumours, pneumonia, congenital or parasitic cysts can develop. Necrotic areas in granulomatous lesions are generally observed in a heterogeneous structure on CT images (Foster and Martin, 2011). The structures interpreted in the CT image of our case are the following: thoracic spine, ribs, sternum, muscular thoracic wall, pleural space and diaphragm, respectively. Subsequently, the mediastinum was

evaluated along with the trachea, oesophagus, thymus and the heart. Finally, bronchi and lungs were examined. As a result, heterogeneous necrotic areas were detected within diffuse granulomatous lesions with severe mediastinal shift. With this systematic examination, not only was the obvious pathological condition determined, but the changes that could affect the treatment and prognosis were also observed. In addition to bacterial, viral, fungal and parasitic pneumonia and bronchopneumonia, allergic and idiopathic inflammatory changes in the lung are often manifested as marked changes in density and different distribution patterns of the alveolar space. Specific CT findings of different inflammatory lung diseases have not yet been established in dogs and cats, but some general characteristics have been identified. CT findings often show a tendency to soft tissue opacification, which is evident in fungal pneumonia, and associated perihilar lymphadenopathy (Roden and Schuetz, 2017). In the present case, the lesions detected by CT imaging are similar to the reported findings of fungal pneumonia.

Feline necrotic pneumonia has been reported before (Sharp, 2013). Although cats and dogs with necrohemorrhagic pneumonia associated with extraintestinal *Escherichia coli* and *Bartonella henselae* (Sharp, 2013; Sumner and Rozanski, 2013) are generally evaluated as subclinical, various clinicopathological abnormalities such as anaemia, fever, neurological dysfunction, endocarditis and pyelogranulomatous myocarditis and diaphragmatic myositis may be evident. In addition, Elsmo et al. (2018) reported that necrotic interstitial pneumonia and suppurative myocarditis developed due to *B. henselae* infection in their study on three Florida pumas, diffuse tracheal haemorrhage, thoracic effusion, lung colour change, pulmonary edema, and duodenal haemorrhage and lymphadenomegaly developed in necropsy findings. They also reported that the most important microscopic finding was acute, fibrinonecrotic, and haemorrhagic interstitial pneumonia. In the present case, alveolar collapse with mediastinal shift, diffuse granulomatous structures and surrounding necrotic areas, severe haemorrhage in the lung parenchyma, mucopurulent leakage in the respiratory tract were detected in the necropsy, and the diagnosis of diffuse granulomatous pneumonia with mediastinal shift was confirmed. In addition, fungal agents were observed with GMS and PAS staining of spheroid structures around the necrotic areas.

Fungal infections are generally characterized by granulomatous or pyogranulamatous pneumonia (Sumner and Rozanski, 2013). The lungs are considered the primary site for human *Cryptococcal* infection. Although pulmonary *Cryptococcal* infections have rarely been reported in cats, pulmonary lesions were reported in 29% of cats with radiographic abnormalities and 38%

in necropsy in a previous study (Sura et al., 2007). In the lung histopathology of pulmonary *Cryptococcus* infection, pathogens can be detected within the transparent areas that are not stained around the necrotic lung tissue. These pathogens appear as an oval fungal organism of various sizes, with a shell-like blue-grey or red staining. The necrotic area surrounded by many macrophages with vacuoles and also fibrosis with minor lymphocyte infiltration may be seen. It was reported that in the necrotic areas, with PAS staining eosinophilic, with GMS staining brown-black stained fungal cell walls can be seen (Vogl et al., 2015). In the present case, the histopathological examination findings of necrotic areas in the lung tissue were consistent with the findings of previously reported *Cryptococcal* infections. Roden and Schuetz (2017) reported that this type of infections may be accompanied by necrotized or non-necrotized granulomas. In some cases, the organism may not be detected in GMS or PAS staining and, in these cases, polymerase chain reaction (PCR) or fungal serology can be performed from tissue samples. Lack of BALF sampling, PCR analysis, or fungal serology are the limitations of this clinical report.

Dyspnoeic cats are usually cause emotional distress to their owners. Minimizing patient stress is very important and it can be difficult to apply the appropriate diagnostics to decide the best treatment protocol for the patient. A composed, logical approach that involves clinical observation, laboratory analysis, thoracic radiography and ultrasonography, CT and, where necessary, echocardiographic examinations are vital. In most cases, the protocol that was followed in our case is sufficient - applying empirical treatment, achieving successful stabilization and retrenching the list of diseases to be considered in differential diagnosis.

In this clinical report, the diagnosis of diffuse granulomatous pneumonia, which was established after imaging techniques were performed following by non-specific clinical and laboratory findings, was confirmed in histopathological examination and it was found that it was complicated with fungal pneumonia as a result of positive GMS and PAS staining. The histopathological findings were consistent with *Cryptoccocosis* infection. No bacteria were detected in the culture of tissue samples. Although a systemic examination protocol is important for a successful diagnosis and treatment in dyspnoeic cats, this is very difficult for clinicians. In this presented manuscript, the diagnostic difficulty of dyspnoeic cats was stated over a cat case with respiratory distress due to diffuse granulomatous pneumonia accompanied by necrotic areas and complicated with *Cryptoccocosis* infection. It was concluded that the management protocol which was followed in the present case would be useful for the differential diagnosis and management of dyspnoea in cats for clinicians.

Author's Contribution:

E.G. performed the ultrasonographic examinations and together with H.G., interpreted the haemotochemical results and wrote the manuscript; M.Y. performed radiographic and computed tomography examinations; M.B.A. and Z.Y. performed necropsy and did histopathologic examinations; B.B.E. and S.S.İ. did the examination and collection of the data of the cat; the final version of the manuscript was drafted by E.G. with the assistance of all co-authors who revised the manuscript.

Competing interest

The authors declare that they have no competing interests for a work presented in the manuscript.

REFERENCES

1. Bart M., Guscetti F., Zurbriggen A., Pospischil A., Schiller I. 2000. Feline infectious pneumonia: a short literature review and a retrospective immunohistological study on the involvement of Chlamydia spp. and distemper virus. Veterinary Journal, 159, 220–230. doi:10.1053/tvjl.1999.0451.
2. Blaxter A. 1986. Differential diagnosis of dyspnoea in the cat. In Practice, 8, 225-227. doi:10.1136/inpract.8.6.225.
3. Cohn, L.A. 2009. Pulmonary parenchymal disease. In. Textbook of Veterinary Internal Medicine. Eds. Ettinger S., and Feldman E., Elsevier, Saunders, St. Louis, USA, 7th edition, eBook ISBN: 9781437702828.
4. Elsmo E.J., Fenton H., Cleveland C.A., Shock B., Cunningham M., Howerth E.W., Yabsley M.J. 2018. Necrotizing interstitial pneumonia and suppurative myocarditis associated with Bartonella henselae infection in three Florida pumas. Journal of Veterinary Diagnostic Investigation, 30, 728-732. doi:10.1177/1040638718789226.
5. Foster S. and Martin P. 2011. Lower Respiratory Tract Infections In Cats Reaching beyond empirical therapy. Journal of Feline Medicine and Surgery, 13, 313–332. doi:10.1016/j.jfms.2011.03.009.
6. Luna, L.G. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. Blakiston Division, McGraw-Hill, New York, 3rd edition.
7. Newman R. and Schaible M. 2019. Isolation of Cryptococcus gattii from feline chronic stage lipoid pneumonia. Medical Mycology Case Reports, 25, 19-21. doi:10.1016/j.mmcr.2019.06.002.

8. Pavelski M., Seixas S.V., Warth J.F.G., de Souza C., Dittrich R.L., Froes T.R. 2018. Fungal pneumonia in dogs and cats with pulmonary clinical signs in southern Brazil. Pesquisa Veterinaria Brasileira, 38, 696-702. doi:10.1590/1678-5150-PVB-5066.
9. Roden A.C. and Schuetz A.N. 2017. Histopathology of fungal diseases of the lung. Seminars in Diagnostic Pathology, 34, 530-549. doi:10.1053/j.semdp.2017.06.002.
10. Samii V.F, Biller D.S., Koblik P.D. 1998. Normal cross-sectional anatomy of the feline thorax and abdomen: comparison of computed tomography and cadaver anatomy. Veterinary Radiology and Ultrasound, 39, 504–511. doi:10.1111/j.1740-8261.1998.tb01640.x.
11. Sharp C.R. 2013. Feline lower airway disease: Presentation and diagnosis. Today's Veterinary Practice, 20, 3, 28-31. Available at: https://todaysveterinarypractice.com/diagnosis-of-feline-lower-airway-disease/ Accessed 01.08.2021.
12. Sumner C. and Rozanski E. 2013. Management of Respiratory Emergencies in Small Animals. Veterinary Clinics of North America - Small Animal Practice, 43, 799–815. doi:10.1016/j.cvsm.2013.03.005.
13. Sura R., Van Kruiningen H.J., DebRoy C., Hinckley L.S., Greenberg K.J., Gordon Z., French R.A. 2007. Extraintestinal pathogenic Escherichia coli-induced acute necrotizing pneumonia in cats. Zoonoses Public Health, 54, 307–313. doi:10.1111/j.1863-2378.2007.01067.x.
14. Vogl T.J., Reith W., Rummeny E.J. (Eds.) 2015. Chest and Mediastinum. Diagnostic and Interventional Radiology, 12, 479-587. Springer-Verlag, Berlin, Heidelberg, 2016. doi:10.1007/978-3-662-44037-7_19
15. Wang J., Zhou Q., Cai H., Zhuang Y., Zhang Y., Xin X., Meng F., Wang Y. 2014. Clinicopathological features of pulmonary cryptococcosis with cryptococcal titan cells: a comparative analysis of 27 cases. International Journal of Clinical and Experimental Pathology, 7, 4837-4846. PMID: 25197354.

Received: 26.06.2021.
Accepted: 09.08.2021.

Note for Contributors

ARCHIVES OF VETERINARY MEDICINE (AVM) is a journal of the Scientific Veterinary Institute "Novi Sad" in Novi Sad. The journal publishes original research papers and review articles, short communications and case reports. The journal is devoted to the advancement and dissemination of scientific knowledge concerning veterinary sciences, biotehnology and related academic disciplines. The journal is published in the English language, with an abstract in English and Serbian language. All manuscripts are sent for a review and evaluation. In the case the reviewer suggests additional changes, the manuscript will be sent to the first author with a kind request to change the manuscript. In the case the author does not want to change, appropriate argumentation should be given. Final decision on accepting the manuscript is given by the Editor in Chief or Deputy Editor.

By submitting a manuscript, Authors warrant that their contribution to the Journal is their original work, that it has not been published before, that it is not under consideration for publication elsewhere, and that its publication has been approved by all co-authors, if any, and tacitly or explicitly by the responsible authorities at the institution where the work was carried out. Authors are exclusively responsible for the contents of their submissions, the validity of the experimental results and must make sure they have permission from all involved parties to make the data public. Authors wishing to include figures or text passages that have already been published elsewhere are required to obtain permission from the copyright holder(s) and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the Authors. Authors must make sure that only contributors who have significantly contributed to the submission are listed as authors and, conversely, that all contributors who have significantly contributed to the submission are listed as authors.

Peer review process is managed electronically. Authors have the possibility to provide the contact details of one or two potential peer reviewers, but the final decision on the choice of reviewers is on Editor decision without any obligation to contact any of the Author-recommended peer reviewers. The Editor nominates peer reviewers (at least two). The submitted manuscripts are sent to the reviewers together with the instructions for reviewers and accompanying reviewers' evaluation form. Acceptance of the manuscript is based on the reviews and on the final decisions of the reviewers regarding the acceptability of the manuscript. A reviewer's decision is one of following: "Accepted as it is", "Accepted after minor revision", "Accepted after major compulsatory revision" or "Rejected". If there is marked discrepancy in the decisions between the reviewers, the Editor can send the manuscript to another reviewer for additional comments and further decision, or make the decision based on the existing information. Names of the reviewers are not transmitted to the author (single blind review). The

peer review process is expected, in general, to start within two weeks after the submission of the manuscript. The Corresponding Author must indicate clearly in a separate letter what corrections have been made in response to the reviewers' comments point by point, and giving the relevant new line numbers using *Final show markup* view under Track Changes in MS Word Review ribbon. Corrected manuscripts are expected to be returned within three weeks. Accepted manuscripts are published on **https://niv.ns.ac.rs/e-avm/index.php/e-avm/issue/current** and then selected for a subsequent print issue.

The Journal *Archives of Veterinary Medicine* (AVM) uses open-source software for the management of peer-reviewed academic journals, the *Open Journal System (OJS)*, created by the Public Knowledge Project (PKP), released under the GNU General Public License. Instructions for the submission using OJS 3.0 are available on the Open Journal Systems web page (OJS 3.0 User Guide" © by PKP **https://openjournalsystems.com/ojs-3-user-guide/**).

General notes

The manuscript must be written in clear and concise English, and should be checked by a native-English speaker or certified English instructor with a good understanding of scientific terminology. The complete manuscript must be prepared according to instructions given in this section. Please read all instructions carefully. A manuscript that has not been prepared in accordance with the Journal's instructions for authors will not be considered for review.

All manuscripts submitted to *Archives of Veterinary Medicine*, must be accompanied by a Cover letter (see below). Also, authors are required to submit a scanned copy of the signed original, Competing interests, Authors contribution, and License to Publish which can be downloaded from the Journal homepage (see below).

Approval of the ethics committee must be obtained prior to the start of the study and be available upon request. Authors report this approval in their manuscript in the first paragraph of the Materials and Methods section, including the name of the regulatory group, reference number and date of the approval.
Examples:

Ethical approval: The research related to animal use complied with all the relevant national regulations and institutional policies for the care and use of animals (name of regulatory group, number and date).

Ethical approval: No ethical approval was obtained because this study did not involve laboratory animals and only involved non-invasive procedures (e.g. collection of waste tissue after surgery, fecal samples, voided urine etc.)

Registration and/or Login are required to submit items online and to check the

status of current submissions. Please note that by signing up and submitting your manuscript to *Archives of Veterinary Medicine* you agree to the Journal's terms and conditions. All correspondence, including notification of the Editor's decision and requests for revision, is done by e-mail. The Journal submission system guides you stepwise through the process of entering your article details and uploading your files. Editable files (Microsoft Word, .doc, or .docx format) are required to typeset your article for final publication. The text of the submitted manuscript should be written in good English language. The Editor reserves the right to amend submitted manuscripts to a suitable standard of formal, written English. Manuscripts are pre-evaluated at the Editorial Office in order to check whether they meet the basic publishing requirements and quality standards, and authors will be notified by email upon receiving their submission. Only those contributions which conform to the following instructions can be accepted for peer-review. Otherwise, the manuscripts will be returned to the authors with comments / suggestions.

Online submission (**https://niv.ns.ac.rs/e-avm/index.php/e-avm**)

- If you already have a username and password for Archives of Veterinary Medicine continue to Login Page (**https://niv.ns.ac.rs/e-avm/index.php/e-avm/login**).
- If you are not registered with this site, you need to fill in and submit the form avalable on Registration Page (**https://niv.ns.ac.rs/e-avm/index.php/e-avm/user/register**).

Layout of the manuscript, Style and Form

The paper should be in Word program, Latin characters, size 12 pt., Times New Roman with justified alignment, double spaced. Left and right margins 20 mm, top and foot margins 30 mm, paper size A4. The journal requires continuous line numbering in submitted manuscripts. Number the pages consecutively with the title page being page 1. If special symbols are used, use font Symbol. The manuscript should be submitted on paper size A4 in electronic form. Pagination on the right side, starting from the title page.

The journal publishes the following types of articles: Original research articles, Review articles, Short communications and Case reports.

Original research articles describe the original research, previously unpublished and includes the following sections: Title Page, Abstract with Key words, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgments, References, Tables/Figures (with legends). The writing style should be concise but clear enough to enable readers to reproduce the experimental methodology as described. The article must present a clear hypothesis and data expressed according to an appropriate experimental design. The Original research article should not contain more than 40 references, and usually have no more than 5000 words.

Review articles represent analysed and discussed summaries of different topics in the area of veterinary medicine, biotechnology and related scientific disciplines which are of broad interest to the target audience of *Archives of Veterinary Medicine*. The authors of a Review articles should be experts on the subject of the review, as proven by their own published peer-reviewed papers and by citations of these papers in the submitted review. It is essential that the authors of a review article are cited as authors of at least five other articles, published in Journals from the SCI list, addressing the same subject as the review article submitted. A review contains: Title Page, Abstract with Key words, Main Text, Conclusion, Acknowledgements, References, Tables/Figures/ Illustrations (with legends). The main text in reviews preferably has less than 6000 words.

Short communications represent the concise but complete descriptions of limited investigations, which will not be included in a later full research article. A short communication usually contains the following sections: Title Page, Abstract with Key words, Introduction, Materials and Methods, Results and Discussion, Conclusion, Acknowledgments, References, Tables/Figures/Illustrations (with legends). A short communication should not contain more than 15 references and preferably has less than 3000 words.

Case Reports are reports describing a novel case (or set of cases) with detailed documentation and diagnostic investigation. Authors are encouraged to describe how the subject of the Case report is novel or unusual in the covering letter that will accompany the submission of the Case report. Case reports usually contain the following sections: Title page, Abstract with Key words, Case presentation, Discussion, Acknowledgements, References, Tables/Figures/Illustrations (with Legends). Case reports usually have no more than 2500 words, and should not contain more than 15 references.

Title page

On the title page the following should be written:

- The title of the paper in capital letters, without underlining and abbreviations
- The names of the authors (first name and surname, followed by a comma).
- Above the surname place a number in superscript (1,2,3) that denotes the institution where the author works (affiliation).
- Full name of the institutions should be given below the list of authors (the following order should be considered: university or organization/institution, department, city, and country for all authors).
- One of the authors must be designated as the Corresponding Author (add an asterisk next to the Author's surname). Full contact details including postal address, and e-mail address for the corresponding author must be provided at the bottom of the Title page.

Abstract

An Abstract not exceeding 300 words must be provided with and three to eight key words. It should contain and summarize the most important facts from the submitted manuscript. For Original research articles, Short Communications and Case reports the material and techniques used must be mentioned without going into methodological detail, and the most important findings must be summarized. An Abstract for a Review paper *should contained* background, key findings and/or conclusions. Citations, tables and specialist abbreviations are not included in any Abstract.

Authors from Serbia must also submit an Abstract and Key words in Serbian, including the title of the paper, list of authors and their affiliations on a separate page after References. Translation of the Abstract and Key words from English to Serbian language will be performed by the Editorial Office for Authors who are not fluent in Serbian.

The main Text

All the text headings should be typed in bold capital letters, and the subheadings, where necessary should be in bold italic with only an initial capital letter. Use short and concise sentences. Name the drugs as their International Nonproprietary Names (so called generic names). If the name of a specific drug is to be stressed, name it together with the producer (in brackets). The names of devices write as used in trade (name of the producer in brackets). When using an abbreviation for the first time, write the words that stand for. Abbreviations cannot be used in the title and summary. Text should not be longer than 8 pages. Avoid long enclosures.

Tables and Figures

Tables and Figures (graphs, images, schemes etc.) must not be integrated into the main text. Instead, they must be submitted as separate, supplementary files. Tables and Figures are numbered consecutively using Arabic numerals (Figure 1, Figure 2, Figure 3, Table 1, Table 2, Table 3, *etc.*). Results which can be described as short statements within the text must not be presented as Figures or Tables. Data must not be replicated in Tables and Figures. The titles and legends help make the tables and figures understandable without the reader having to refer to the main text. However, they must also be concise and are not used to re-describe the methodology. Appropriate numbers and titles for tables and numbers and legends (including titles and explanations of markings) for figures are typed single line spaced using Times New Roman, 12 pt, in the main text and placed next to the relevant text in the article to enable the Editors to place them properly.

Tables are submitted as one document, with one table per page. Tables should be submitted as editable text and not as images. Each table is prepared on a separate page

and is appropriately numbered with a short, descriptive table title placed above. Tables can include footnotes, which are placed below the table and explain any abbreviations. Use superscript Arabic numbers (1, 2, 3 etc., in the correct order of reading the table, from top left to bottom right) to link each footnote to the appropriate item in the table requiring explanation.

Each *Figure* (graphs, images, schemes etc.) is submitted as a separate file. Uppercase letters A, B, C, etc. should be used to identify parts of multipart figure. Images must be at resolution 300 dpi or higher and allowable formats – JPG, TIFF. For microphotographs, scale bars with appropriate units must be provided. Symbols, arrows or letters used in photographs should contrast with the background. Legends for figures do not appear in the figures. However, when uploading the figures in the submission procedures, please ensure that the files are appropriately identified as Figure 1, Figure 2, etc. Legends for figures include both the figure number and title and explanations of any symbol, line or marking used in the figure and are placed at the appropriate location within the main text.

Headings

Headings in the paper are: **INTRODUCTION, MATERIAL and METHODS, RESULTS, DISCUSSION (or RESULTS and DISCUSSION for Short Communications), CONCLUSION and REFERENCES**. For Review articles instead M&M, Results and Discussion section, the Main text section should subdivided by different subheadings describing topics presented/discussed/analysed. For Case studies this part could be the same or replaced by Case presentation part with or without different subheadings.

INTRODUCTION points on the most important, i.e. most recent data regarding the topic with a short presentation of the aims of this research. The essence of the problem and the purpose of the study should be pointed to in this section. The key aspects of the published literature and research should be reviewed. A detailed literature survey or a summary of the results should be avoided. The last part of the Introduction must contain the specific aim(s) of the study.

MATERIAL and METHODS. Here describe the conditions in the experiment, name the used methods, material and animals. The Ethical statement (or description why ethical statement is not applicable) should be provided at the beginning of the section. This section includes, as appropriate, a description of study design, experimental animals or data about the samples used, analytical methods and statistical analyses. Identify the methods and procedures in sufficient details to allow others to reproduce the study. If methods are widely known, they are not described, but appropriate references must be cited. For new methods, the detailed protocols for the method should be included. Authors must provide references for established methods including sta-

tistical methods. Specify any general computer program used. Identify all drugs and chemicals used with generic or chemical names, doses and route of administration. For diagnostic kits/reagents and instruments used in the study provide manufacturer, product number, city and country where applicable.

RESULTS. Results are presented in a logical order and in parallel with the Methods (for every Method, there should be a Result), using text descriptions and Tables and/or Figures without duplicating the results between these formats. To enhance clarity, this section can be divided into subsections, each with a concise subheading in italics and which provides details of findings that are required to support the conclusions made in the manuscript.

DISCUSSION. Here give analyses of the obtained results comparing to the results and opinions of other authors, pointing the importance of this research, without giving a conclusion. The Discussion section is not used to summarize current knowledge. The Discussion should clearly identify the main Conclusions of the study. Authors are to provide a clear explanation of the importance and relevance of these Conclusions. Any new information should be distinguished from the previous findings, and relevant hypotheses can be generated.

CONCLUSION. Here the authors gives his final conclusions. This part consists of a short integration of Results that refer directly to the stated aim(s) of the study and a statement on the practical implications of the results. The statements and conclusions not supported by data in the presented research should be avoided. This section should not exceed one short paragraph. Do not summarize the Discussion or current knowledge in this section.

ACKNOWLEDGEMENT. The source of funding for the study should be stated in this section. Also, those who have made a substantial contribution to the study in terms of design, execution, analysis or manuscript drafting/revision but do not fit the criteria for authorship should be mentioned in this section. It is the responsibility of the Authors to ensure that those being acknowledged have agreed to being named in this part.

REFERENCES

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). The author should list the references, preferably the most recent one. Only essential references should be included in the text, and these are restricted to the necessary minimum. Cite in-text references by surname and year in parentheses (e.g. Petrović, 2014). In the case of two authors, use the word "and" (e.g. Petrović and Savić, 2015). The abbreviation "et al." must be used in all cases where more than two authors are cited (e.g. Lazić et al., 2016). Multiple references within parentheses in the text are cited in chronological order (e.g. Petrović, 2014; Petrović and Savić, 2015;

Lazić et al., 2016). In case the same author has more publications in the same year, additional letters are added next to the year (Savić et al., 2008a; Savić et al., 2008b).

In the list of **REFERENCES,** references should be numbered with Arabic numerals, one under the other, written in alphabetical order (use the A-Z sort tool) according to the surname of the first author. In general, the number of references is not limited, but only essential references should be included in the text, and these are restricted to the necessary minimum.

Examples of references:

Articles in journals:

1. Stojanović D., Maličević Ž., Ašanin R. 2002. The use a new model for the investigation of sepsis. Acta Veterinaria, 52, 2/3, 125-131.
2. Chen J. and McClane B.A. 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infection and Immunity, 83, 2369–2381. doi:10.1128/IAI.03136-14.
3. Williams R.B. 2015. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology, 34, 159-180. doi: 10.1080/03079450500112195.
4. Bailey M.A., Macklin K.S., Krehling J.T. 2013. Use of a multiplex PCR for the detection of toxin-encoding genes *netB* and *tpeL* in strains of *Clostridium perfringens.* ISRN Veterinary Science, Article ID 865702, 1-4. doi:10.1155/2013/865702.

Books:

5. Ficken, M. D. and Wages, D. P. 1997. Necrotic enteritis in Diseases of Poultry, Eds. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, Iowa State University Press, Ames, Iowa, USA, 10th edition, ISBN xxx-xxx-xxxxx-xx-x.

Chapters in books:

6. Plumb J.A. and Hanson L.A. 2011. Sturgeon viruses. In *Health maintenance and principal microbial diseases of cultured fishes*. Eds. J.A. Plumb, L.A. Hanson, 3rd edition, Blackwell Publishing, 219-225.

Articles in proceedings:

7. Giangaspero A., Marangi M., Pati S., Cafiero M.A., Camarda C., Sparagano O.A.E. 2011. Investigating the presence of acaricide residues in laying hens naturally infected by the red mite *Dermanyssus gallinae*. In *Book of Abstracts*, The 12th Asian food conference 2011, BITEC Bangna, Bangkok, Thailand, 27.
8. Vidanović D., Petrović T., Šekler M., Debeljak Z., Vasković N., Matović K., Plavšić

B., Dmitrić M. 2018. Avian influenza in Serbia: epidemiological situation during 2016–2017. In *Programme and Abstract book*, 11th International Congress for Veterinary Virology, 12th Annual Meeting of EPIZONE, 27-30.08.2018, University of Veterinary Medicine Vienna, Vienna, Austria, 118 (p187).

9. Lazić G., Lazić S., Bugarski D., Grubač S., Lupulović D., Samojlović M., Petrović T. 2018. Human enteroviruses in river water and sewage in Vojvodina. In *Book of Abstracts*, International Scientific Conference "Green economy and environment protection", Belgrade, 23-25. April 2018, edited by Larisa Jovanović, Belgrade, Naučno stručno društvo za zaštitu životne sredine "ECOLOGICA", 95-96. ISBN 978-86-89061-11-6.

Lows and Regulations:

10. European Union. 2003. Commission Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official Journal of the European Union, L 268:29. **https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2003R1831:20100901:EN:PDF**

Citations with organisations as authors:

11. European Food Safety Authority. 2016. Peer review of the pesticide risk assessment of the active substance benzoic acid. EFSA Journal, 14(12):4657-n/a. **http://dx.doi.org/10.2903/j.efsa.2016.4657.**

Software:

12. Statistica (Data Analysis Software System). 2006. v.7.1., StatSoft, Inc., USA (**www.statsoft.com**).

Web Links:

13. OIE: Animal Diseases. Available at: **http://www.oie.int/en/animal-health-in-the-world/information-on-aquatic-and-terrestrial-animal-diseases/.** Accessed 07.08.2019.
14. European Centre for Disease Prevention and Control (ECDC). Historical data by year - West Nile fever seasonal surveillance. Available at: https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical Accessed 31.07.2019.

Cover letter

All submitted manuscripts must be accompanied by a cover letter which explains why the manuscript is suitable for publication in *Archives of Veterinary Medicine* and declaring that the manuscript has not been submitted or accepted for publication else-

where. Authors should suggest one or two potential unbiased reviewers who are qualified to review the manuscript (reviewers' affiliations with email addresses, and briefly explain why authors think the suggested scientists would be good reviewers). This letter must warrant that all authors have seen and approved the content.

Authors' contributions

In order to give appropriate credit to each author of a paper, the individual contributions of authors to the manuscript are specified by initials in this section. An author is generally considered to be someone who has made significant contribution to a published study. All contributors who do not meet the criteria for authorship should be listed in the Acknowledgements section.

Competing interest

A competing interest exists when your interpretation of data or presentation of information may be influenced by your personal or financial relationship with people or organizations. Authors are required to complete a declaration of competing interests. All competing interests that are declared will be listed at the end of published articles. Where an author gives no competing interests, the listing will read 'The author(s) declare that they have no competing interests'.

Note

A paper that is not in accordance to the aforementioned instructions will not be sent for a review and will be returned to the authors for corrections.

Copyright

Once the manuscript is accepted for publication, authors assign a *License to Publish*. Authors retain copyright of the published papers and grant the publisher the right to publish the article, to be cited as its original publisher in case of reuse, and to distribute it in all forms and media. The final proof will be sent to the Corresponding Author, together with a *License to Publish*, which all authors have to sign. When an issue is completed, a hard copy of the issue will be produced as well as electronic copy on the Journal webpage. If the submitted manuscript is not accepted for publication by the journal, all rights to the manuscript will be retained by the author(s).

Articles published in *Archives of Veterinary Medicine* will be Open-Access articles distributed under a **Creative Commons Attribution International License** (CC BY 4.0).

Address of the journal

Scientific Veterinary Institute "Novi Sad",
21000 Novi Sad, Rumenački put 20
Tel. 381 (0)21 4895 321
E-mail: **arhiv@niv.ns.ac.rs**
Web: **https://niv.ns.ac.rs/e-avm/**
Account No: 355-1006444-18 Vojvođanska banka,
ID No.: 08608857,
PIB 100236555

Privacy Statement

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purpose or to any other party.

List of reviewers:

Sava Lazić, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Tamaš Petrović, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Milica Živkov Baloš, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Dubravka Milanov, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Dr Ljiljana Spalević, Research Fellow	Scientific Veterinary Institute of Serbia, Belgrade (Serbia)
Sara Savić, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Vladimir Polaček, Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Aleksandar Potkonjak, Professor	Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad (Serbia)
Nada Plavša, Professor	Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad (Serbia)
Ljubo Barbić, Professor	Faculty of Veterinary Medicine, University of Zagreb (Croatia)
Ivan Toplak, Assoc. Professor	Faculty of Veterinary Medicine, University of Ljubljana (Slovenia)
Maja Velhner, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Dejan Vidanović, Senior Research Fellow	Veterinary Specialist Institute „Kraljevo", Kraljevo (Serbia)
Dušan Petrić, Professor	Faculty of Agriculture, University of Novi Sad (Serbia)
Vladimir Petrović, Professor	Faculty of Medicine, University of Novi Sad (Serbia)
Neđeljko Karabasil, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Milorad Mirilović, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Jelena Petrović, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Tamara Ilić, Assoc. Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Igor Stojanov, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)

List of reviewers:

Sandra Jakšić, Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Jakov Nišavić, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Ksenija Nešić, Senior Research Fellow	Scientific Veterinary Institute of Serbia, Belgrade (Serbia)
Diana Lupulović, Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Nikolina Novakov, Assoc. Professor	Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad (Serbia)
Jasna Prodanov Radulović,Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Vesna Milićević, Research Fellow	Scientific Veterinary Institute of Serbia, Belgrade (Serbia)
Vladimir Radosavljević, Principal Research Fellow	Scientific Veterinary Institute of Serbia, Belgrade (Serbia)
Gospava Lazić, Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Vladimir Ivović, Assoc. Professor	The Faculty of Mathematics, Natural Science and Information Technologies, University of Primorska (Slovenia)
Dragana Ljubojević Pelić, Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Branislav Kureljušić, Senior Research Fellow	Scientific Veterinary Institute of Serbia, Belgrade (Serbia)
Milanko Šekler, Senior Research Fellow	Veterinary Specialist Institute „Kraljevo", Kraljevo (Serbia)
Sandra Stefan Mikić, Professor	Faculty of Medicine, University of Novi Sad (Serbia)
Ivana Hranjaković Cvjetković, Professor	Faculty of Medicine, University of Novi Sad (Serbia)
Juan Carlos Saiz, Principal Research Fellow	INIA, Madrid (Spain)
Kiril Krstevski, Assoc. Professor	Faculty of Veterinary Medicine, University Ss. Cyril and Methodius, Skopje (North Macedonia)
Vladimir Savić, Principal Research Fellow	Croatian Veterinary Institute, Zagreb (Croatia)
Lorena Jemeršić, Principal Research Fellow	Croatian Veterinary Institute, Zagreb (Croatia)
Aleksandar Mašić, Senior Research Fellow	ZOETIS, Global Bios Research (USA)

List of reviewers:

Alessio Lorusso, Principal Research Fellow	IZSAM, Teramo (Italy)
Dejan Bugarski, Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Tamas Bakonyi, Professor	European Centre for Disease Prevention and Control (ECDC), Solna, (Sweden)
Teufik Goletić, Professor	Faculty of Veterinary Medicine, University of Sarajevo (Bosnia and Herzegovina)
Igor Džadžovski, Assoc. Professor	Faculty of Veterinary Medicine, University Ss. Cyril and Methodius, Skopje (North Macedonia)
João R. Mesquita, Assoc. Professor	Institute of Biomedical Sciences Abel Salazar, University of Porto (Portugal)
Jasna Kureljušić, Research Fellow	Scientific Veterinary Institute of Serbia, Belgrade (Serbia)
Radmila Resanović, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Igor Jajić, Professor	Faculty of Agriculture, University of Novi Sad (Serbia)
Dušan Mišić, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Marija Zgomba, Professor	Faculty of Agriculture, University of Novi Sad (Serbia)
Vera Gusman, Professor	Faculty of Medicine, University of Novi Sad (Serbia)
Neđeljko Karabasil, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Dragan Šefer, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Ljiljana Suvajdžić, Professor	Faculty of Medicine, University of Novi Sad (Serbia)
Abdeljelil Ghram, Research Fellow	Pasteur Institute of Tunis, Department of Veterinary Microbiology (Tunis)
Nadir Alloui, Assist. Professor	Veterinary Department, University of Batna (Algeria)
Dragica Stojanović, Professor	Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad (Serbia)
Mirjana Đukić Stojčić, Professor	Faculty of Agriculture, University of Novi Sad (Serbia)
Ljiljana Janković, Assoc. Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)

List of reviewers:

Stanimir Simin, Assist. Professor	Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad (Serbia)
Jovan Bojkovski, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Nevanka Aleksić, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Marija Lesjak, Assoc. Professor	Faculty of natural science, University of Novi Sad, (Serbia)
Žolt Bečkei, Assist. Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Olivera Bjelić Čabrilo, Professor	Faculty of natural science, University of Novi Sad, (Serbia)
Jelena Nedeljković Trailović, Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Kazimir Matović, Senior Research Fellow	Veterinary Specialist Institute „Kraljevo“, Kraljevo (Serbia)
Adnan Jažić, Professor	Faculty of Veterinary Medicine, University of Sarajevo (Bosnia and Herzegovina)
Goran Marković, Assoc. Professor	Faculty of Agronomy Čačak, University of Kragujevac (serbia)
Marko Stanković, Assist. Professor	Faculty of Agriculture, University of Belgrade (Serbia)
Peter Hostnik, Professor	Faculty of Veterinary Medicine, University of Ljubljana (Slovenia)
Vladimir Kukolj, Assoc. Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)
Bojan Toholj, Professor	Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad (Serbia)
Milan Hadži-Milić, Assist. Professor	Faculty of Veterinary Medicine, University of Belgrade (Serbia)

UDK 619(051) ISSN 1820-9955

Archives of Veterinary Medicine
Arhiv veterinarske medicine

Vol. 14 No. 1 str. 1-132 Novi Sad, 2021.

Scientific Veterinary Institute „Novi Sad" Novi Sad
Naučni institut za veterinarstvo „Novi Sad" Novi Sad