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Tel. 381 (0)21 48 95 321
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IN VITRO STUDY OF THE EFFICACY OF MYCOTOXINS DEGRADATION BY FEED ENZYMES

Sandra Jakšić[1*], Milica Živkov Baloš[1], Nenad Popov[1], Željko Mihaljev[1], Bojan Zloh[2], Vladimir Polaček[1]

[1] Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia
[2] INBERG ltd, Boljevci, Republic of Serbia

Abstract

Providing healthy and safe food in terms of mycotoxicological safety is an imperative for not only good and sustainable livestock production, but also for the population that consumes food of both plant and animal origin. Climate change in the temperate regions of southern Europe has led to frequent occurrence of aflatoxins, deoxynivalenol and zearalenone in cereals. In order to reduce harmful effects of these toxins on animal health as well as to avoid large economic losses, various feed additives are increasingly being used. All of them must first of all be safe, and then have certain efficiency in the fight against mycotoxins. Although *in vivo* experiments are mandatory to assess the efficacy, *in vitro* test offers the advantage of rapid screening of the efficacy of a large number of food additives. In this paper, the efficiency of two commercial products belonging to the enzyme group for animal nutrition was investigated for degradation of aflatoxin B1, zearalenone and deoxynivalenol using *in vitro* experiments. For this purpose, two different methodologies were used according to the recommendation of the enzyme manufacturer. The percentage of mycotoxin degradation was recorded by high pressure liquid chromatography and ELISA methods. One of the tested enzymes showed a very high efficiency in zearalenone degradation being as much as 96%. Both tested enzyme samples showed similar percentage of aflatoxin B1 degradation (about 35%). Deoxynivalenol was not significantly degradable under the applied test conditions.

Key words: aflatoxin, deoxynivalenol, zearalenone, degradation, *in vitro*, enzyme

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

IN VITRO ISPITIVANJE EFIKASNOSTI RAZGRADNJE MIKOTOKSINA POMOĆU ENZIMA ZA ISHRANU ŽIVOTINJA

Sandra Jakšić[1], Milica Živkov Baloš[1], Nenad Popov[1], Željko Mihaljev[1], Bojan Zloh[2], Vladimir Polaček[1]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija
[2] INBERG d.o.o., Boljevci, Republika Srbija

Kratak sadržaj

Obezbeđivanje zdrave i bezbedne hrane u smislu mikotoksikološke bezbednosti imperativ je ne samo dobre i održive stočarske proizvodnje, već i brige o stanovništvu koje konzumira hranu biljnog i životinjskog porekla. Klimatske promene u umerenoj klimi južne Evrope dovele su do česte pojave aflatoksina, deoksinivalenola i zearalenona u žitaricama. U cilju smanjenja štetnog uticaja ovih toksina na zdravlje životinja, kao i izbegavanja velikih ekonomskih gubitaka, sve više se koriste različiti aditivi za hranu. Svi oni moraju pre svega biti bezbedni i imati određenu efikasnost u borbi protiv mikotoksina. Iako su *in vivo* eksperimenti obavezni za procenu efikasnosti, *in vitro* test nudi prednost brzog skrininga efikasnosti velikog broja aditiva za hranu. U ovom radu je ispitivana efikasnost dva komercijalna proizvoda iz grupe enzima za ishranu životinja za razgradnju aflatoksina B1, zearalenona i deoksinivalenola u *in vitro* eksperimentima. U tu svrhu korišćene su dve različite metodologije prema preporuci proizvođača enzima. Procenat razgradnje mikotoksina je izmeren metodama tečne hromatografije visokog pritiska i ELISA metodom. Jedan od testiranih enzima, pokazao je veoma visoku efikasnost u razgradnji zearalenona od čak 96%. Oba ispitana uzorka enzima su pokazala sličan procenat razgradnje aflatoksina B1 (oko 35%). Deoksinivalenol nije bio značajno razgradiv u primenjenim uslovima ispitivanja.

Ključne reči: aflatoksin, deoksinivalenol, zearalenon, razgradnja, *in vitro*, enzim

INTRODUCTION

Enzymes are considered biological catalysts; they are proteins capable of accelerating the speed of chemical reactions, which are essential for the proper cellular functioning of all living beings. Their use has shown benefits in the food industry, while in animal feed could improve the consistency and nutritional value of feed, increase digestibility, animal performance and reduce the effect of antinutrients. (Velázquez-De Lucio et al., 2021). The purpose of adding enzymes in animal feed is to improve food efficiency, production, and consequently to reduce the cost of feeding (Bedford 2018). Enzymes can be obtained from animals, plants or microorganisms. The development of recombinant DNA technology has allowed for the isolation and expression of genes of some microorganisms, and production of enzymes for animal feed (Sarder et al., 2005). Enzymes used in the production of animal feed are considered zootechnical additives in the Republic of Serbia, their use is allowed by the Rulebook on the quality of animal feed (Official Gazette of the Republic of Serbia, 2010).

Mycotoxins are a numerous group of secondary metabolic products of fungi or molds that pose a serious risk to human and animal health. Mycotoxin contamination is widespread in animal feed, especially in cereals (Wu et al., 2015). Currently, more than 300 mycotoxins have been identified, and scientific attention has been focused mainly on mycotoxins that have been shown to be carcinogenic and/or toxic to human and animal health. Aflatoxins (AF), zearalenone (ZEA) and deoxynivalenol (DON) are of great public health concern due to their high prevalence, teratogenic, carcinogenic, mutagenic and immunosuppressive effects (Oueslati et al., 2012). Increased amounts of mycotoxins in animal feed can result in huge economic losses on an annual basis, including declining livestock production as well as increasing human and animal mortality (Zain, 2011). An eight-year study based on the determination of mycotoxins (AF, ZEA, DON, fumonisin and ochratoxin A) in animal feed and feed materials worldwide showed that 72% of the samples were positive for at least one mycotoxin, and 38% is simultaneously contaminated with multiple mycotoxins (Streit et al., 2013).

Prevention of mycotoxin contamination begins in the field. However, when speaking of the protection of cereals from mold and mycotoxins during storage, further measures are necessary. Different treatments (physical, chemical and biological) are used for this purpose (Stoev, 2013; Jevtić et al., 2021). Mycotoxin adsorbents are often used as feed additives (Nešić et al., 2020). Although mycotoxins are stable compounds, research on their degradation is

very topical today. Effective degradation of mycotoxins must provide irreversible degradation of mycotoxins to less toxic or non-toxic products. One of the frequently studied techniques is the biological detoxification of mycotoxins, using microorganisms and/or enzymes to degrade mycotoxins into non-toxic or less toxic compounds (Taylor and Draughon, 2001). The advantage of mycotoxin degradation using enzymes is the simplicity of the process, without the potential risk of contamination and operator safety compared to the use of live microorganisms (Loi et al., 2017). The main conversion paths are hydroxylation, hydrogenation, hydrolysis, oxidation, esterification, glucuronidation and glucosidation, de-epoxidation, methylation, sulfation, demethylation and deamination (Nešić et al., 2021). The following enzymes are used for this purpose: oxidase, peroxidase, laccase, reductase (AF), carboxylesterase and aminotransferase (fumonisins), glucosyltransferase (trichotecenes), laccase, lactonohydrolase (ZEA), lypase, protease (ochratoxin) (Loi et al., 2017). Europen Union (EU, 2014; 2017; 2018) and European Food Safety Authority (EFSA, 2020) established the regulations regarding fumonisin esterase (the enzyme that degrade fumonisins) as a feed additive for animal species in accordance with rules for additives for use in animal nutrition (EU, 2003).

Climate change in the temperate climate of southern Europe has led to frequent occurrences of AF, DON and ZEA in cereals and in Serbia. In order to reduce the harmful effects of these toxins on animal health, as well as to avoid large economic losses, various feed additives are increasingly being used. All of them must first of all be safe, and then have a certain efficiency in the fight against mycotoxins. Although *in vivo* experiments are mandatory to assessing the efficacy, the advantage of the *in vitro* test is its capacity to rapidly screen the efficacy of a large number of food additives. In this way, the reduction of mycotoxin toxicity is indirectly confirmed. In this work, the degradation efficiency of AFB1, ZEA and DON was investigated *in vitro* using two commercial products belonging to the enzyme feed additives.

MATERIAL AND METHODS

Chemicals and enzymes

Two samples of different feed enzymes were provided by INBERG ltd (Belgrade, Republic of Serbia). The tested enzymes are intended for use as feed additives in order to reduce the harmful effects of mycotoxins.

Standard substances were used for degradation tests: AFB1 Cat No A6636, ZEA Cat No Z2125, and DON Cat No D0156. All standards were purchased from "Sigma Aldrich", Saint Louis, USA.

In vitro experiments

For the purpose of in vitro testing of the possibility of mycotoxin degradation by enzymes, two different methodologies were used. The efficiency of sample No. 1 was tested according to the manufacturer's recommendation applying the methodology No. 1. Its efficiency for the degradation of AFB1, ZEA and DON was examined. The efficiency of sample No. 2 for the degradation of the same mycotoxins was tested according to the manufacturer's recommendation and using the methodology No. 2.

The first methodology involved incubation of mycotoxin standards and enzyme in phosphate buffer solution (0.1M PBS, pH 6.5). The test solution for ZEA degradation assay consisted of 1980 µL (1900 µL) of buffer (with appropriate enzyme weighing in suspension), in which 20 µL (100 µL) of ZEA standard solution (100 µg/mL) was added. For testing the degradation of DON 100 µL of standard solution (100 µg/mL in a mixture of ethyl acetate and methanol) was evaporated and reconstituted in 2000 µL buffer (with appropriate weighing of enzymes in suspension). The test solution for AFB1 degradation assay consisted of 1980 µL of buffer (with appropriate enzyme weighing in suspension), in which 20 µL of standard solution of AFB1 (10 µg/mL) was added. All tested solutions were incubated with shaking for 4 h at 37 °C and then centrifuged, filtered, and mycotoxins were determined by liquid chromatography.

The second methodology included incubation of mycotoxin standards and enzyme on LB medium 20 mL (in 1l: peptone 10 g, NaCl 10 g, 5 g yeast extract, adjusted to pH 7.4, autoclaved for 20 min), at 37 °C for 24 h (0.1 µg/mL AFB1 and 1 µg/mL ZEA) or 1 h (2.5 µg/mL DON). After stopping the reaction, purification was done by solid phase extraction. Purification of samples after treatment on LB medium was performed by using MycoSep 224 AflaZon, and MycoSep 225 TrichMultifunc columns (RomerLabs, USA).

In both cases, the experiment consisted of a toxin and enzyme assay and a control assay with toxins only. The degradation efficiency expressed with standard deviation (STD) is the result of measurement in three replications.

Mycotoxins analysis

After *in vitro* tests, the efficiency of the tested enzymes for mycotoxins degradation was evaluated. The percentage of degradation was recorded by quantitative measurement of residual mycotoxin in the supernatant by using optimized and validated methods. In the case of ZEA and AFB1, there high pressure liquid chromatography with fluorescence detection (HPLC-FLD) was

used, while HPLC-DAD and ELISA were applied for DON.

HPLC Dionex UltiMate 3000 Series system with FLD 3100 and DAD detector (Thermo Scientific, Germany) was used for quantitative measurement of mycotoxins in the solution before and after adsorption. The system was controlled with Chromeleon®7 software (Thermo Scientific, Germany).

For the determination of AFB1 Supelcosil column, 250 x 4.6 mm, 5μm, was used for separation with mobile phase 50% ACN and flow rate 1.2 mL/min. Fluorescence detection was done on λex 365 nm, and λem 435 nm. For ZEA determination Hypersil Gold aQ column, 150 x 3 mm, 3 μm, (Thermo Scientific, Germany), with mobile phase 60% ACN, and flow rate 1 mL/min was used, while for detection the wavelengths λex 275 nm and λem 455 nm were set. The method for the determination of DON used the same column as for ZEA, mobile phase 10% ACN, flow rate 1 mL/min, and detection was done at λ 220 nm.

Determination of the percentage of toxin degradation

After HPLC determination, the peak areas of the determined mycotoxins in the test samples were compared with the corresponding areas of control samples without added enzyme. The percentage of adsorption was calculated by using the equation:

$$\% \text{ degradation} = (1 - PI / P0) \times 100\%$$

Where: PI = peak area of toxins after incubation with enzyme; P0 = surface area of toxin peaks in control solution without enzyme addition.

RESULTS

The tested enzymes are commercially available as feed additives. Their use is based on the effect of reducing the harmful effects of mycotoxins, that is, they degrade mycotoxins in the conditions of the digestive tract of animals. There were no available data on their activity and efficiency, the exact composition and origin. The only available information was about the conditions under which those enzymes work, i.e., the pH values, in which medium, and at what mycotoxins concentrations. These data were used for *in vitro* testing thereof.

Because of different testing conditions as well as different concentrations of mycotoxins and enzymes, the results for each enzyme were presented separately.

Efficiency of mycotoxin degradation by enzyme No. 1

The results of the study of the influence of enzyme number 1 on the degradation of ZEA are shown in Table 1. It can be seen that increasing the mass of the enzyme increases the degradation efficiency to a significant 93%. Given such a high efficiency with a high amount of enzyme, and in order to optimize the ratio of enzyme and ZEA, the possibility of degradation of a larger amount of ZEA was tested (Table 2). As can be seen, a 96% degradation of ZEA was achieved at a toxin: enzyme ratio of 1 μg: 1.8 mg.

Table 1. Results of testing the degradation efficiency of 2 μg ZEA by using enzyme No. 1.

	Enzyme (mg)	ZEA degraded (%)	STD (%)
TEST 1	5	49.4	4.5
TEST 2	10	66.1	5.5
TEST 3	100	93.6	0.6

Table 2. Results of testing the degradation efficiency of 10 μg ZEA and 10 μg DON using enzyme No. 1.

	Enzyme (mg)	ZEA degraded ± STD (%)	DON degraded (%)
TEST 1	1.8	53 ± 2	2.7
TEST 2	18	96 ± 0.2	6.4

The efficacy of the same enzyme for AFB1 degradation was examined with three different enzyme amounts, and the results showed maximum efficiency of 34.8% (Table 3).

Table 3. Results of testing the degradation efficiency of 0.2 μg AFB1 by using enzyme No. 1.

	Enzyme (mg)	AFB1 degraded (%)
TEST 1	3.27	0
TEST 2	6.53	4.5
TEST 3	32.67	34.8

Efficiency of mycotoxin degradation by enzyme No. 2.

Testing the efficacy of enzyme sample No. 2 was examined by incubating the enzyme and toxin in a nutritionally rich medium Lysogeny Broth (LB). The degradation efficiency of ZEA alone and ZEA and AFB1 in the mixture was examined. The results are shown in Figure 1. There was no difference in the efficiency of ZEA degradation in case where ZEA alone was present in the reaction mixture (13 ± 6%) as compared to the mixture with AFB1 (11%).

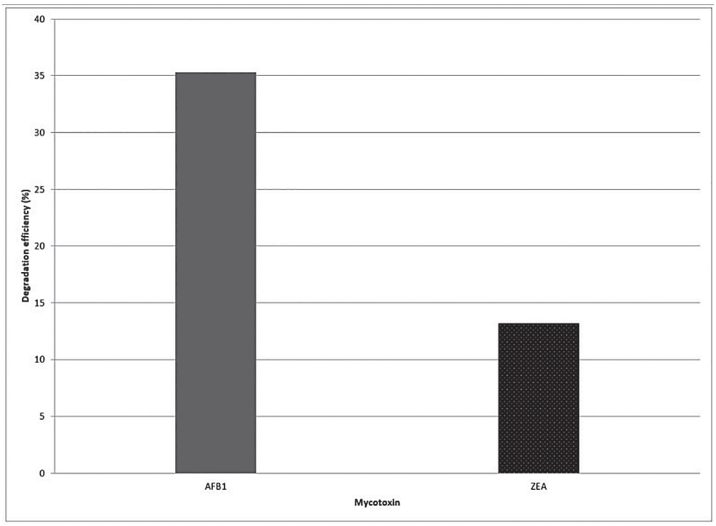

Figure 1. Results of testing the degradation efficiency of 20 μg ZEA and 2 μg AFB1 by using enzyme No. 2. (40 mg).

The enzyme degradation assay of DON was performed in the same medium, but with a shorter incubation time (1 h) and a higher amount of enzyme (200 mg) in comparison to ZEA and AFB1. Due to the low concentration of DON the determination by HPLC-DAD method was not possible, so semi-quantitative determination of toxin in the control and solution with enzyme was determined by ELISA. As it can be seen from Figure 2, there is no significant difference in the colour of the cells with the control and test samples. These results reveal no significant difference in DON concentration between control and test samples after enzyme degradation. Enzyme No. 2 has no effect on the degradation of DON. Poor degradation of DON is expected, given its chemical nature (small pollar moiety) and literature data on difficulties in the development of agents for their irreversible detoxification (Nešić et al., 2021).

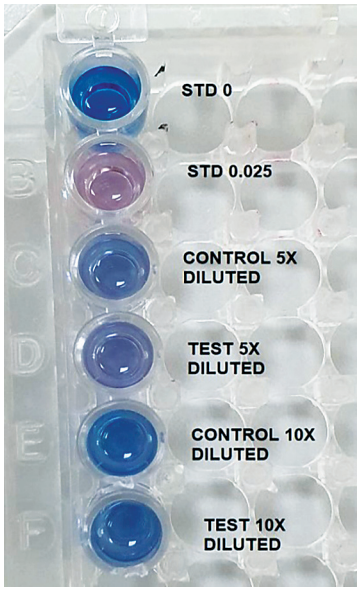

Figure 2. Determination of DON after treatment 50 µg with enzyme No. 2 (200 mg) 1 h at 37 °C.

DISCUSSION

A modern and economically justified approach to the fight against mycotoxins also involves the use of additives that enzymatically lead to their degradation. Before using these supplements, it is necessary to confirm their activity or efficiency for the decomposition of mycotoxins. Although regulations in the EU on enzymes as additives against mycotoxins are available, national regulations in Serbia and other countries do not include requirements pertaining to the quality and degradation efficiency of enzymes used against mycotoxins in animal feed. Also, there are no unique methodologies for analyzing enzyme efficiency. In *in vitro* tests, it is important to define the conditions under which these experiments are performed. Experimental conditions should mimic the biotransformation of toxins in the animal's body. The levels of toxin and enzyme to be tested in *in vitro* reaction system are also important. A range of various experiments to check the activity of enzymes for the degradation of mycotoxins is described in the literature. Two different methodologies, with different concentrations of toxins and enzymes, were used in this study. Therefore, it is difficult to compare the obtained results with each other. The conclusions of experiments on the efficacy of enzymes should be stated with reference to the

conditions under which they were obtained. According to the available literature, effective degradation of AFB1 and ZEA (86% and 100% respectively) is achieved by laccase, using redox mediators, while under the same conditions the degradation of DON was not possible (Loi et al., 2018). A high percentage of AFB1 degradation (90 – 100%) using the enzymes peroxidase and oxidase, while ZEA reduction was achieved by using lactono hydrolase (100%) has also been reported in the literature (Loi et al., 2017).

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

The result of this research is the confirmation of a quality of the enzyme that has been proven to be highly efficient for the decomposition of ZEA, a toxin often present in cereals and responsible for reproductive disorders in livestock production. Based on the results obtained in *in vitro* studies, the optimal ratio of enzyme and ZEA was obtained, which gives maximum efficiency in application.

Control of enzymes, similar to adsorbents used as feed additives, before mixing into complete mixtures is necessary, and conscientious producers are aware of the cost-effectiveness of such approach and conscious production strategies. The significance of ensuring the health of animals and humans by using proven components of animal feed to prevent the occurrence of mycotoxicosis and mycotoxin-contaminated food is an imperative in the production of safe food.

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Author's Contribution:

SJ -conducted the experiment, design of the study, and paper concept and writing, MZB- revising the manuscript critically, NP- experiment performance, ZM-conducted the experiment, BZ-initial idea and providing material, VP-experiment organization.

Competing interest

The authors declare that they have no competing interests.

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Review article

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THE MOST COMMON INTENTIONAL POISONING OF DOGS AND CATS ON THE TERRITORY OF THE REPUBLIC OF SERBIA

Jelena Aleksić Radojković[1*], Ivana Davidov[2],
Aleksandra Aleksić Agelidis[1], Jelena Vranešević[3], Saša Ivanović[1]

[1] Faculty of Veterinary Medicine, University of Belgrade, Republic of Serbia
[2] Department of Veterinary Medicine, Faculty of Agriculture,
University of Novi Sad, Republic of Serbia
[3] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia

Abstract

The paper presents the most common toxic substances used in malicious poisoning of dogs and cats in the territory of the Republic of Serbia, mechanisms of their action, symptoms that occur in poisoned animals, antidote therapy and in the case of death, pathomorphological changes. The understanding of the mechanisms of toxic action of the most common substances used and the clinical symptoms in poisoned dogs and cats contribute to a faster diagnosis and the prompt suitable therapy application. The participation of forensic veterinarians in official procedures prior to criminal proceedings is necessary, considering its importance in the recognition and prosecution of acts defined in Article 269 of the Criminal Code (Official Gazette of RS, No. 85/2005, 88/2005 - amended, 107/2005 - amended, 72/2009, 111/2009, 121/2012, 104/2013, 108/2014, 94/2016 and 35/2019).

Key words: animal poisoning, poisons, forensic veterinarian, crime

[1*] Corresponding Author: alexjellena@yahoo.com

NAJĆEŠĆA NAMERNA TROVANJA PASA I MAČAKA NA TERITORIJI REPUBLIKE SRBIJE

Jelena Aleksić Radojković[1], Ivana Davidov[2],
Aleksandra Aleksić Agelidis[1], Jelena Vranešević[3], Saša Ivanović[1]

[1] Fakultet veterinarske medicine, Univerzitet u Beogradu, Beograd, Republika Srbija
[2] Departman za veterinarsku medicinu, Poljoprivredni fakultet,
Univerzitet u Novom Sadu, Novi Sad, Republika Srbija
[3] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija

Kratak sadržaj

U radu su prikazane toksične supstance koje se najčešće koriste prilikom zlonamernog trovanja pasa i mačaka na teritoriji Republike Srbije, mehanizmi njihovog delovanja, simptomi koji se javljaju kod otrovanih životinja, antidot terapije, a u slučaju uginuća i patomorfološke promene. Poznavanje mehanizama toksičnog dejstva najčešće korišćenih supstanci i karakteristike kliničkih simptoma kod otrovanih pasa i mačaka doprineće bržoj dijagnostici i blagovremenom sprovođenju adekvatne terapije. Učešće veterinara forenzičara u službenim radnjama koje prethode krivičnom postupku je neophodno, uzimajući u obzir njegov značaj u otkrivanju i procesiranju dela definisanih članom 269. Krivičnog zakonika (Službeni glasnik RS, br. 85/2005, 88/2005 - ispr., 107/2005 - ispr., 72/2009, 111/2009, 121/2012, 104/2013, 108/2014, 94/2016 i 35/2019).

Ključne reči: trovanje životinja, otrovi, veterinar forenzičar, krivično delo.

INTRODUCTION

Chemical injuries (*Laesio valetudinis violenta chemica*) or animal poisoning are a global worldwide problem (Wang et al., 2007; Ladislav et al., 2011). They have become an increasingly frequent occurrence on the territory of the Republic of Serbia. Toxins are substances that, depending on the concentration, amount and manner of reaching the body, lead to various toxic effects (Aleksić and Aleksić, 2019). Regarding the intentions of poisoning, they can be unintentional (accidental) and intentional (murderous). Accidental animal poisonings have been documented worldwide (Berny et al., 2010; Guitart et al.,

2010a, Guitart et al., 2010b), they cannot be prevented and their percentage is low compared to intentional poisoning (Giorgi et al., 2007; Berny et al., 2010). Intentional poisoning means the abuse of toxic substances and malicious intent of the perpetrator, but also an act of revenge against a certain animal or its owner or keeper (Merck, 2007).

The Animal Welfare Law, among other things, prohibits the use of poisons and other chemical agents that cause pain, suffering and death of animals, except for the purpose of controlling rodent populations, i.e. rodent control and animal testing for scientific research purposes (Animal Welfare Law, Article 7, Official Gazette No. 41/2009). Along with the criminal offense defined in Article 269 of the Criminal Code (Official Gazette of RS, No. 85/2005, 88/2005 - corrigendum, 107/2005 - corrigendum, 72/2009, 111/2009, 121/2012, 104/2013, 108/2014, 94/2016 and 35/2019), the cases of intentional poisoning of animals often take account of criminal offense against the protection of the general public stated in the Article 278 as well. According to the provisions of the Criminal Code, any person that causes danger to a human life by means of fire, flood, explosion, poison or poisonous gas, radioactive or other ionizing radiation, electricity, motor force or any other dangerous action that has a potential to endanger human life or habitat, will be prosecuted. In addition to a fine, a prison sentence of six months to five years is imposed for this crime. If the crime was committed in a place where a large number of people gather (e.g., in a park, on the street, in a square), and there are signs of a more severe form of poisoning, then a stricter prison sentence is prescribed, from one to eight years, along with a fine.

In most cases, according to the clinical course, intentional poisonings are peracute or acute, so in order to respond in a timely manner and implement appropriate therapy, it is important that veterinarians have information on the most commonly used types of poisons used by perpetrators. According on our case law, perpetrators have most commonly been using anticoagulant rodenticides, organophosphate and carbamate insecticides, creosote and molluscicides (metaldehyde) in the recent years.

The prevalence of poisoning is higher in dogs compared to cat poisoning, which has been established in the countries of the European Union: Belgium, France, Greece, Italy, Spain, Austria (Berny et al., 2010; Wang et al., 2007). Dogs account for about 75% of cases, and cats for about 15% of reported cases of intentional poisoning (Gwaltney-Brant, 2012). The higher incidence of dog poisoning is understandable given their nonselective eating habits compared to cats (Medeiros et al., 2009).

During a six-year period (2006-2012), the Department of Veterinary Forensic Medicine and Legislations of the Faculty of Veterinary Medicine in Belgrade autopsied 48 corpses of dogs with suspected poisoning. Anticoagulant rodenticide poisoning was suspected in 20 cases, creosote poisoning in 17 cases, and organophosphate and carbamate pesticides in 11 cases. According to the data provided by the Public Utility Company "Veterina Belgrade", in 2018, 58 death cases of animals with suspicion of poisoning were reported in Belgrade, while 41 cases were recorded in 2019. There are no official data on the number of intentionally or accidentally poisoned dogs and cats on the territory of the Republic of Serbia (RS), on an annual basis (Aleksić J. et al., 2014).

The diagnosis of poisoning is based on anamnestic data, clinical, autopsy, histopathological and toxicological findings (Jubb et al., 2007), and in medical-legal cases the chemical-toxicological findings play a crucial role. The aim of this paper is to point out the most commonly used agents for the purpose of intentional poisoning of dogs and cats in the territory of the Republic of Serbia, the characteristics and mechanism of toxic effects of the most commonly used poisons, the clinical picture of poisoned animals, antidote therapy and autopsy findings.

ANTICOAGULANT RODENTICIDE POISONING

Rodenticides are used to control the population of harmful gophers and are the most commonly used type of poison for the purpose of intentional poisoning. The reasons for frequent poisonings by these compounds are their pleasant taste (due to sucrose which is added to make them more attractive to gophers) and lack of odors (Eason et al., 2002; Endepols et al., 2003; Binev et al., 2005; Svendsen et al., 2002). There are different first and second-generation anticoagulants. The first generation includes indandione derivatives (difacion, chlorofacion) and coumarin (warfarin, coumachlor, coumatetralyl). This generation of anticoagulants is characterized by the need for repeated oral administration in order for non-target species to be poisoned. Over time, due to the emergence of rodent resistance to first-generation anticoagulants, second-generation coumarin derivatives have also been developed, which are very toxic to dogs and cats, and therefore poisoning can occur even after single consumption. Difenacoum is the first in a series of second-generation coumarin anticoagulants, and bromadiolone and brodifacoum are also used. Brodifacoum is more recent and has several times higher toxicity than bromadiolone. The oral LD_{50} of brodifacoum for cats is 25 mg/kg and from 0.25 to 3.6 mg/kg of body weight for dogs (Eason and Wickstrom, 2001). In our country, the most used coumarin derivatives are warfarin, bromadiolone and brodifacoum.

Anticoagulant rodenticides act as antagonists of vitamin K and the enzyme vitamin K-epoxide reductase, which participates in the recycling of vitamin K. factors) (Sheafor and Couto, 1999; Merola, 2002). As a result, the synthesis of blood coagulation factors is blocked: II (prothrombin), VII (proconvertin), IX (antihemolytic factor B) and X (Stuart's factor) (Sheafor and Couto, 1999; Merola, 2002). In terms of chemical structure, coumarin is similar to vitamin K_1, and for that reason it competes with vitamin E for its place on the enzyme epoxy reductase (competitive inhibition). There is a reduced regeneration of vitamin K_1, which is essential for the synthesis of coagulation factors. Coumarin is also thought to have a direct toxic effect on capillaries, but the mechanism of action has not yet been sufficiently clarified (Ćupić, 2015).

The latent period depends on the type and amount (dose) of anticoagulant taken, but poisoned individuals usually do not show clinical symptoms in the first 24 to 48 hours after ingestion. Newer anticoagulants have a longer biological half-life and therefore prolonged toxic effects, which requires prolonged treatment. The plasma half-life of warfarin is 15 hours, it is 5 days for diphacinone, and 6 days for bromadiolone. Brodifacoum can be detected in blood serum for up to 24 days. After a drop in a serum concentration, all anticoagulants can be identified in the liver (Khan and Schell, 2014). When a clinical manifestation occurs, the most common signs of poisoning are lethargy, dyspnea, cough and blood in the sputum (Merola, 2002). Clinical signs depend on the site of bleeding, which may be from the oral cavity, nose, vulva, foreskin or rectum. Internal bleeding in the lungs, mediastinum, thymus or trachea can manifest itself in the form of acute dyspnoea, and bleeding in the muscles or subcutaneous tissue in the form of larger hematomas. Bleeding into the joint cavities causes lameness, and in cases of bleeding in the brain or spinal cord, neurological symptoms can develop. Extensive bleeding in the abdomen leads to pallor of the visible mucous membranes, weakness and lethargy of the animal. Bleeding has also been reported in various structures of the eye - subconjunctivally, in the eye cavity, retina, and the presence of blood in the anterior chamber of the eye, between the iris and the cornea has been recorded (Petterino et al., 2004; Cullen et al., 2013, Griggs et al., 2015).

Haematological examination revealed a decrease in hematocrit and blood plasma proteins, as well as a violation of coagulation parameters, namely prothrombin time (PT), activated partial thromboplastin time (aPTT), activated coagulation time (ACT) and protein induced by vitamin K deficiency (PIVKA) (Murphy, 2007).

In the cases of suspected anticoagulant rodenticide poisoning, treatment is based on general, supportive and specific therapy. General therapy includes the use of emetics or gastric lavage, the use of adsorbents and laxatives. In

cases of heavy bleeding or a significant drop in hematocrit, supportive therapy is applied, which is based on the use of fresh plasma or whole blood transfusion every 4 to 8 hours (Chalermchaikit et al., 1993). Specific therapy is the use of vitamin K_1. The recommended doses are 1.5 – 2.5 mg/kg/twice daily, orally, for 3 - 4 weeks. Prolonging the therapy for an additional week will not result in side and harmful effects, and premature cessation of treatment can be a vital threat to poisoned dogs and cats. The most reliable way to determine when therapy should be performed is to check the prothrombin time, 72 hours after the last dosing. If the prothrombin time in that period has a physiological value, vitamin K_1 should be excluded from the therapy, and if the prothrombin time is still extended, the treatment with vitamin K_1 should be given for another week. Vitamin K_1 should be applied with small amounts of foods rich in fats (milk, meat, cheese), because fats improve its absorption. Applying half of the total daily dose every 12 hours ensures a constant level of vitamin K_1. When coagulation factors are not within physiological value levels and the animal shows clinical signs of poisoning, parenteral administration should be avoided due to the risk of bleeding and/or hematoma formation at the injection site, unless vitamin K_1 cannot be administered orally to the animal. Anaphylactic reactions are possible with parental administration of vitamin K_1 (Khan and Schell, 2014).

Anticoagulant rodenticides, especially coumarin, lead to generalized bleeding in various organs (liver, kidneys, intestines, heart and lungs). The autopsy shows bleeding in the meninges, thymus, larynx, kidneys, liver, pericardium, gastrointestinal tract, nasal cavities, joints, muscles and mediastinum, in the chest and abdomen. Petechiae and ecchymoses are often present on the skin, mesentery and mucous membranes of the gastrointestinal tract. The most common postmortem findings are hemoperitoneum, hemothorax, and bleeding in the lung parenchyma (DuVall et al., 1989). Histopathologically, degeneration of the heart muscle, inflammation of the bladder and hepatic dystrophy can be found in dogs (Srebočan and Glomerčić, 1996).

ORGANOPHOSPHATE INSECTICIDE POISONING

Organophosphates are phosphoric acid esters by chemical composition. They include some of the most important compounds for the development of life processes such as nucleic acids (DNA and RNA) and essential cofactors, and some compounds from this group are used in human medicine in the treatment of glaucoma (echothiophate, isoflurophate), Alzheimer's disease, *myasthenia gravis* and dysfunction urinary tract. In veterinary medicine,

they are used as anti ectoparasitics (diazinon) and anthelmintics (trichlorfon). They are often used as pesticides for plant protection in agriculture and forestry (malathion, parathion, diazinon, fenthion, dichlorphos, chlorpyrifos). Throughout history, they have also been used as nerve agents (sarin, soman, tabun, VX) (Gupta and Milatović, 2012).

They can be taken orally, through the skin and by inhalation. After absorption, they are distributed in the body, and the highest concentration due to lipophilicity is in adipose tissue and the brain (Gupta, 2012). The order of the most frequently used organosphosphate pesticides from extremely toxic to less toxic is the following: disulfoton, terbufos, forate, parathion, chlorpyrifos, fenthion, diazinon, malathion, tetrachlorvinphos. Chlorpyrifos is particularly toxic to cats, with an oral LD_{50} of 10 to 40 mg/kg (Fikes, 1992). Organophosphates act by inhibiting acetylcholine esterase (AChE), an enzyme that breaks down the neurotransmitter acetylcholine (ACh) within the synapses of the autonomic nervous system, neuromuscular synapses, and cholinergic synapses of the CNS. Inhibition of AChE results in accumulation of ACh and overstimulation of postsynaptic neurons or muscle cells (Ivanović et al, 2016). Organophosphate compounds have the property of "aging complex" with AChE molecules, which results in irreversible inhibition of this enzyme, which is why the effects of organophosphate are much longer-lasting and more pronounced compared to the toxic effects of carbamates (Merck, 2007).

Clinical signs of organophosphate poisoning are the result of excessive stimulation of nicotine and muscarinic receptors. Signs of excessive stimulation of nicotinic receptors are tremor of the muscles, tetanic spasms, stiffness accompanied by general weakness of the animal, paresis and paralysis. Peripheral muscarinic signs are salivation, lacrimation, frequent urination and defecation, miosis, increased bronchial secretion, dyspnoea, bradycardia, and abdominal pain. Central cholinergic signs are anxiety, restlessness, generalized convulsions, and in the later course of CNS depression and in the terminal phase coma. In some cases, not all symptoms are present, and their intensity varies depending on the dose administered, the mode of exposure, the type of animal, and the type of organophosphate compound (Merck, 2007). In dogs and cats, CNS stimulation usually progresses to convulsions. In dogs, disorders of the gastrointestinal tract often occur with diarrhea, vomiting and abdominal pain, and in cats, muscarinic effects dominate. The onset of clinical symptoms after exposure to organophosphates usually occurs within a few minutes to several hours. In some cases, delayed onset of symptoms may follow after a few days. Death is a result of respiratory disorders (bronchoconstriction, bronchosecretion, laryngospasm) or paralysis of respiratory muscles (diaphragm, intercostal muscles).

An unavoidable procedure in the diagnosis of organophosphate poisoning is the determination of AChE activity in erythrocytes, which is structurally similar to AChE in nerve tissue and as a surrogate marker reflects its activity in synapses. However, inhibition of AChE in erythrocytes is not always closely correlated with the intensity of the clinical picture. Signs of poisoning are manifested when erythrocyte AChE activity is inhibited >70%. In order to reliably diagnose poisoning, the activity of AChE erythrocytes is determined immediately before and a few minutes after the application of oximes used in the therapy of poisoning with these compounds. If after the application of oxime there is a noticeable increase in AChE activity, poisoning with these compounds is confirmed. This method also confirms those poisonings in which the initial values of AChE were within physiological limits (Izraeli et al., 1986).

Proving the presence of organophosphates in biological materials is uncertain, because these compounds are degradable and do not remain in their original form in tissues for long. In order to identify and quantify the organophosphate compound, a sample of gastric contents is delivered to the laboratory and analyzed by gas-mass chromatography (GC-MS) or with more advanced instrumental techniques (e.g. LC/MS/MS). Blood/serum and urine residues can also be analyzed for organophosphate residues or their metabolites. More than 70% of organophosphates produce one or more dialkyl phosphates (dimethyl phosphate, diethyl phosphate, dimethyl thiophosphate, diethyl thiophosphate, dimethyl dithiophosphate and diethyl dithiophosphate).

Three groups of drugs are used in the treatment of organophosphate poisoning: (1) emetics and adsorbents in order to reduce further absorption; (2) muscarinic receptor antagonists; (3) AChE reactivators. Atropine sulfate blocks the central and peripheral muscarinic effects of organophosphate. In dogs and cats, it is administered in a dose of 0.2 to 2 mg/kg (lower limit of the dose range for cats), every 3 to 6 hours or as often as the severity of the clinical picture requires. Atropinization is adequate when mydriasis occurs, salivation stops, and the animal appears more conscious (awake). Animals initially respond well to atropine sulfate, but after repeated treatments, the intensity of the response decreases, so excessive use of atropine should be avoided. However, since atropine does not reduce the nicotinic cholinergic effects (fasciculations and paralysis of the intercostal muscles and diaphragm), lethal outcome is still possible due to respiratory insufficiency. Experimental studies in primates have shown that the inclusion of diazepam in therapy reduces the frequency of muscle convulsions and increases survival rates. The efficacy of the treatment is increased by combining atropine with oximes (2-PAM, pralidoxime chloride) that reactivate inhibited AChE. The dose of 2-PAM is 20 - 50 mg/kg and is applied as a 5% solution i.m. or slow i.v. (for 5 to 10 minutes),

with a repeated half dose as needed. The i.v. administration of 2-PAM must be carried out slowly to avoid skeletal muscle paralysis and respiratory arrest. As the possibility of AChE reactivation weakens with time after exposure, oxime application must be started as soon as possible, no later than 24 to 48 hours. The rate at which the enzyme-organophosphate complex reacts to reactivators varies depending on the type of organophosphate compound (Gupta and Milatović, 2012; Gupta, 2014a).

There are no specific pathoanatomical changes at autopsy. The hair coat or stomach contents may smell of kerosene, sulfur or garlic. There may be pale mucous membranes, bleeding in the digestive tract, congestion of the stomach, especially the fundus. The liver is pale with multifocal fields of necrosis, and congestion and hemorrhage are present in the lungs. Splenomegaly, mild meningeal congestion, and multifocal necrosis fields in the kidney have been observed (Ola-Davies et al., 2018). Pathohistologically, pulmonary edema and pancreatitis can be established (Merck, 2007; Srebočan and Glomerčić, 1996).

CARBAMATE INSECTICIDE POISONING

Carbamates are esters of carbamic acid and have a less complex chemical structure compared to organophosphates. Regarding the total consumption in the world, they are ahead of organophosphates, because they are considered safer to use. In veterinary medicine, they are available as anti ecto parasitics in various pharmaceutical formulations (powders, concentrated emulsions, sprays, shampoos, flea and tick collars). They are used in agriculture for plant protection, and due to improper or malicious use, they are often the cause of acute poisoning of domestic animals, birds, fish and wild animals (Gupta, 2012). In terms of toxicity, this group of insecticides includes substances with a wide range of LD_{50} values. In rats, carbaryl has an oral LD_{50}>300 mg/kg, and aldicarb, which is a highly toxic LD_{50}, is 0.9 mg/kg. Highly toxic carbamates include methomyl (oral LD_{50} for rats 17 mg/kg) and carbofuran (oral LD_{50} for rats 8 mg/kg, for dogs 19 mg/kg), and propoxur has several times lower toxicity than the previous two compounds (oral LD_{50} for rats 95 mg/kg). Animals usually ingest carbamates by ingestion, but percutaneous and inhalation routes of poisoning are also possible. After absorption, this group of compounds is distributed in most tissues, it passes through the placental barrier and leads to inhibition of fetal AChE. In young animals, they are metabolized more slowly, which is why they are more toxic to them compared to older categories of animals. About 80% of the resorbed compound is excreted in the urine in the first 24 hours after ingestion (Gupta, 2014b).

In our country, in order to intentionally poison animals from the carbamate group, the preparation "Furadan 35 ST" (FMC Corporation) whose active substance is carbofuran is most commonly used. Its trade and use has been legally prohibited in our country since December 31st 2013, but the perpetrators are still used for the purpose of deliberate poisoning of dogs and cats.

In some parts of the world, intentional poisoning of dogs with the carbamate pesticide aldicarb is becoming more common (Frazier et al., 1999; Motas-Guzman et al., 2003; Verster et al., 2004). On the world market, one of the most famous preparations containing aldicarb is "Temik" (Bayer CropScience). It is used in agriculture against harmful insects and plant parasitic nematodes. It contains 15% aldicarb and is most often in the form of small black granules. The cases of intentional poisoning by this compound are still present, although aldicarb-based preparations are prohibited in phyto-pharmaceutical formulations. EU Directive 2003/199/EC of 2003 prohibits aldicarb and it cannot be used in plant protection products in the European Union. The derogation period referred to France (for vines and sugar beet) with a ban on sales after the May 30th, 2007 and a ban on use after December 31st, 2007 (EU Directive 2003/199/EC). Poisoning in the course is often peracute, since it is an extremely toxic carbamate compound that leads to lethal outcome within a few minutes after ingestion, due to respiratory failure (Goswamy et al., 1994; Jokanović, 2009; Ragoucy-Sengler et al., 2000).

In the cases when clinical symptoms develop in dogs, muscle tremor, hypersalivation accompanied by vomiting, miosis, bradycardia, convulsions, and difficulty breathing are observed (Verster et al., 2004). Frequent urination, paresis and paralysis may also occur. Death is a result of respiratory failure due to bronchospasm, paralysis of the diaphragm and intercostal muscles, and depression of the respiratory center (Fikes, 1990; Goswamy et al., 1994; Jokanović, 2009). In the acute course of poisoning, the appearance of acute necrotic-hemorrhagic pancreatitis is possible. Excessive cholinergic stimulation results in spasm of the Odi's sphincter and the consequent enzyme pathway in the pancreatic ducts, which increases intraductal pressure and creates the potential for enzyme transfer to the interstitium (Aslan et al., 2010; Makridges et al., 2005). In peracute cases of poisoning, this pathohistological finding is most often absent.

Carbamates act by the same mechanism as organophosphates - by inhibiting AChE at neuro-neuronal and neuro-muscular synapses. In the case of poisoning with carbamate compounds, the inhibition of AChE is reversible, because the formed bonds of carbamate with the enzyme are much weaker, and thus shorter, which is why the inhibition of AChE in the blood (erythro-

cytes) during laboratory analysis is often not evident. Clinical signs of poisoning last shorter compared to organophosphate poisoning. They include hypersalivation, gastrointestinal hypermotility, abdominal cramps, vomiting, diarrhea, sweating, dyspnoea, cyanosis, miosis, muscle fasciculations (in extreme cases, tetany accompanied by weakness and paralysis) and convulsions. The most pronounced clinical manifestations of carbamate and organophosphate poisoning are salivation, lacrimation, urination, diarrhea (SLUD). Death is a result of hypoxia due to respiratory insufficiency caused by paralysis of the respiratory muscles, bronchoconstriction and tracheobronchial hypersecretion (Gupta, 2014b).

The diagnosis of poisoning is based on the anamnesis and a positive response to atropine therapy. However, when the history is unknown, and cholinergic signs and a clear positive response to atropine suggest carbamate or organophosphate poisoning, it is necessary to determine AChE activity in erythrocytes, whole blood (for live animals) or in the cerebral cortex (for dead animals). Enzyme activity that is significantly inhibited (>50%) confirms the suspicion of poisoning by these compounds. Clinical signs of hypercholinergic activity are observed when AChE inhibition is >70%. Identification and quantification of a particular carbamate and differential diagnosis of organophosphate insecticide poisoning is possible by examining the contents of the gastrointestinal tract using GC-MS (Gupta, 2014a).

The recommended dose range of atropine for dogs and cats is from 0.2 to 2 mg/kg, parenterally, with one-quarter of the dose administered i.v. and the remainder s.c. (lower dose range is recommended for cats). Dosing is repeated as needed. The use of oxime (2-PAM) alone is contraindicated in carbamate poisoning, because it is not effective, and it can also increase the toxic effect of carbamates. In combination with atropine, 2-PAM may also worsen the clinical picture, depending on the dose administered, and in the best outcome the combination with atropine gives only a slightly better therapeutic effect compared to atropine alone. Because of all this, the use of 2-PAM is useful only if the poisoning is caused by a mixture of organophosphates and carbamates or when there are symptoms of excessive cholinergic activity, which is the case with organophosphate poisoning. 2-PAM can be fatal if applied too quickly, so its careful and slow application is necessary, i.e. in 5% saline for 10 minutes, as described in the section on organophosphate poisoning therapy. It is important that the 2-PAM solution is fresh during application, because solutions that have been unused for a long time can lead to the formation of cyanide (Gupta and Milatović, 2012; Gupta, 2014a). As part of symptomatic therapy, fluid, electrolyte replacement and vitamins B, C and E are used, because the

mechanism of toxic action of organophosphates and carbamates partly takes place through oxidative stress. The use of morphine or barbiturates in carbamate poisoning is contraindicated.

The autopsy report is not specific. Congestion of parenchymal organs and pulmonary edema may be observed. The contents of the stomach or suspect substance may have the smell of oil, sulfur or garlic. For the purpose of pathohistological analysis, the tissue of the lungs, heart, liver, kidneys, pancreas and lumbar part of the spinal cord is sampled. The contents of the stomach, intestines, bladder and feces are sampled for chemical and toxicological analysis. Pathohistological changes are diverse and include lung congestion, hyperemia and degenerative changes of myocardial cells, renal hyperemia and renal tubular degeneration, hyperemia and necrotic fields in the liver parenchyma. Examination of pancreatic tissue samples shows acute pancreatitis with wider fields of necrosis involving the parenchyma and interlobular connective tissue. In the ventral horns of the lumbar part of the spinal cord, lysis of the nuclei of motor neurons, loss of the tigroid substance and pericellular edema are noted. Sensitive neurons in the dorsal horns of the spinal cord are usually morphologically preserved (Aleksić et al., 2011).

CREOSAN POISONING

Creosan (4-6 dinitro-ortho-cresol - DNOC) is a derivative of cresol and belongs to the chemical group of dinitrophenol, which includes dinoseb and dinotherb. It is used in agriculture as an insecticide, herbicide and fungicide, and due to its characteristic yellow color, it is known as "yellow powder", which can be seen in the dog shown in Figure 1, which was submitted to the Department of Forensic Veterinary Medicine and Legislation, Faculty of Veterinary Medicine, University of Belgrade. It was withdrawn from use in the countries of the European Union in 2000 (EU Directive 1999/164/EC), and in our country it was banned in 2003. The oral LD_{50} for cats is 50 mg/kg TM (World Health Organization, Geneva, 2000).

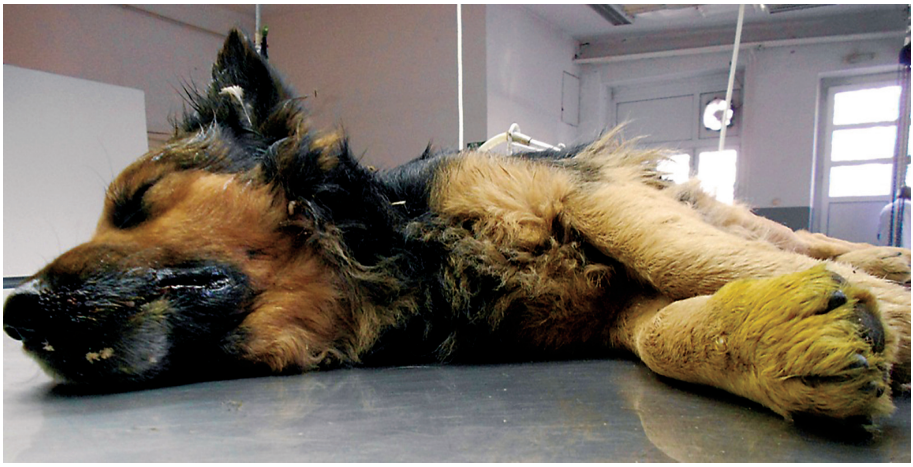

Figure 1. Canine coat colored intensely yellow in the area of the lower jaw and front right paw

It enters the body orally, percutaneously and by inhalation. In terms of physical and chemical properties, it is less hydrosoluble, i.e., it has a more pronounced liposolubility, which is why it is characterized by rapid resorption (Agency for Toxic Substances and Disease Registry, 2018). The effect is achieved by separating the process of oxidation and phosphorylation in the respiratory chain in mitochondria. Oxidation cannot take place in the respiratory chain and the accompanying phosphorylation of ADP and the creation of the energy-rich adenosine triphosphate compound ATP are absent. As a result, there is a sharp increase in oxygen consumption and the release of a large amount of energy that is converted into heat (hyperthermia). In the organism of poisoned individuals, catabolic processes (glycolysis, glycogenolysis and metabolism of fatty acids) increase sharply. Due to the lack of ATP in vital organs (heart, respiratory muscles), their function may cease. The dominant symptom is high fever (malignant hyperthermia), which reaches a value of up to 42 °C. Dyspnoea, convulsions, coma, and lethal outcome with rapid development of corpse stiffness are also present. Death is most often a result of cardiac arrest or paralysis of the respiratory center (Decision Guidance Document, 2005; Ćupić, 2015).

There is no specific antidote and nonspecific therapy is used. If the poisoning occurred by ingestion, and the animal is conscious and actively manifests signs of anxiety, vomiting should be induced. If the animal has CNS depression, gastric lavage should be performed and activated charcoal should

be used. In order to control hyperthermia, the procedure of physical cooling (cold baths, cold compresses) is recommended, without the use of antipyretics. Diazepam (not barbiturates) should be used to sedate the animal. Phenothiazines are contraindicated. Infusions of saline and/or dextrose solution in combination with diuretics contribute to the alleviation of dehydration and faster elimination of creosone from the body, since it is excreted in the urine. The success of therapy is significantly contributed by i.v. sodium bicarbonate administration, parenteral vitamin A administration, and oxygen administration (Gupta, 2020).

Pathoanatomical changes are not specific. The contact of the animal with this compound is indicated by the discoloration of the dog's coat, skin and mucous membranes with an intense yellow color that is present for several weeks. Urine has a characteristic fluorescent yellow color. After death, corpse stiffness develops rapidly. The dominant macroscopic findings are round-shaped particles in gastric contents, intensely yellow in color. The gastric mucosa is hyperemic and wrinkled (Đurđević et al., 2018). The presence of this compound in the stomach contents is confirmed by GC-MS.

MOLLUSCICIDES POISONING (METALDEHYDE)

Metaldehyde is a tetramer of acetaldehyde and belongs to the group of pesticides intended for the control of snail populations in the areas with wet soil. Although most poisonings with this neurotoxic substance have been reported in dogs, poisoning is also possible in other species of domestic and wild animals, and is associated with careless or malicious placement of baits. In commercial molluscicides, metaldehyde may be present in combination with other pesticides such as carbamates to make them more effective. Also, molluscicides can contain bran or molasses in order to be more attractive to snails, but in that way, they become more attractive for dogs and other types of animals. Metaldehyde is not considered a stable substance, but it may remain effective for 10 days. The preparations are usually in the form of blue-green granules or pellets with a mild odor of aldehyde and contain 1.5 to 5% of metaldehyde. During an autopsy of a dead dog, blue-green granules were found at the Department of Forensic Veterinary Medicine and Legal Regulations, Faculty of Veterinary Medicine, University of Belgrade, which indicates poisoning with molluscicidal preparations of metaldehyde (Figure 2). In terms of toxicity, the oral LD_{50} of metaldehyde is 100 mg/kg for dogs and about 200 mg/kg for cats (Dolder, 2003).

Metaldehyde after ingestion, under the action of gastric acid, undergoes partial hydrolysis to form acetaldehyde, and then both compounds are rapidly

resorbed from the gastrointestinal tract. The properties of the stomach contents and the speed of its emptying significantly affect the speed of absorption, and thus the beginning of the clinical manifestation of poisoning. After absorption, metaldehyde is rapidly metabolized. Enterohepatic circulation can prolong the retention of metaldehyde in the body, but eventually both metaldehyde and acetaldehyde are excreted in the urine (Blakley, 2013). Clinical manifestations are primarily attributed to metaldehyde, because studies in mice have shown that metaldehyde crosses the blood-brain barrier and that its presence is detected in the brain (Puschner, 2001). Signs of toxicity may be due to a decrease in the concentration of γ-amino-butyric acid (GABA) in the brain as a major inhibitory amino acid, resulting in CNS excitation. As the concentration of GABA in the brain decreases, the mortality rate increases (Osweiler, 1996). Another factor that contributes to morbidity and mortality is hyperthermia. It most often appears secondary to neurological manifestations. Muscle tremor also occurs. When the body temperature exceeds 41.6 °C in all organ systems, cell necrosis begins in a few minutes. Metaldehyde also affects electrolyte balance and acid-base status by causing metabolic acidosis, which is often associated with central nervous system depression and hyperpnea (Puschner, 2001). In dogs, the signs of this toxicosis can occur from a few minutes to three hours after ingestion. Neurological symptoms are predominant, and muscle tremors, anxiety, hyperesthesia, ataxia, tachycardia, and hyperthermia may occur. Metabolic acidosis is present and as it is more pronounced, depression and hyperpnea can be further intensified. Typical signs of advanced toxicosis are opisthotonus and continuous tonic convulsions that do not respond to external stimuli (unlike in cases of strychnine poisoning). Symptoms often include vomiting, diarrhea, hypersalivation, colic, cyanosis, mydriasis, and feline nystagmus. Deaths due to respiratory failure can occur within hours of ingestion (Blakley, 2013; Beasley, 1999; Booze and Oehme, 1985).

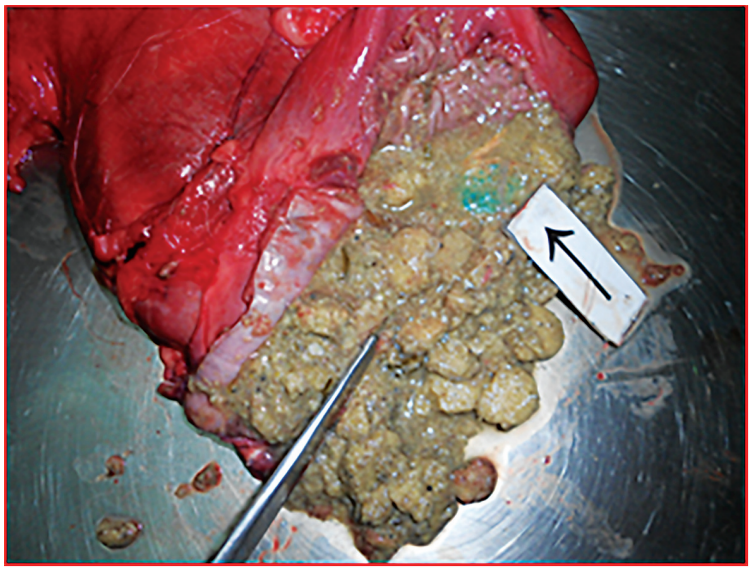

Figure 2. Molluscicide preparation of metaldehyde in gastric contents

The diagnosis is based on anamnestic data and clinical symptoms. Gastric contents, gastric lavage fluid, and expired air may have an acetaldehyde odor that is similar to formaldehyde or acetylene but less intense. To confirm the diagnosis of poisoning, it is important to analyze the contents of the stomach for metaldehyde and acetaldehyde.

Although there is no specific antidote, timely and intensive symptomatic therapy during the first 24 hours allows most poisoned animals to recover within the next 2 to 3 days. The goals of symptomatic therapy are prevention of metaldehyde absorption, control of clinical symptoms, monitoring and correction of metabolic acidosis and dehydration. If no more than 30 minutes have elapsed since ingestion and if there are no contraindications in dogs and cats, vomiting with hydrogen peroxide (1 to 5 mL/kg, maximum 45 mL) or apomorphine hydrochloride should be induced. Otherwise, gastric lavage with animal anesthesia and endotracheal intubation should be undertaken to prevent aspiration (Plumb, 1999; Dorman, 1995). In dogs and cats, the use of activated charcoal in a dose of 1 to 4 g/kg TM is recommended, and repeated application of half of the original dose every 6 to 8 hours contributes to a better therapeutic effect. Enema with warm water is also used to eliminate metaldehyde from the gastrointestinal tract. To control convulsions, i.v. diazepam at a dose of 1 to 5 mg/kg TM. If necessary, other anticonvulsants can be used, such

as inhalation anesthesia (for severe and persistent convulsions) or barbiturates, which must be used with caution because during biotransformation in the liver as a substrate may compete with enzymes involved in acetaldehyde metabolism (Plumb, 1999 Carson and Osweiler, 1997). Hyperthermia resulting from muscle tremors and seizures is usually corrected when tremor and seizures are kept under control. Therefore, aggressive physical cooling measures such as ice baths should not be used, as they can cause hypothermia. Of essential importance for the correction of metabolic acidosis and electrolyte imbalance is i.v. application of sodium lactate or sodium bicarbonate, and i.v. administration of dextrose or calcium borogluconate may reduce liver damage. Prolonged excessive muscle activity (tremor, convulsive seizures) can cause myoglobinuria and secondary renal dysfunction. In such cases, the use of diuretics is recommended to prevent kidney damage (Dolder, 2003; Blakley, 2013).

In poisoned dogs, the autopsy finding is nonspecific. Hyperemia of the liver, lungs, and kidneys, inflammation of the gastric mucosa, and subendocardial and subepicardial hemorrhages may be found (Beasley, 1999).

CONCLUSION

In order to reduce the frequency of malicious poisoning of dogs and cats on the territory of our country, it would be important to conduct strict control of the sale of agricultural preparations whose active substances have high toxicity for both humans and animals. In order to monitor the frequency of this phenomenon in our society, which is sanctioned by Article 269 of the "Criminal Code" ("Official Gazette of RS", No. 85/2005, 88/2005 - amended, 107/2005 - amended, 72/2009, 111/2009, 121/2012, 104/2013, 108/2014, 94/2016 and 35/2019), it is important to introduce a central register of confirmed cases of poisoning of owner dogs and stray dogs.

The processing of cases of intentional poisoning of animals should be based on reliable findings of forensic veterinarians and toxicological confirmation of poisoning. Judicial practice indicates that such cases are difficult to process due to the lack of evidence linking the perpetrator to the abuse of toxic substances and in this sense better cooperation and coordination of state administration bodies (police, public prosecutor's office, and veterinary inspectors), veterinarians and accredited laboratories performing chemical-toxicological analyses is required.

Author's Contribution:

JAR and SI made contributions to the idea of the publication, organisation of work and writing the manuscript; JV, ALL and ID were involved in the writing of the manuscript, JV reviewed the manuscript; JAR and SI gave the final approval of the manuscript to be published.

Competing interest

The authors declare that they have no competing interests.

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Case report

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DEEP PECTORAL MYOPATHY IN BROILER CHICKEN MEAT OBTAINED FROM A SUPERMARKET - CASE REPORT, LITERATURE REVIEW AND PREVENTIVE MEASURES

Dragana Ljubojević Pelić[1*], Miloš Pelić[1], Jelena Vranešević[1], Suzana Vidaković Knežević[1], Bojana Prunić[1], Nikolina Novakov[2], Milica Živkov Baloš[1]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia
[2] Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Republic of Serbia

Abstract

The fast growth rates in commercially reared chickens could lead to the changes in their muscle tissue structure and cause breast muscle myopathies, including deep pectoral myopathy (DPM). The incidence of DPM depends on various factors including rearing conditions, age, sex, weight and genetic strain. The aims of the present paper were to report a case of DPM in broiler chickens bought in a supermarket and review important information regarding this disease from the available literature, especially its effect on meat quality parameters and consumer preferences.

Key words: poultry meat, breast muscle myopathies, meat quality, consumer acceptability

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

DUBOKA PEKTORALNA MIOPATIJA MESA BROJLERSKIH PILIĆA IZ SUPERMARKETA – PRIKAZ SLUČAJA, PREGLED LITERATURE I PREVENTIVNE MERE

Dragana Ljubojević Pelić[1], Miloš Pelić[1], Jelena Vranešević[1], Suzana Vidaković Knežević[1], Bojana Prunić[1], Nikolina Novakov[2], Milica Živkov Baloš[1]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija
[2] Departman za veterinarsku medicinu, Poljoprivredni fakultet, Univerzitet u Novom Sadu, Novi Sad, Republika Srbija

Kratak sadržaj

Velika brzina rasta kod komercijalno gajenih pilića može dovesti do promena u strukturi mišićnog tkiva pilića i izazvati miopatije grudnih mišića, uključujući i duboku pektoralnu miopatiju. Incidenca duboke pektoralne miopatije zavisi od različitih faktora uključujući uslove gajenja, starost, pol, telesnu težinu i genetske faktore. Cilj ovog rada je da prikaže slučaj duboke pektoralne miopatije kod brojlerskih pilića kupljenih u supermarketu i da prikaže pregled važnih informacija vezanih za ovu bolest iz dostupne literature, sa posebnim osvrtom na efekat ove bolesti na kvalitet mesa i prihvatljivost od strane potrošača.

Ključne reči: živinsko meso, miopatije grudnih mišića, kvalitet mesa, prihvatljivost od strane potrošača

INTRODUCTION

Broiler chicken meat industry is of great importance and it is well known that poultry meat is one of the main sources of protein for the people worldwide. The use of novel technologies and rapid development of genetic, farm management and nutrition are the main reasons why poultry meat industry is growing so fast. Some of the reasons for increasing demand for poultry meat are also modern trends and growing interest in healthy eating habits. However, the fast growth rate in commercially reared chickens could lead to the changes in their muscle tissue structure and cause breast muscle myopathies.

Deep pectoral myopathy (DPM) is also known as Green Muscle Disease and Oregon Disease. DPM was first described in adult turkeys by Dickinson et al. (1968), while in young broiler chickens it was first described by Richardson

et al. (1980). The appearance of unusual green color in breast meat of commercially reared poultry is typical for this condition. It has been reported mainly in turkeys, but in recent years this disease has become more common in broiler chickens. DPM is not connected with any infectious diseases or harmful substances (Pastuszczak -Frak and Uradziński, 2009). It doesn't represent food safety concern. However, it significantly affects the visual appearance of the chicken breast meat. Furthermore, alterations in visual sensory properties of the breast meat could negatively affect consumer acceptance of chicken meat (De Carvalho et al., 2020). Also, it could lead to meat quality losses (Yalcin et al., 2018). This condition is also a significant economic problem in the poultry farming. It should be pointed out that breast muscles of broiler chickens are their economically most profitable part. Due to the fact that DPM significantly affects meat quality of commercially reared chickens and has a negative economic impact, it represents a challenge to the broiler industry.

The aims of this paper were to describe a case of DPM in broiler chickens obtained from a supermarket and review important information from the available literature. This is very important, especially because consumers need to be able to recognize this disease. Finally, some recommendations for preventive measures and monitoring of DPM in poultry meat are proposed.

CASE PRESENTATION

No ethical approval was obtained because this study did not involve laboratory animals. It only involved non-invasive procedures.

Case report - consumer complaint report

Consumers bought four "ready to grill" chickens in a supermarket and, after realizing that the breast meat had a strange color and texture, they contacted the laboratory of Scientific Veterinary Institute "Novi Sad". All chickens available at the supermarket are produced on the same broiler chicken farm. The consumers were very distressed and concerned. They found green flesh inside chicken breasts and they described it as "atypical, greenish, breast resembling vomit in color that smelled strange". They explained their surprise at the discovery, describing the meat as "absolutely disgusting". Furthermore, they said that the changes they encountered were like "finding a foreign body, a possible biological weapon" and "the emergence of a new parasite". They bought four originally packed chickens "ready to grill" and found the changes in two, while cutting the chickens into pieces, to freeze them. They claimed that they won't shop at that supermarket ever again. Additionally, they said that they had been raising chick-

ens for years and had never encountered a similar problem. Obviously, consumers identified this condition as an important health and aesthetic issue.

Organoleptic analysis

The consumers brought two carcasses of chicken meat that they bought at a supermarket to the laboratory of Scientific Veterinary Institute "Novi Sad". Sensory assessment was carried out in the laboratory.

Organoleptic characteristics of chicken meat

The changes in color were notable as green discoloration. There were also the changes in the texture of breast muscles. Bilateral macroscopic changes of breast muscle that was green in color, as well as dry, crumbly, friable and solid consistency were observed during the examination. Dry appearance was also detected on the section. Progressive degeneration of the *Pectoralis minor* muscle and the damaged muscle tissue were found. Also, the unpleasant smell of the affected tissue was detected. The meat was repulsive. The diagnosis of DPM was established on the basis of distinctive green color of the muscle tissue and overall organoleptic characteristics (Figure 1).

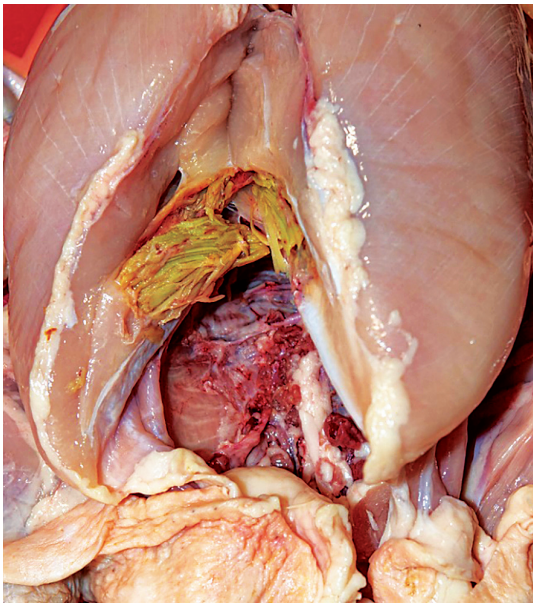

Figure 1. Green muscle disease after opening the breast muscles

DISCUSSION

The affected muscle tissue should be removed and the rest of the carcass is fit for human consumption. Due to the fact that the most valuable part of the chicken carcass is affected and removed, the quality of the product is significantly lower, which results in economic losses.

All the above mentioned indicates that there is a need to properly inform the public about such cases and conduct researches in order to prevent the unnecessary spread of fear and panic.

The main risk factors associated with the occurrence of DPM

Slaughter weight, rearing conditions, age, sex, genetics and mobility of chickens are the main factors associated with the occurrence of this disease (Petracci and Cavani, 2012). A major factor for the occurrence of DPM is undoubtedly rearing conditions. It is well known that the live weight of broiler chickens and turkey increased by more than two times over the last 50 years mainly due to the size of pectoral muscles (Semenova et al., 2019). An increase in incidence of DPM is connected with the increase in growth rate and muscle size. Petracci and Cavani (2012) came to the conclusion that the main problems related to occurrence of DPM in broilers are related to the selection of the chickens for growth rate and breast yield. It is known that heavier turkeys and broiler chickens have a higher incidence of this disease. Pajohi-Alamoti et al. (2016) found a significant link between chicken's age, weight and occurrence of the disease. Furthermore, broiler chickens are mostly inactive during the growing period in commercial farming conditions. The consequence is that the breast muscles are not active enough and therefore they cannot expand due to the lack of physical activity. Consequently, some rapid and immoderate wing activity could lead to degeneration and death of the cells in muscle tissue. Discoloration of the flesh is caused by swelling and represents a result of deficiency of oxygen in the breast muscle due to improper blood supply. Distinctive swollen reddish-brown lesions appear first and they are followed by lesion changes in color to green. Eventually, the lesions become pale green (Stangierski et al., 2019). Earlier findings showed that pathological changes affect both major and minor pectoral muscles (Pastuszczak et al., 2002).

Mechanical stresses which could occur during contracting of myofibers and inadequate energy metabolism are listed as the cause of muscular degeneration. Some other chicken breast meat abnormalities such as spaghetti meat, white-striping and wooden breast are also associated with intensive broiler production conditions as a result of pushing biological boundaries (Petracci et al., 2019).

It is very interesting that DPM was observed in free-range broiler chickens. Bilgili and Hess (2002) reported that DPM was more frequently found in males than in females. However, Lien et al. (2012) reported that DPM was more frequently found in females. Bianchi et al (2006) concluded that genetics plays an important role in the development of this condition.

Incidence and intensity of DPM

Kijowski et al. (2014) reported that the number of cases of DPM in commercial genetic lines increased in the USA, Italy, Greece and Bulgaria. In Poland, the number of cases in broilers aged five to seven weeks was in the range from 0.02 to 1.9% (Kijowski et al., 2014). Bianchi et al. (2006) noted that DPM was estimated to be below 1%. One case was reported in Romania in a household reared broiler chicken (Stancu et al., 2015). Pajohi-Alamoti et al. (2016) reported that 0.033% carcasses in slaughtered broiler chickens from the west of Iran were affected with DPM. In Bulgaria, Dinev and Kanakov (2011) reported 0.51% of carcasses with DPM.

Only one study related to DPM has been conducted in Serbia so far, at a slaughterhouse for fattening chickens from intensive housing conditions (Maslić-Strižak et al., 2014). The incidence did not depend on sex and in the examined hybrids it was 0.36% and 0.60%. No research has been conducted on the prevalence of DPM in chicken carcasses on the market.

Effects of DPM on poultry meat quality

The nutritional quality is the main reason for the fact that chicken meat is appealing to consumers worldwide, so the measures to preserve the quality of chicken meat are very important. The most significant quality attributes for poultry meat are appearance and texture (Giampietro-Ganeco et al., 2022). Meat color is important for consumers when they decide to buy raw meat in the marketplace. Meat texture is also very important when choosing poultry meat. Dransfield and Sosnicki (1999) reported that toughness and poor cohesiveness, color and water holding capacity are the main meat quality problems connected with the selection for muscle growth.

Pastuszczak-Frak and Uradziński (2009) examined the hygienic and technological value of turkey meat originating from flocks with DPM and concluded that the meat was suitable for consumption from microbiological viewpoint. However, the technological value of the affected meat was diminished. Deviations in pH value, water binding capacity, color and chemical composi-

tion were observed. They reported that the meat affected with the DPM shows significant differences in cross-section color, juiciness and taste after thermal processing.

Cavalcanti et al. (2021) concluded that the severe condition of DPM causes variation in the quality of turkey muscle. They observed color changes in affected meat samples. Also, they observed a greater water-holding capacity, pH, length of sarcomere, fat content and lower shear force and moisture content in the affected samples in comparison to nonaffected samples. This is significant from the viewpoint of the manufacturing of processed products since fat content and water holding capacity represent crucial meat properties. They suggest that the processing represents a proper alternative for exploitation of affected meat. DPM affects the color and partially a reduction of texture of the breast meat which are the main attributes for consumers and chicken's most valuable part, which consequently has a negative economic impact on broiler meat industry (Giampietro-Ganeco et al., 2022).

Cavalcanti et al. (2021) concluded that the processing is an economically feasible potential for the commercialization of affected breast meat.

Consumers' acceptability of DPM

The main problem (with DPM) is the absence of symptoms (while chickens are) on the farm. It can be identified only during carcass dissection. The additional problem occurs when whole chickens are sold. The consumers normally detect the changes when cutting the chickens into pieces. They observe changes in color and texture of the muscles. Consumers recognize the observed changes as signs of spoilage. The main problem could be the fact that consumers will no longer trust the producer and they will refrain from buying chickens and other products in the future (Kijowski et al., 2014).

Consumers' attitude towards green muscle diseases should be regarded as an important and relevant issue. They have the right to know and choose what they want to eat. Consumers identify these changes as uncommon for chicken meat. The changes reduce the visual acceptability of chicken breasts and even the whole carcass. Unpleasant green color which appears when the chickens are cut scares the consumers despite the fact that meat is safe for consumption. De Carvalho et al. (2020) noted that the consumption of fresh poultry meat in some European countries decreased. The image of modern poultry farming system and a changed perception of poultry meat quality and safety are the important reasons for the above-mentioned decreasing trend in poultry meat consumption. The recognition of DPM may have negatively affected the consumer's attitude by associating the green muscle diseases with unhealthy and unsafe meat.

In the future, these conditions could lead to an increasing number of consumer complaints mainly due to the fact that it is regarded as an unpleasant "surprise" during preparation. The affected meat is unfit for consumption, it is sensory – organoleptic unacceptable.

Preventive measures and detection of DPM

The main problem is the fact that DPM is usually undetected until processing. Also, it is very difficult to predict the occurrence of this condition. DPM leads to significant economic losses due to the fact that it affects chicken breast which is the most valuable part of the carcass. During processing of chickens, the affected muscle tissue is removed.

Management practice on the farm is the most important preventive measure against DPM. Minimizing wing-flapping is the best preventive measure. It is very important to monitor health conditions of chickens, ventilation, air quality, ammonia level, temperature, etc. Also, the time that people spend in broiler farm should be limited. Noise levels and amount of light that could frighten the chickens should be minimized.

Recently, creatine kinase was identified as a blood enzyme that could be a noninvasive tool for breeders to screen birds for susceptibility to the disease. However, this method is not applicable in commercial poultry farms due to the time needed for analysis and high costs (Kijowski et al., 2014). Also, genetic selection against DPM could be an effective tool for reducing the future occurrence of DPM (Petracci et al., 2015). Petracci and Cavani (2012) reported that genetic selection against DPM has been undertaken by poultry companies. They also reported that developments in whole-genome selection using dense DNA - markers could be significant in reducing the occurrence of this disease in the future.

A non-destructive sensor able to detect DPM in whole carcasses has been developed (Traffano-Schiffo et al., 2018). The sensor measures the permittivity of chicken whole carcass with skin in depth. This method could be commercially available in future.

The presented data are very important for consumers, poultry farmers, poultry abattoirs, poultry suppliers, veterinary practitioners, inspectors and scientific community. There are no clinical signs of DPM and it could be detected only after dissection of carcasses. The diagnosis is confirmed by macroscopic examination. The main problem is the fact that this condition is only noticed after the broilers are slaughtered. DPM has important effect on meat quality. Such meat is aesthetically undesirable so the changed parts of meat should be removed and the rest of carcass is fit for human consumption. The

fact that DPM affects the most valuable parts of poultry carcass leads to significant economic losses.

This the only case of DPM which has been reported and confirmed in the laboratory for microbiological and sensory analysis of food of the Scientific Veterinary Institute "Novi Sad" so far. Due to the extreme concern of consumers, we considered it important to describe this case and draw attention to this disease. The presented data is of public interest and it could significantly contribute to the consumers' knowledge about the disease.

A proper solution to this disease is not currently commercially available.

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Author's Contribution:

DLJP and MP made substantial contributions to basic idea, conception and design, acquisition of samples and data, analysis of the data and interpretation of results; JV, BP, NN and SVK were 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.

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THE GLOBAL SPREAD AND EPIDEMIOLOGICAL CHARACTERISTICS OF *SALMONELLA* SPP., *ESCHERICHIA COLI* AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA): RELATED RESEARACH STUDIES IN SERBIA

Maja Velhner[1*], Dubravka Milanov[1], Nevenka Aleksić[2]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia
[2] Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Republic of Serbia

Abstract

It has been established that some clones of pathogenic bacteria such as *Salmonella* spp., *Escherichia coli* ST131, and methicillin-resistant *Staphylococcus aureus* (MRSA) tend to spread worldwide. Therefore, epidemiological surveys have been conducted to identify the source of infection and to break the chain of infection. In this article, it was pointed out that common international clones of *Salmonella* are represented with the serotypes Typhimurium, Kentucky, Infantis and Enteritidis. Serovars Typhimurium and Kentucky display multidrug-resistant phenotypes more frequently. Several sequence types of *E. coli* and the international clone ST131 are described, including clades C1 and C2 with the extended-spectrum cephalosporin-resistance genes (*bla*CTX-M-15 or *bla*CTX-M-27). These pathogens are often found in both humans and animals. It is noted that *Staphylococcus aureus* became resistant to methicillin almost instantly after its introduction into clinical practice. Soon afterwards, MRSA found its way to farm animals and wildlife. The cycles of infection are bidirectional: humans can disseminate MRSA in the environment but animals may also be sources of infection for humans. Comprehensive work has been done by epidemiologists to introduce all necessary measures to eliminate MRSA from hospitals. Also, much effort has been made in MRSA control to prevent infections on animal farms and contamination in the primary food production chain. As the struggle with pathogenic bacteria continues, we face the incessant

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

threat of new resistance and virulence mechanisms, which bacteria use to resist the hostile environment and enhance their survival in their natural habitats including humans and animals. Therefore, the capacity of certain bacteria to spread due to their virulence mechanisms and resistance phenotypes is presented, and a brief description of the research conducted in Serbia is included.

Key words: *Salmonella*, *E. coli*, MRSA, virulence, epidemiology, clonal spread

GLOBALNA RASPROSTRANJENOST I EPIDEMIOLOŠKE KARAKTERISTIKE POJEDINIH KLONOVA *SALMONELLA* SPP., *ESCHERICHIA COLI* I METICILIN-REZISTENTENTNIH *STAPHYLOCOCCUS AUREUS* (MRSA) I PRIKAZ SLIČNIH ISTRAŽIVANJA U SRBIJI

Maja Velhner[1*], Dubravka Milanov[1], Nevenka Aleksić[2]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija
[2] Fakultet veterinarske medicine, Univerzitet u Beogradu, Beograd, Republika Srbija

Kratak sadržaj

Pojedine vrsta bakterija, kao što su *Salmonella* spp., *Escherichia coli* ST131 i meticilin-rezistentni *Staphylococcus aureus* (MRSA), rasprostranjene su širom sveta. Zbog toga se sprovode brojna epidemiološka istraživanja s ciljem otkrivanja izvora i prekida lanca infekcija koje izazivaju. Istaknuto je da su serovarijeteti Typhimurium, Kentucky, Infantis i Enteritidis, najznačajniji globalno rasprostranjeni klonovi *Salmonella* spp. kao i da su Typhimurium i Kentucky češće od drugih serovarijeteta, rezistentni na više klasa antibiotika. Opisano je nekoliko različitih vrsta *Escherichia coli* uključujući i podvrste *E. coli* ST131, i to C1 i C2 sa genima rezistencije na cefalosporine proširenog spektra (*bla*CTX-M-15 or *bla*CTX-M-27). Navedene bakterije izolovane su i od ljudi i od životinja. Meticilin rezistentni sojevi *Staphylococcus aureus* (MRSA) ustanovljeni su vrlo brzo nakon uvođenja meticilina u kliničku praksu. Ubrzo su MRSA nađeni i kod domaćih životinja i divljači. Ciklusi infekcije ovom vrstom bakterija odvijaju se u oba pravca: ljudi mogu izlučivati MRSA u životnu sredinu, a životinje mogu biti rezervoari infekcije za ljude. Sveobuhvatan rad epidemi-

ologa doprineo je primeni strogih mera za kontrolu i eliminaciju MRSA u bolničkim sredinama, a značajna pažnja je usmerena i na otkrivanje MRSA infekcija kod farmski gajenih životinja, u cilju sprečavanja kontaminacije u lancu proizvodnje hrane. Uprkos naporima koji se ulažu u kontroli širenja patogenih vrsta bakterija i prevenciji infekcija koje izazivaju, suočeni smo sa konstantnom pretnjom od pojave novih mehanizama rezistencije i virulencije koje bakterije koriste da bi preživele u nepovoljnim uslovima životne sredine, uključujući tu i organizme ljudi i životinja. Iz navedenih razloga u ovom radu prikazani su mehanizmi virulencije i rezistencije za koje se smatra da značajno doprinose globalnoj rasprostranjenosti pojedinih klonova navedenih vrsta bakterija, uz kratak opis sličnih istraživanja u Srbiji.

Ključne reči: *Salmonella*, *E. coli*, MRSA, virulencija, epidemiologija, klonalna

INTRODUCTION

Coping with multidrug-resistant bacteria has been among the ultimate goals of medicine. Apart from having intrinsic resistance, bacteria can also acquire antibiotic resistance genes and mobile genetic elements and induce the exchange of genetic material within their counterparts. The long-lasting bacterial evolution prompts the question whether it is possible to create efficient therapy for humans and animals in the future (Dandekar et al., 2015). Bacteria use specific metabolic pathways to increase their fitness, colonize the gut and organize themselves in biofilm communities. For example, even highly pathogenic bacteria with prominent virulence and resistance mechanisms, which may present a burden to themselves, tend to become well-established in natural environments (Beceiro et al., 2013). All these features together result in the endless dissemination of resistant and virulent clones worldwide (Pitout and Finn, 2020). Therefore, it is very important to detect and prevent the dissemination of pathogenic and multidrug-resistant bacteria in hospital settings and food production chains. Control strategies aiming to deal with bacterial infections are more comprehensive in more economically developed countries as compared to developing countries. However, mass primary production and utilization of antibiotic feed additives as growth promoters has led to an increase in resistance to antibiotics, even in developed countries (Silbergeld et al., 2008). Those were the reasons why as early as since 1970 some antibiotics have been sequentially banned as feed additives in the EU and from 1 January 2006 antibiotics may no longer be used as growth promoters (Castanon,

2007). Consequently, we have witnessed a decrease in antimicrobial resistance in pathogenic and commensal bacteria which originate from the environment and food-producing animals in some European countries (Aarestrup et al., 2001). However, the antibiotic withdrawal from food-producing animals has not significantly changed the resistance levels in human pathogens (Pradella et al., 2006). The fear of continuous development of new mechanisms of resistance in bacteria is still an important global issue, while trade and travel continuously increase the risk from dissemination of multidrug-resistant and virulent bacteria with limited fitness cost. In this brief review, some of the prominent examples of the worldwide spread of bacterial pathogens are discussed and the brief presentation of related research in Serbia is included as well.

CLONAL SPREAD OF *SALMONELLA* SPP.

There are several examples of the successful clonal spread of pathogenic bacteria from food to humans. Non typhoidal *Salmonella* spp. is at the top of the list of foodborne pathogens (Majowicz et al., 2010). Poultry meat and products including table eggs have long been recognized as the main reservoirs of non-typhoidal *Salmonella* (Antunes et al., 2016). The diseases are manifested as a self-limiting food poisoning or take a course of severe infection that requires antibiotic therapy. These are the reasons why *Salmonella* spp. is targeted in countless studies aiming to discover epidemiological trends in infection especially in serovars that become well-established international clones. The most prominent examples of the successful clonal spread in *Salmonella enteric* serovars are *Salmonella* Typhimurium DT104 (Threlfall, 2000), *Salmonella* Kentucky ST198 (Le Hello et al., 2013), *Salmonella* Infantis (Aviv et al., 2014) and *Salmonella* Enteritidis (Pijnacker et al., 2019, Li et al., 2021). It is important to bring up the fact that serovar Enteritidis can also be vertically transmitted, which renders them even more dangerous for public health due to the increased potential for dissemination across the food chain and subsequent increase of their spread in communities. Therefore, one of the important goals of contemporary agricultural production is to eliminate dangerous *Salmonella* serovars from poultry and other food-producing animals around the globe. Subsequently, the implementation of enforced biosecurity measures in the entire food chain including pre-harvest has become important both in developed and developing countries (EFSA, 2019).

Epidemiologists who are focused to trace back the source of *Salmonella* spp. outbreaks, to minimize the likelihood of infection in humans, are trying to help to break the chain of infection at the very bottom of the production

pyramid (Pijnacker et al., 2019). Indeed, a comprehensive genome analysis of *Salmonella* originating from poultry has revealed that *S.* Enteritidis from breeder flocks has been spreading through global trade for decades (Li et al., 2021). The exact reasons for persistent infection with serovars Enteritidis and Typhimurium can be revealed using experimental animals. It was shown that invasive serovars have more virulence factors, invade internal organs of the host more efficiently and possess mechanisms prompting their survival inside the macrophages of the host (Velhner et al., 2018). For instance, the global spread of *Salmonella* Enteritidis phage type 4 (PT4) was attributed to successful infection of the hens' reproductive tract most likely by inheriting additional virulence factors which facilitate internal contamination of eggs (Velge et al., 2005). Therefore, the enhanced management practice, vaccination and utilization of prebiotics and probiotics have been enforced in many countries of the world, which has led to a much better epidemiological situation regarding *Salmonella* spp. infection of food-producing animals. Many of these strategies have helped to eliminate some other serovars besides Enteritidis and Typhimurium (two serovars that are primarily targeted by vaccines) and thus reduce the burden of infection at the farms worldwide.

The clonal spread of *Salmonella* Infantis in Serbia was in part attributed to strains which become resistant to ciprofloxacin (Velhner et al., 2014). However, in 2018, it was established that strain Infantis continues to be resistant to nalidixic acid but not longer to ciprofloxacin, which is possibly due to restrictions on the use of enrofloxacin for the therapy of day-old chickens. It is interesting to note that brief monitoring of antimicrobial resistance in *Salmonella* isolates in Serbia reveals that *Salmonella* Typhimurium is frequently susceptible to antibiotics unlike the isolates reported in other European countries (Jovčić et al., 2020). In Serbia, *Salmonella* Enteritidis (SE) was not examined by any of the available genetic methods including phage typing and the whole-genome sequencing approach. Thus, factual information about the possible clonal spread of SE from humans and animals is still lacking.

GENETIC BASIS OF THE PROGRESSION OF THE *E. COLI* WITH THE DISTINCTIVE SEQUENCE TYPES AND PHENOTYPE

E. coli sequence type-ST131 is primarily a human pathogen often causing urinary infection (Giedraitiene et al., 2016). However, due to the high dissemination rate, it can infect all animal species and contaminate the environment. The exact reasons for the successful global spread of the clone ST131 have not been entirely elucidated. Namely, the virulence factors, including their

resistance determinants, could be assumed as moderate, while the transmission rates could be minimized with proper hygiene and disinfection in hospital facilities. Therefore, the combination of all virulence properties perhaps contributes to the good fitness and widespread nature of the ST131 clone but none of these factors alone is significantly pronounced to cause such persistent infection and significant contamination rates (Pitout and Finn, 2020). There are several well-established *E. coli* ST131 clades. The clades A and B comprise strains susceptible to antibiotics but possess different *fimH* genes, which are a part of a *fim* operon. The *fim* operon encodes fimbria which determines the success of bacterial attachment to the host tissue receptors (Pitout and Finn, 2020). Clade C is represented by three major groups: C0, C1 and C2. The major differences between these are the resistance to fluoroquinolones, which is present in isolates belonging to clades C1 and C2 but absent in those from clade C0. Fluoroquinolone resistance has been developed due to the multiple mutations in topoisomerase genes (the *gyrA* and the *parC* gene). During genomic evolution of the *E. coli* ST131 isolates from the clades C1 and C2, the acquisition of the extended-spectrum beta-lactamase resistance genes took place, also. All of these genetic changes are attributed to the new plasmid or the presence of insertion elements required for the successful integration of other genes in already existing plasmids. However, even if these properties have helped scientists with the phylogenetic classification of the ST131 clones, none of them is the key reason for the successful spread of these bacteria. The following additional factors are further influencing the abundant pathways of the *E. coli* ST131 strains: the level of compensatory mutations required to obtain plasmid stability and decreased fitness cost, the horizontal integration of genomic islands and prophages including a variety of virulence genes carried on by chromosomes and plasmids (Beceiro et al., 2013; Pitout and Finn, 2020).

The ESBL resistance gene *bla*CTX-M-15 becomes one of the most distinctive and worldwide spread genes in *E. coli* clones of various sequence types. However, the CTX-M-15 carriers are more prevalent in humans than in animals or the environment (Irrgang et al., 2017). The *bla*CTX-M-15 gene is often found in the multireplicon IncF plasmids or, in some rare cases, it could be integrated into the chromosome owing to the occurrence of several independent events (Irrgang et al., 2017). The CTX-M-15-producing *E. coli* encompasses 5.2% of all ESBL/AmpC producing *E. coli* isolates from food animals and products in Germany. The most frequent sequence types were ST167 and ST410. Four ST410 isolates except one carried the *bla*CTX-M-15 gene on a chromosome, while in strains ST167, the *bla*CTX-M-15 gene was found on IncF multireplicon plasmids. In both the chromosome and the plasmid,

*bla*CTX-M-15 gene was associated with the IS*Ecp1* element, confirming the high tendency of the transferability. The *E. coli* sequence type ST410 was also detected in humans, implicating the possibility of clonal transfer (Irrgang et al., 2017).

The recent increase in infections caused by the extra intestinal pathogenic *E. coli* in Japan, in human patients, was attributed to the worldwide clone ST131, resistant to extended-spectrum cephalosporins due to the inheritance of the *bla*CTX-M-27 gene (Matsumura et al., 2016). Based on a core genome sequence analysis this clone is genetically distinctive and forms the C1-M27 clade. The pangenome analysis identified a specific region in the clade C1-M27 that closely resembles a prophage-like genomic island originally identified in an *E. coli* isolate from a pig. This region was termed M27PP1. Upstream the M27PP1, in the two C1-M27 isolates, an additional insertion region was identified showing homology to a prophage sequence found in a chromosome of the g-proteobacterium HdN1 (Matsumura et al., 2016). The reservoirs of the CTX-M27 are found worldwide in non-human and environmental specimens and thus present an important clade among the ST131 variants with the capacity of further global spread.

Unlike *Salmonella*, which tends to disseminate as a common clone, *E. coli* is genetically quite diverse. The exceptions are the above-mentioned sequence types, which persist in various niches and disseminate thanks to their enhanced fitness. During the investigation of the diverse, multidrug-resistant commensal *E. coli* from a poultry farm practicing frequent use of antibiotics in Serbia, three independent clones with the common virulence type and resistotype were determined by pulse-field gel electrophoresis (Velhner et al., 2018). The multilocus sequence type of only a small number of *E. coli* isolates from gulls was done as well. It was discovered that isolates resistant to extended-spectrum cephalosporins were ST38, ST2307, ST224 or ST162 while ST131 was not detected (Velhner et al., 2021). However, more comprehensive multilocus sequence typing of commensal and pathogenic *E. coli* isolates from animals was not performed, and therefore, the data on the existence of particular ST is not available.

EVOLUTION OF THE METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen. This worldwide epidemic clone emerged in 1961, soon after methicillin had been introduced for the therapy of infections caused by peni-

cillin-resistant *Staphylococcus aureus*. MRSA is characterized by the *mecA* gene that is incorporated in a staphylococcal cassette chromosome SCC*mec,* which differs in size and genetic composition. The *mecA* gene encodes the penicillin-binding protein PBP2a of the bacterial cell wall, which has a low affinity for beta-lactam antibiotics (Enright et al., 2002). MRSA isolates are multidrug-resistant and often only glycopeptide antibiotics such as vancomycin are effective against them. However, some strains became resistant also to these antibiotic classes (such isolates are called GISA), which renders successful treatment impossible (Enright et al., 2002). MRSA emerged primarily as a hospital pathogen, but it is now widespread in the community and can be isolated from animals and the environment. The evolution of MRSA is still a puzzle. Since genetic analysis has confirmed genetic divergence between strains, it has been proposed that independent inheritance of the *mecA* gene contributed to the evolution of MRSA rather than the diverse inheritance of genetic material that has arisen in a single clone (Enright et al., 2002). The multilocus sequence typing (MLST) utilizes analysis of the seven housekeeping genes to obtain the specific allelic profile. This method is beneficial in the classification of various MRSA lineages that have appeared in the past decades and more recently. It was elucidated that early MRSA strains evolve from the ST250 ancestor, who in turn arose from the methicillin-susceptible (MSSA) ST8 linage. The ST247 clone that emerged independently from ST250 is currently circulating in European hospitals. Several MRSA clones have the same allelic composition as their methicillin-susceptible (MSSA) counterparts indicating the independent horizontal acquisition of the *mecA* gene. This fact is in a way supported by the independent introduction of the four SCC*mec* types within MRSA strains of the same sequence type (Enright et al., 2002).

The community-acquired MRSA strains (CA-MRSA) possess more virulence determinants than hospital strains. The production of the Panton-Valentine leucocidin (PVL) exotoxin is pronounced in community-acquired strains and its detection could be used to distinguish CA-MRSA from the healthcare MRSA (HA-MRSA). PVL toxin is recognized in those strains that cause necrotizing pneumonia and invasive skin diseases although the mechanism of these virulence factors is not known. Other toxins produced primarily by the CA-MRSA are toxic shock syndrome toxin-1, staphylococcal enterotoxin B or C, α-haemolysin and phenol-soluble modulins (Hassoun et al., 2017).

Livestock-associated MRSA (LA-MRSA) are also very important pathogens (Deiters et al., 2015). Not only may farm workers or those in contact with raw meat become infected with LA-MRSA but they might also transmit this pathogen to hospitals. In the vice versa manner, humans infected with LA-

MRSA can transfer the pathogens to animals. This type of two-way directional transmission makes it even more difficult to keep infections under control in both human and veterinary medicine. However, with the proper treatment and management in hospitals and on farms it is possible to eliminate and prevent the spread of MRSA. The principle "search and destroy" is very effective in healthcare settings in eliminating this pathogen. Firstly, the MRSA carriers are to be detected, and then the medical treatment is to be applied to eliminate the pathogen and interrupt its transmission pathways. A similar principle is applied on farms, but there it is also important to prevent the purchase of positive herds to keep the MRSA infection under control (Crespo-Piazuelo and Lawor, 2021).Therefore, effective management programmes are required to be rewarded with a healthier environment and better treatment options for both human and animal patients.

It was shown that MRSA isolates among patients and health care workers from the Serbian University Clinical Hospital were mostly of the CC-5-SCC-*mec* type I. These isolates were predominantly found in patients from emergency departments and medical departments. In addition, various genetic lineages were found in isolates from healthcare workers in surgical departments. MRSA isolates from healthcare workers carried mostly smaller SCC elements of type IV and V. This was the most comprehensive MRSA detection in Serbia and, as noted, has shown the increased risk of MRSA carriage not only among patients but also among the healthcare workers (Cirkovic et al., 2015). The presence of MRSA in pigs was estimated on one farm from the north of Serbia, revealing that out of 84 nasal swabs, six tested positive for MRSA. Those isolates belonged to the ST45, *spa* type t015 and in addition possessed SCC*mec* IVa. The MRSA sequence type 45 is a global MRSA lineage of particular strain diversity and common nosocomial isolate in Europe (Velebit et al., 2010).

CONCLUSION

The important goal in human and veterinary medicine is to follow up the work of epidemiologists to break the infection cycles of all persistent and dangerous pathogens. If collective efforts in improving management are introduced in everyday practice, it will be possible to reduce the burden of infections with pathogenic bacteria.

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Author's Contribution:

MV wrote the manuscript and reviews the literature, DM reviewed the manuscript, NA reviewed the manuscript and did the English editing.

Competing interest

The authors declare that they have no competing interests.

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ISOLATION, IDENTIFICATION AND ANTIMICROBIAL PROFILE OF *CORYNEBACTERIUM BOVIS* FROM SELECTED DAIRY FARMS IN BISHOFTU, CENTRAL ETHIOPIA

Muhabaw Yimana[1,2] and Juhar Tesfaye Bekele[3*]

[1] Addis Ababa University, College of Veterinary medicine and Agriculture, Ethiopia
[2] Sekota Agriculture Bureaue, Amhara, Ethiopia
[3] Ethiopian Institute of Agricultural Research, Werer Agricultural Research Center, Ethiopia

Abstract

From January to May 2018, a cross-sectional study was undertaken on lactating dairy cows in Bishoftu town to isolate *Corynebacterium bovis*, determine the prevalence and risk factors, and evaluate the effectiveness of several antibiotics in lactating dairy farms. Study animals were selected randomly from selected dairy farms in the area. Collecting milk samples from mastitic cows, cultivating, and then performing an antibiotic sensitivity test were the procedures followed. A total of 384 lactating dairy cows were examined with inspection and California Mastitis Test (CMT), in which 86 of them were found to be CMT positive. Accordingly, prevalence was 3.9% and 18.5% for cows affected by clinical and subclinical mastitis respectively. The prevalence of mastitis showed statistically significant difference between, lactation stage, breed, age and washing ($p > 0.05$). However, there was no statistically significant difference noted in animal husbandry practice ($p > 0.05$). A total of 384 lactating dairy cows were examined with inspection and CMT, in which 86 of them were found to be CMT positive. Out of the 86 mastitis positive samples (sample indicates milk from one cow) sent to microbiology laboratory for microbiological examination, 7 bacterial isolates were identified as *Corynebacterium bovis.* The biochemical and morphological characteristics of 7 (1.8%) *C. bovis* isolated from bovine milk samples and the *C. bovis* reference strains were found to be uniform. Valuable criteria for identification were presence of catalase and oxidase,

[1*] Corresponding Author: juhar_t@yahoo.com

production of acid from glucose and a requirement for enriched basal media. *C. bovis* isolates have revealed a higher sensitivity to the kanamycin and streptomycin (71.4% each). A certain resistance has been noted to oxytetracycline (71.4%) and nalidixic acid (42.8%). Higher number of isolates showed moderate sensitivity or resistance to amoxicillin (51.1%). Regarding to multidrug resistance, the study reflects that only one isolate (14.3%) shows multidrug resistance to four drugs namely kanamycin, amoxicillin, nalidixic acid and oxytetracycline. This study demonstrated that mastitis due to *C. bovis* is rare in lactating dairy farms in Bishoftu. Some of the risk factors for mastitis can be addressed by practical management of dairy cows. Farm owners should selectively use the antibiotics to which the bacteria do not show resistance, such as streptomycin and kanamycin.

Key words: *Corynebacterium bovis*, CMT, dairy farm, mastitis, prevalence

IZOLACIJA, IDENTIFIKACIJA I ANTIMIKROBNI PROFIL *CORYNEBACTERIUM BOVIS* SA ODABRANIH FARMI MLEČNIH KRAVA U BISHOFTU, CENTRALNA ETIOPIJA

Muhabaw Yimana [1,2*] and Juhar Tesfaye Bekele[3]

[1] Univerzitet u Adis Abebi, Koledž za veterinarsku medicinu i poljoprivredu, Etiopija
[2] Kancelarija za poljoprivredu Sekota, Amhara, Etiopija
[3] Etiopski institut za poljoprivredna istraživanja,
Poljoprivredni istraživački centar Werer, Etiopija

Kratak sadržaj

U periodu od januara do maja 2018. sprovedena je studija preseka na mlečnim kravama u fazi laktacije u gradu Bishoftu. Ciljevi istraživanja bili su izolacija *Corynebacterium bovis*, određivanje prevalence i faktora rizika, kao i procena efikasnosti nekoliko antibiotika na mlečnim farmama tokom perioda laktacije. Ispitivane životinje odabrane su sa farmi u datoj oblasti metodom slučajnog izbora. Ispitivanje je vršeno u skladu sa procedurama koje su obuhvatile prikupljanje uzoraka mleka od krava sa mastitisom, kultivaciju i sprovođenje testa osetljivosti na antibiotike. Ukupno je ispitano 384 mlečne krave u laktaciji, što je podrazumevalo pregled i California Mastitis Test (CMT), gde je ustanovljeno da je 86 životinja bilo CMT-

pozitivno. Shodno tome, utvrdjen je stepen prevalencije od 3,9% kod krava sa kliničkim i 18,5% kod krava sa subkliničkim mastitisom. Utvrđene su statistički značajne razlike u pogledu stope prevalentnosti mastitisa u odnosu na stadijum laktacije, rasu, starost i pranje vimena ($p > 0.05$). Statistički značajne razlike nisu ustanovljene u odnosu na način uzgoja ($p > 0.05$).Od ukupno 86 uzoraka pozitivnih na mastitis (uzorak se odnosi na mleko od jedne krave) poslatih na ispitivanje u mikrobiološku laboratoriju, 7 bakterijskih izolata identifikovano je kao *Corynebacterium bovis.* Ustanovljeno je da se biohemijske i morfološke karakteristike 7 (1,8%) izolata *C. bovis* izolovanih iz uzoraka kravljeg mleka podudaraju sa referentnim sojevima *C. bovis*. Prisustvo katalaze i oksidaze, produkcija kiselina iz glukoze i potreba za obogaćenim hranljivim podlogama primenjeni su kao validni kriterijumi za identifikaciju bakterijskog mikroorganizma. Izolati *C. bovis* pokazali su veću osetljivost na kanamicin i streptomycin (71,4% na oba). Uočen je određeni stepen rezistencije na oksitetraciklin (71,4%) i nalidiksičnu kiselinu (42,8%). Veći broj izolata pokazao je umerenu senzitivnost ili rezistentnos na amoksicilin (51,1%). Što se tiče multiple rezistencije na lekove, studija je pokazala da je samo jedan izolat (14,3%) bio rezistentan na četiri leka – kanamicin, amoksicilin, nalidiksičnu kiselinu i oksitetraciklin. Ovo istraživanje je pokazalo da je mastitis izazvan *C. bovis* retka pojava na farmama mlečnih krava u fazi laktacije u oblasti grada Bishoftu. Neki od faktora rizika za mastitis mogu se kontrolisati kroz adekvatno upravljanje na farmi. Vlasnici farmi bi trebalo selektivno da koriste antibiotike na koje bakterije ne pokazuju rezistenciju, na primer streptomicin i kanamicin.

Ključne reči: *Corynebacterium bovis,* CMT, farma mlečnih krava, mastitis, prevalentnost

INTRODUCTION

Ethiopia has high livestock production potential and is a home for around 61.6 million cattle (55.23% female and 44.77% male), 69.7 million sheep and goats, 11.7 million equines, 48.2 million poultry and 3.8 million camels. However, the country is not benefiting from the livestock sector as its production potential. Along with drought, feed and water shortage, and genetic factors of the animals, animal diseases are the most common constraints to the sector in particular and the country in general. Livestock diseases can cause death of animals, loss of weights, slow down growth, poor fertility performance, decrease in physical power and the likes (CSA, 2020). Bovine mastitis is the sec-

ond most frequent disease next to reproductive disorders and one of the major causes for economy failure in Ethiopia. It affects both the quantity and quality of milk. Mungube (2001) calculated the cost of mastitis in Addis Ababa's urban and peri-urban districts to be 210.8 Ethiopian Birr per cow per lactation. Aside from the financial implications, there is a risk that bacterial contamination of milk from infected cows will make it unfit for human consumption by causing food poisoning or providing a pathway for disease transmission to humans. Brucellosis and tuberculosis can be transmitted to humans in this way (Radostits et al., 2000).

In Ethiopia, mastitis prevalence rate was 85.6% and 81.2% using CMT and somatic cell count (SCC), respectively (Husien et al., 1999). An overall prevalence of 30.2% and 5.5% was obtained for subclinical and clinical mastitis, respectively, in a study conducted in urban and per-urban dairy production system in and around Addis Ababa. In addition, 43 and 75% prevalence rates of bovine mastitis were reported in different parts of Ethiopia (Mekibib et al., 2010).

Mastitis induced via pathogenic microorganisms generally come from two sources: the environment such as *Escherichia coli*, *Enterobacter* and *Klebsiella* acquired by exposure of the teat to contaminated environment, or the animal itself like *Staphylococcus aureus* and *Streptococcus agalactiae*, Coagulase negative *Staphylococcus, Micrococcus* species, *Corynebacterium* species, *Bacillus* species, *Pasteurella* species, *Mycoplasma* etc. (Workineh et al., 2002). *Corynebacterium bovis* is the most frequently isolated *Corynebacterium* spp. from bovine intramammary infections (IMI) (Woodward et al., 1990). *C. bovis* readily colonizes the teat canal of dairy cows and has been used as an indicator of milking hygiene. It is not uncommon for *C. bovis* to be isolated from over 60% of quarter milk samples in herds where post milking teat antisepsis is not used. Indeed, the rate of new *C. bovis* IMI was about 30 times higher than that of *Streptococcus agalactiae* under experimental challenge settings. However, rather than real IMI, this high infection rate was thought to be attributable to teat canal colonization and subsequent contamination of milk samples (Woodward et al., 1990). This high reliance on presumptive identification has limited the ability of most mastitis microbiology laboratories to recognize *Corynebacterium* spp. (Hogan et al., 1999).

In recent years, antimicrobial resistance has been a growing concern worldwide (WHO, 1997, 2000). Acquired antimicrobial resistance in bacteria is an increasing threat in human as well as in veterinary medicine. Hence, monitoring antimicrobial susceptibility in pathogenic as well as in commensal bacteria in animals is recommended by OIE (Acar and Rostel, 2001). Such monitoring

generates data of importance for therapeutic decisions and provides information on trends in resistance that might be cause for interventions regarding antimicrobial use. Mastitis is one of the most costly diseases for the dairy industry (Kossaibati and Esslemont, 1997) and antimicrobials are important parts of therapy of the disease. Susceptibility tests of milk samples submitted to state diagnostic laboratories that use the disk-diffusion method have demonstrated remarkable agreement but vary from results of a small survey processed using broth dilution (Constable and Morin, 2003).

Ethiopia holds large potential for dairy development due to its large livestock population the favorable climate for improved high-yielding animal breeds, and the relatively disease free environment for livestock. Given the considerable potential for smallholder income and employment generation from high value dairy products, development of the dairy sector in Ethiopia can contribute significantly to poverty alleviation and nutrition in the country (Ahmed, et al., 2003). Various authors have indicated that mastitis is a major problem in Ethiopia. However, works on pathogen specific mastitis particularly that of *C. bovis* and their effect on milk production is insufficient. Therefore, the objectives of this study were: To isolate and characterize *C. bovis* and test its antimicrobial susceptibility from dairy cows with mastitis, and to estimate prevalence of mastitis in lactating dairy cows in Bishoftu dairy farms.

MATERIAL AND METHODS

Study Area

The study was conducted in Bishoftu town from January to May 2018. Bishoftu is located at 9°N and 40°E, in Oromia National Regional State about 47 km southeast of the capital city of Ethiopia, Addis Ababa. The altitude is about 1850m above sea level. It experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (of which 84% of rain is expected) and a short rainy season from March to May with an average annual rainfall of 800mm. The mean annual minimum and maximum temperatures are 12.3°C and 27.7°C, respectively, with an overall average of 18.7°C. The highest temperatures recorded in May and the mean relative humidity is 61.3%. Bishoftu is the center of Ada‟a Liben woreda. The Woreda has a total land area of about 1610.56 Km^2 and is divided into three agro-ecological zones: the midland (94%), highland (3%) and lowland (3%) (ADARDO, 2011).

Study Animals

The study populations included milking cows found in Bishoftu, selected from smallholder (< 5 cows), medium sized (5-16 cows) and large sized (>16 cows) private and government-owned dairy farms. The sampling animals were selected randomly from the selected dairy farms in the town.

Study Design

A cross-sectional study was carried out from January 2018 to May 2018 and bacteriological analysis of milk samples from mastitis infected dairy cattle found in Bishoftu town was performed.

Sample size

The total numbers of study animals required for the present study were calculated based on the formula given by Thrusfield (1995). As there was no previous information available in the study area, 50% expected prevalence was taken for sample size determination. Moreover, 5% level of precision and 95% of confidence interval were used to calculate the sample size. Accordingly, a total of 384 animals were considered as sample size during the study period.

Milk Sample Collection

Composite samples of approximately 10 ml of fresh milk were collected from each cow before milking using sterile tight-seal sampling bottles to avoid leakage and contamination. From each farm, one sample per animal was taken by mixing the milk from all quarters. Before beginning with sample collection, loose dirt, bedding, and hair from the udder and teats were brushed with a hand and excessively dirty udder, the teats were washed with lukewarm water thoroughly dried with a towel, and disinfected with 70% ethyl alcohol. Before collecting milk samples from each quarter, the first two streams of milk were discarded, and the milk and udder were examined for evidence of clinical mastitis. Between milking two cows, hands were cleansed in a sanitizing solution, and gloves were used if contagious diseases were anticipated. (Quinne et al., 1999). The sample was taken and stored in an ice box before being transported to the Microbiology Laboratory of the College of Veterinary Medicine and Agriculture for bacterial culture and isolation.

CMT Screening

Abnormal milk, milk clots, gland swelling, and cow disease are the signs of clinical mastitis, while CMT was used to identify subclinical mastitis. CMT was carried out according to the method described by Quinne et al., (1999). Each of the four shallow cups in the CMT paddle received roughly 2 ml of milk sample from each quarter. On each cup, an equal amount of CMT reagent was added, and the mixtures were gently rubbed together in a horizontal plane for no more than 15 seconds. The existence of subclinical mastitis was revealed by the coagulation and viscosity of the mixture.

Bacteriological techniques

Positive milk samples (clinical and sub-clinical) were collected from cows and cultured bacteriologically following standard microbiological procedures (Quinn et al., 1994). For primary isolation, cultivation, and detection of bacterial hemolytic reaction, blood agar (BBL^{R}, Becton Dickinson, USA) was prepared. Bacteria were isolated by streaking one standard loop of milk (0.01ml) over the surface of blood agar supplemented with 5% sheep blood. The inoculated blood agar plates were placed in an incubator at 37°C for 48 h. Identification of Corynebacteria isolated from bovine mammary glands has been largely based on colony morphology, hemolysis, and growth requirements. The presence of small, white and non-hemolytic colonies on 5% sheep blood agar after 48 h of incubation at 37°C indicates *C. bovis.* Furthermore, *C. bovis* tends to grow nicely only in regions of visible milk fats because it requires oleic acid. From subculture blood agar plates, colonies were streaked onto nutrient agar (Oxoid, Hampshire, England) for better colony characterization, biochemical checks and sample preservation. The presumptive Corynebacterium colonies, Gram positive, catalase positive and oxidase positive bacilli that passed via Gram stain, catalase and oxidase checks have been similarly purified by sub culturing onto nutrient agar and the plates were incubated aerobically at 37°C for 24 h.

The differentiation between *Corynebacterium* and other mastitis-causing microorganisms were performed using a tube triple sugar iron (TSI) test and Methyl Red broth. Corynebateria can overcome the buffering potential of the media by producing massive quantities of a stable acid end product from glucose fermentation, hence lowering the PH.

Antimicrobial susceptibility test

The antimicrobial resistance patterns of the isolates were determined according to Kirby-Baur disc diffusion method (Quinne et al., 1999, NCCLS, 2012). Antimicrobial susceptibility tests were performed using a broth micro dilution method (Sensititre, Westlake, Ohio), except that the Mueller-Hinton agar was not supplemented with 1% Tween 80 (Watts and Rossbach, 2000). The antimicrobial susceptibility test panel contained the following antimicrobial agents: kanamycin (30µg), streptomycin (10µg), amoxicillin (10µg), nalidixic acid (30µg), oxytetracline (30µg), and ceftriaxone (30µg) (Thermo Scientific™, Oxoid™) were tested by disc diffusion on Mueller-Hinton agar plates (BBL®, Becton Dickinson, USA).

A suspension with a density equivalent to that of a 0.5 McFarland standard inoculums was prepared in 0.9% saline to achieve a final density of the standard. The suspension was applied onto the surface of the Mueller-Hinton agar plates with a swab, and antibiotic disks were applied onto the surface of the inoculated Mueller-Hinton agar plates using aseptic technique (Quinne et al., 1994). The results were recorded after 24 h of incubation at 37°C and interpreted according to the guideline (NCCLS, 2012).

Data Management and Analysis

The data obtained from this study were compiled, entered to Microsoft Excel work sheet and analyzed with Statistical Package for Social Science (SPSS) 20. Descriptive statistics such as percentage, frequency and cross tabulation distribution were used to describe the nature and characteristics of generated data on the rate of bacterial isolation and resistant patterns of the bacterial isolates. A confidence level of 95% was used to interpret statistical associations. Categorical variables were compared by using chi-square tests. *P* - Value was calculated and $p > 0.05$ was taken as statistically significant.

RESULTS

A total of 384 lactating dairy cows were examined with inspection and CMT for detection of clinical and subclinical mastitis, respectively. Of the total lactating cows examined, overall mastitis prevalence in the area was 22.4% (68/384). The results showed that the prevalence rates of clinical and subclinical mastitis were 3.9% and 18.5%, respectively (Table 1).

Table 1. The overall prevalence of bovine mastitis

Mastitis condition	No. cows Examined	No. of positive (%)
Clinical mastitis	384	15 (3.9)
Subclinical	384	71 (18.5)

The result showed that the effect of lactation stage was statistically significant ($p > 0.05$) for the prevalence of bovine mastitis, and the infection rate was high in animals in early (49.1%) and late (68.4%) lactation stage as compared to the mid lactation stage (3.1%). Animals managed in semi-intensive husbandry practice showed high rate of infection (27.8%) as compared to those managed intensively (21.8%). The infection rate was lower in crossbred dairy cows (7.1%) than in those of Holstein Friesian (75%) and local breeds (11.8%). The other variable in the study was the age, and the study revealed the following values for dairy cows < 3 years (1.2%), 4-8 years (11.4%) and those > 8 years (81.9%), thus, as the age increases the incidence of mastitis also increases. In the present study, from selected potential risk factors the breed ($p > 0.05$), stage of lactation ($p > 0.05$), and age ($p > 0.05$) had statistically significant effect, but animal husbandry practice had no significant effects on the prevalence of mastitis ($p > 0.05$) (Table 2).

Table 2: Prevalence of bovine mastitis in relation to lactation stage, breed, husbandry and age

Risk factors		No. of cows examined	No. of positive (%)	X^2	*P* - value
Lactation Stage	Early	53	26 (49.1)	167.8	0.00
	Mid	255	8 (3.1)		
	Late	76	52 (68.4)		
Husbandry	Intensive	348	76 (21.8)	0.662	0.406
	Semi-intensive	36	10 (27.8)		
Breed	Holstein Friesian	84	63 (75)	171.56	0.00
	Cross-bred	266	19 (7.1)		
	Local	34	4 (11.8)		
Age	<3 years	83	1 (1.2)	184.41	0.00
	4-8 years	229	26 (11.4)		
	>8 years	72	59 (81.9)		

In the majority of mastitis, bacteriological techniques are used for phenotypic characterization to presumptively identify *C. bovis*. Basically, the organisms that exhibit a small white non-hemolytic colony type after 48 h of incubation in the area where butterfat was deposited on agar surface were presumptively considered to be *C. bovis*.

Out of 86 CMT positive samples, 57 (66.3%) organisms were identified presumptively as coryneform bacteria and had similar biochemical reactions and were consistent with the *C. bovis* reference strain. All or almost all were small, circular with regular edges, white to cream in color, and non-hemolytic after 48 h incubation on blood agar enriched with 5% sheep blood. Colonies usually appear on the first strike, which is due to the lipophilic nature of the bacterium.

All strains were Gram positive, catalase positive and oxidase positive. The remaining 29 strains were identified as coryneforms based on Triple Iron Sugar (TSI) test. The study showed that coryneform bacteria ferment glucose and cause acid production, but no acid production was detected using carbohydrates lactose and sucrose as substrates (Table 3). The isolates identified as coryneform bacteria were further identified based on Methyl Red and Voges-Proskauer (MR-VP) test. Gas production was detected in 2 isolates.

Table 3: Summary of the metabolic and physiological characteristics of presumptive *C. bovis*

Test or Substrate	***C. bovis* isolates**	**No. of isolates**
Growth on blood agar with 5% sheep blood	+	
Morphology on blood agar	Small white, non-hemolytic colony	54
Gram stain	Gram positive, rod shaped	45
Catalase test	+	40
Oxidase test	+	36
Acid production from		
Glucose	+	29
Lactose	-	
Sucrose	-	
Methyl Red	-	7

Key: (+) = positive, (-) = negative

Information on the susceptibility of coryneform bacteria to antimicrobial agents is scarce. The study of the frequency of susceptibility of *C. bovis* (n = 7) to antibiotics revealed higher sensitivity to kanamycin and streptomycin (71.4% each). A certain resistance has been noted to oxytetracycline (71.4%) and nalidixic acid (42.8%). Higher number of isolates showed moderate sensitivity or resistance to amoxicillin (51.1%). Regarding to multidrug resistance, the study reflects that only one isolate (14.3%) shows multidrug resistance pattern. Susceptibility and resistance patterns of each bacterial isolates are shown in Table 4.

Table 4: Frequency of susceptibility of *Corynebacterium bovis* (n = 7)

Bacterial strains		***Corynebacterium bovis* (n=7)**	
Antibiotic agent	S %	IM %	R %
Kanamycin	71.4	14.3	14.3
Streptomycin	71.4	0	28.6
Amoxicillin	14.3	51.1	28.6
Nalidixic acid	28.6	28.6	42.8
Oxytetracycline	14.3	14.3	71.4
Ceftriaxone	28.6	42.8	28.6

Key: S=Susceptible, I= Intermediate, R=Resistant

From the total *C. bovis*, 14.3% were resistant to one drug, 57.14% to two drugs and 14.3% were to more than two drugs.

DISCUSSION

This study showed the overall prevalence of mastitis associated with *Corynebacterium bovis* in lactating dairy farms to be 1.8%, which is lower than most of the previous reports in Ethiopia and is in agreement with the bovine mastitis reported by Dabash et al. (2014) with the prevalence of bovine mastitis of 2% in North Showa of Ethiopia. This finding is also comparable with the findings of Belayneh et al., (2014) in East Showa zone, Akaki district, Ethiopia (1.2%); Moges et al., (2011) in and around Gonder (2.4%) and Oalekish et al. (2013) in northern Jordan (3.9%). This finding slightly differed from earlier investigations. In other regions in Ethiopia, Lidet et al. (2013) reported 0.52% isolation rate. The high isolation rate in this study could be associated with lowered resistance of the cow due to teat injury.

Clinical mastitis rate was low in all breeds as compared to subclinical mastitis. The prevalence of clinical mastitis in the present study (3.9%) was comparable to the reports from different dairy farms: 6.3% in Bahir Dar (Gizat et al., 2007), and 7.9% in commercial farms in Ethiopia (Abaineh and Sintayehu, 2001). However, the present finding is by far lower than the reports of Kerro and Tarekegn (2003) in local, Friesian and Jersey cows in Sothern Ethiopia (12.1%), and 14.2% in lactating cows in smallholder farms in Tanzania (Kivaria et al., 2004). Mastitis is a complex disease and the difference in results could be due to variations in herd size, management practices, proportion of exotic gene inheritance, and agro-climates. Other risk factors might also have contributed to the observed differences in prevalence rates of mastitis among the findings of various authors.

Significant differences between the high-grade Holstein-Friesian, Holstein indigenous zebu crossbred and local zebu might be associated with their high milk yield. Radostits et al. (2007) stated that high-yielding cows are more susceptible to mastitis than the low-yielding ones.

Significant effect of stage of lactation on the prevalence of mastitis was confirmed in this study, being 49.15%, 3.1% and 68.4% in early, mid and late lactation, respectively, also reported by Nesru (1999), Mungube et al. (2005) and Kerro and Tarekegn (2003) in Ethiopia. The former two authors reported high prevalence of subclinical mastitis in cows at early and late stage of lactation as it is the case in this finding, while the last two reported higher prevalence at early stage of lactation. The variations in the effect of lactation stages between different studies could be related to the disparities in age, parity and breed of the sampled animals.

The present study was also undertaken to determine the resistance pattern of bovine mastitis due to *C. bovis* to commonly used antimicrobials in the study area and to provide information to concerned animal health professionals. The selection of the types of antimicrobial agents was based on clinical considerations including frequent use of the drug in the study area and availability. Oxytetracycline was commonly used antimicrobial for the treatment of mastitis in the study area.

The poor inhibitory effect of oxytetracycline against *C. bovis* strains identified in this study is in agreement with the data reported by Oalekish et al. (2013). The latter reported sensitivity to oxytetracycline in 12% of *C. bovis* isolates from subclinical mastitis in northern Jordan. In our study, *C. bovis* isolates showed most susceptibility to kanamycin and streptomycin (71.4% each). Nibret et al. (2011) obtained comparable results, where kanamycin was effective against *C. bovis* (100%). In the present study, unlike Oalekish et al. (2013), streptomycin was effective against *C. bovis* (71.4%). Out of the seven isolates

investigated in the present study, only one isolate (14.3%) showed multidrug resistance to kanamycin, amoxicillin, nalidixic acid and oxytetracycline.

In general, streptomycin and kanamycin showed very good efficacy, nalidixic acid and ceftriaxone showed moderate efficacy, whereas oxytetracycline and amoxicillin showed poor efficacy in almost all isolates.

CONCLUSION AND RECOMMENDATIONS

Bovine mastitis is an inflammatory response of the mammary gland. It has a major impact on animal production, animal welfare and milk quality. Mastitis is one of the biggest problems for dairy industry because of high morbidity and significant economic losses. Even though, the prevalence of *C. bovis* is lower than that of other common pathogens, it causes mostly subclinical mastitis and has substantial impact on the dairy industry. Milk from cows with subclinical mastitis accidentally mixed into bulk milk enters the food chain, and poses a threat to human health. The low prevalence of C. *bovis* did not restrict the organism to show resistance to antibiotics such as oxytetracycline, nalidixic acid and others, emphasize the need for serious and immediate attention towards consumers of raw milk. On the other hand, some antibiotics such as kanamycin and streptomycin were found to be effective against *C. bovis.* The appropriate and principled use of those antibiotics might help in the control and limitation of resistance against those strains. Based on the above finding and closing remarks, the following recommendations are forwarded:

- Future work need to be done in the field of isolation, characterization and prevalence of coryneform bacteria and consequently, the responsible important strains could be identified.
- Antimicrobial susceptibility test should be conducted at regular intervals to understand the development of resistance against the commonly used antibiotics.
- Proper hygienic and improved management practices should be introduced at farm level.

Author's Contribution:

MY made contributions to conception and design of the study, involved in data collection, data curation, biochemical test and culturing the bacteria, data analysis and drafting the manuscript. JT revised the manuscript critically and together with MY prepared the final draft of the manuscript. Both authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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SEROPREVALENCE OF *CHLAMYDOPHILA ABORTUS* IN SHEEP IN THE BELGRADE EPIZOOTIOLOGICAL AREA DURING 2019-2021

Zorana Zurovac Sapundžić[1*], Milan Ninković[1], Dragica Vojinović[1], Branislav Kureljušić[1], Jadranka Žutić[1]

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

Abstract

As one of the most important infective agents of abortion, *Chlamydophila abortus* takes an important place in pathology of ruminant reproductive tract. In sheep, the disease known as enzootic abortion of ewes or ovine enzootic abortion, and is manifested as abortion and accompanied reproductive disorders, thus resulting in significant economic losses worldwide. The characteristics of the pathogen and its zoonotic potential make this disease significant both for animal and public health. Therefore, the presented study aimed to obtain *Chlamydophila abortus* seroprevalence in sheep population in Belgrade epizootiological area. The study was done by testing 552 sheep sera samples from 10 municipalities of Belgrade city, during the 2019-2021 period. Serological examination was performed using ELISA assay (ID Screen® *Chlamydophila abortus* Indirect Multi-species, IDvet, Grabels, France). The obtained results showed *Chlamydophila abortus* seroprevalence of 6% in Belgrade epizootiological area. The largest number of seropositive sheep was found in municipality of Palilula. The detected antibodies against *Chlamydophila abortus* confirmed the circulation of the pathogen in sheep population in Belgrade epizootiological area. The obtained results show the need for further studies and continuous implementation of measures for detection, control, prevention and eradication of the disease.

Key words: *Chlamydophila abortus*, enzootic abortion of ewes, sheep, seroprevalence, Serbia

[1*] Corresponding Author: zorana991@hotmail.com

SEROPREVALENCIJA *CHLAMYDOPHILA ABORTUS* KOD OVACA NA BEOGRADSKOM EPIZOOTIOLOŠKOM PODRUČJU U PERIODU 2019-2021 GODINE

Zorana Zurovac Sapundžić[1*], Milan Ninković[1], Dragica Vojinović[1], Branislav Kureljušić[1], Jadranka Žutić[1]

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

Kratak sadržaj

Kao jedan od najznačajnijih infektivnih agensa koji uzrokuju pobačaj *Chlamydophila abortus* zauzima značajno mesto u patologiji reproduktivnog trakta preživara. Kod ovaca, ovo oboljenje je poznato kao enzootski pobačaj ovaca (EAE ili OEA) i manifestuje se pobačajima i pratećim reproduktivnim poremećajima, praveći značajne ekonomske gubitke širom sveta. Karakteristike patogena i njegov zoonozni potencijal čine ovu bolest od velikog značaja ne samo za životinje, već i za javno zdravlje. Stoga je cilj ove studije utvrditi seroprevalenciju *Chlamydophila abortus* u populaciji ovaca na beogradskom epizootiološkom području. Ukupno su testirana 552 uzorka seruma ovaca sa 10 opština grada Beograda, od 2019. do 2021. godine. Serološka dijagnostika je rađena primenom komercijalnog ELISA testa (ID Screen® *Chlamydophila abortus* Indirect Multi-species, IDvet, Grabels, Francuska). Dobijeni rezultati su pokazali seroprevalenciju Chlamydophila abortus od 6% na beogradskom epizootiološkom području. Najviše seropozitivnih ovaca ustanovljeno je na opštini Palilula. Utvrđeno prisustvo antitela protiv *Chlamydophila abortus* potvrdilo je cirkulisanje ovog patogena u populaciji ovaca na teritoriji beogradskog epizootiološkog područja. Dobijeni rezultati ukazuju na potrebu za daljim istraživanjima i kontinuiranom sprovođenju mera za detekciju, kontrolu, prevenciju i eradikaciju ove bolesti.

Ključne reči: *Chlamydophila abortus*, zarazni pobačaj ovaca, ovce, seroprevalencija, Srbija

INTRODUCTION

Chlamydophila abortus is an obligate, intracellular, gram-negative bacteria from the *Chlamydiaceae* family, recognized as the species responsible for enzootic abortion since 1950 (Stamp et al., 1950). The pathogen was previously identified as *Chlamydia psittaci*, serotype 1 (Sachse et al., 2015). *Chla-*

mydophila abortus is one of the most common causes of infectious abortion in ruminants present worldwide, except in Australia and New Zealand. Only in the UK, the disease in sheep is accounted for 44% of diagnosed abortions due to infectious agents, and more than 56% of small ruminant abortions in Spain (García-Seco et al., 2016). In the UK, resulting economic losses are estimated at nearly £20 million each year (Longbottom et al., 2002).

In sheep, the disease is known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), causing various reproductive failures, such as abortion, premature or stillbirths, birth of dead or weak lambs with low body weight (Aitken and Longbottom, 2007). Mummification and maceration of the foetuses were also observed. The abortions occur mostly in the last 2-3 weeks of pregnancy, while ewes that have aborted once do not normally abort again as a result of this infection. In non-gravid animals the disease passes as persistent subclinical form. Serohemorrhagic vaginal discharge can be the only clinical symptom, present just occasionally, so the infection often passes unnoticed allowing continuous spread of the pathogen. *Chlamydophila abortus* can cause orchitis and seminal vesiculitis in rams, hence males participate in shedding the pathogen through semen, too (Longbottom and Coulter, 2003). Newborns get infected during the passage through the birth canal of the infected mother (in second gestation), or congenitally in-utero. The transmission of the pathogen is through direct contact with aborted foetuses or genital tract secretions of infected animals, as infected placenta and uterine discharge contain the highest amounts of the pathogen. Moreover, after abortion the pathogen can be shed for several weeks (Rodolakis et al., 1998). Indirectly, the pathogen can be disseminated through contaminated feed and water.

The infection is most commonly diagnosed either by the detection of the specific antibodies using commercially available ELISA kits (or even CFT), or directly by detecting the pathogen, using molecular techniques (conventional and real-time PCR).

Considering its zoonotic potential, all occupationally related staff that are in contact with infected animals or infected material, are at risk of the infection that can be expressed through various manifestations ranging from subclinical infection to influenza-like illness. The disease is particularly dangerous for pregnant women, who are at the highest risk regarding the ability of *Chlamydophila abortus* to colonize the human placenta (Longbottom and Coulter, 2003).

To this day, the data regarding *Chlamydophila abortus* infection on the territory of Serbia are scarce. Therefore, aim of the presented study was to obtain the seroprevalence of *Chlamydophila abortus* in sheep population on Belgrade epizootiological area during the 2019-2021 period and provide novel, updated information about this significant disease.

MATERIAL AND METHODS

In total, 552 sera samples collected from sheep residing in Belgrade epizootiological area, including 10 municipalities, were taken from sera bank between 2019 and 2021 and consequently tested in order to obtain the seroprevalence of *Chlamydophila abortus*. For the purpose of detection of antibodies against *Chlamydophila abortus,* ELISA assay (ID Screen® *Chlamydophila abortus* Indirect Multi-species, IDvet, Grabels, France) was performed, according to the manufacturers' instructions. The optical density was measured with ELISA reader (Tecan), and the samples were considered as positive when S/P ratio was above the cut-off value of 60%. The specificity and sensitivity of the used ELISA kit for small ruminants are 99.5% and 80%, respectively.

RESULTS AND DISCUSSION

The results of the study revealed the presence of the pathogen in Belgrade epizootiological area. The obtained overall *Chlamydophila abortus* seroprevalence in sheep population in Belgrade epizootiological area is 6%. The largest number of *Chlamydophila abortus* seropositive animals (14) was confirmed in municipality of Palilula, while no *Chlamydophila abortus* seropositive animals were found in Obrenovac municipality. The *Chlamydophila abortus* seroprevalence results are presented in Table 1 and 2.

Table 1. The seroprevalence of *Chlamydophila abortus* in sheep population in the Belgrade epizootiological area from 2019 to 2021

Municipality	No. of tested sera	Positive reactions	
		No.	%
Barajevo	54	3	5.5
Grocka	55	4	7.3
Lazarevac	58	2	3.4
Mladenovac	54	2	3.7
Obrenovac	60	0	0
Palilula	60	14	23.3
Sopot	57	1	1.7
Surčin	57	1	1.7
Voždovac	50	2	4
Zemun	47	4	8.5
Total	**552**	**33**	**6.0**

Table 2. The seroprevalence of *Chlamydophila abortus* in sheep population in the Belgrade epizootiological area per year

Year	No. of tested sera	Positive reactions	
		No.	%
2019	184	17	9.2
2020	184	7	3.8
2021	184	9	4.9

As one of the most important infectious agents causing abortions, *Chlamydophila abortus* has severe consequences for both animal health and economy. The available data regarding *Chlamydophila abortus* infection on the territory of Serbia are relatively poor. The study covering the three-year-period reported the *Chlamydophila abortus* seroprevalence of 18.6% in 367 tested sera samples from sheep that have aborted (Vidić et al., 2007). The mentioned study was carried out on the territory of AP Vojvodina, in the north of the country. Studies covering other parts of the country have not recently been conducted.

Reported seroprevalence data on *Chlamydophila abortus* in sheep population throughout Europe are various. In Slovak Republic the reported seroprevalence was 11.7% (Cisláková et al., 2007), while in Italy it ranged from 21% to 46.7% (Masala et al., 2005). In neighbouring Bosnia and Herzegovina, the reported *Chlamydophila abortus* seroprevalence in sheep was 43.3% (Krkalić et al., 2016), while in Croatia, the seroprevalence detected in sheep was 19.6%, i.e. 20.5% depending of the kit used (Špičić et al., 2015). The results of our study have shown the *Chlamydophila abortus* seroprevalence in sheep at a lower level of 6%. The results between different municipalities varied from 0 to 23.3%. The most seropositive animals were found in municipality Palilula (14), while no *Chlamydophila abortus* seropositive animals were detected in Obrenovac.

A group of authors, using the same ELISA test kit in Belgium have established seroprevalence of *Chlamydophila abortus* in sheep in the range from 0 to 4.05% in nine different Belgian regions. The established overall seroprevalence rate was of 0.68%, while it was of 6.15% on the herd level (Yin et al., 2014). It was interesting that only herds with fewer than 50 sheep were seropositive, indicating a higher seroprevalence rate in smaller herds (Yin et al., 2014). Contrary to this, in the UK the number of reported cases in sheep was higher on bigger farms with more than 150 animals, compared to those in smaller herds (Longbottom et al., 2012).

Urban environment of Belgrade city and its municipalities, where most of the herds are of smallholder type, could potentially play an important role in explaining lower seroprevalence. Relatively small sample size should also be taken into account. Still, correlation between the herd size and established seropositivity should be investigated further.

Although low *Chlamydophila abortus* seroprevalence was established, the necessity for further continuous implementation of measures for detection, control, prevention and eradication of the disease on the territory of Serbia is advised. Furthermore, according to the current legislation of Veterinary Directorate, Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, every abortion in sheep must be notified and examined for *Chlamydophila abortus* (Official Gazette of the Republic of Serbia, No. 36/2021). Animal owners are required to report every abortion, whereby the further, prescribed testing will be consequently performed. Although not implemented in our country, vaccines against EAE have shown various efficiency. However, outbreaks have been reported even in vaccinated flocks (Jones et al., 1995). Nevertheless, the incidence of abortions and the period of shedding the pathogen were reduced (Montbrau et al., 2020).

Along with its endemic persistence, the disease is difficult to control regarding its periodic recurrence and maintenance of the pathogen in the flock and host animals. Further studies regarding the infection in ruminants on the territory of Serbia are essential in order to ensure more detailed information about *Chlamydophila abortus* infection. Beside serology, molecular diagnostic should provide additional important information.

CONCLUSION

The presented results confirmed the presence of *Chlamydophila abortus* in sheep population in Belgrade epizootiological area. The obtained *Chlamydophila abortus* seroprevalence is of 6%. The results of our study provided novel and updated information about this significant infection regarding the territory of our country.

Further continuous implementation of measures for detection, control, prevention and eradication of *Chlamydophila abortus* on the territory of Serbia are significant for both animal and public health.

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Author's Contribution:

ZZS and MN drafted the manuscript and performed the laboratory tests. JŽ designed the study and together with DV and BK coordinated the work. JŽ and BK revised the manuscript critically and prepared the final draft of the manuscript.

Competing interest

The authors declare that they have no competing interests.

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SEROPREVALENCE OF PORCINE RESPIRATORY CORONAVIRUS AND TRANSMISSIBLE GASTROENTERITIS VIRUS INFECTIONS ON COMMERCIAL PIG FARMS IN CENTRAL SERBIA

Dimitrije Glišić[1*], Ljubiša Veljović[1], Jelena Maksimović Zorić[1], Branislav Kureljušić[1], Milan Ninković[1], Bojan Milovanović[1], Vesna Milićević[1]

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

Abstract

Porcine respiratory coronavirus is an enzootic, viral, respiratory disease of pigs, which manifests with mild clinical signs, but it takes part in the etiopathogenesis of the porcine respiratory disease complex. The virus was first discovered in Belgium in 1984 as a deletion mutant of the transmissible gastroenteritis virus. The two viruses are strongly antigenically related which is why they cross-react in serological tests. In this study, we tested 276 serum samples from different categories of pigs using ELISA test, which allows differentiation between the porcine respiratory coronavirus infection and transmissible gastroenteritis. The seroconversion for coronavirus infection was determined in 80.4% of tested samples. Out of 222 positive samples, 219 samples (98.6%) were positive for porcine respiratory coronavirus antibodies, while 3 (1.01%) samples were positive for transmissible gastroenteritis virus antibodies. Depending on the production category, 97.7% of piglets, 83% of sows, and 35% of gilts tested positive for porcine respiratory coronavirus antibodies. In total, 2.3% of piglets tested positive for transmissible gastroenteritis virus antibodies. Taking into account the characteristics of the ELISA test, its sensitivity and specificity, this result can be considered a false positive, because of a cross-reaction between the porcine respiratory coronavirus antibodies and the transmissible gastroenteritis virus. Specific antibodies in other swine production categories against the transmissible gastroenteritis virus were not determined.

Key words: Porcine respiratory coronavirus, transmissible gastroenteritis virus, seroprevalence, Serbia

[1*] Corresponding Author: dimitrije.glisic@nivs.rs

SEROPREVALENCIJA INFEKCIJE SVINJA RESPIRATORNIM KORONA VIRUSOM I VIRUSOM TRANSMISIVNOG GASTROENTERITISA NA KOMERCIJALNIM FARMAMA SVINJA U CENTRALNOJ SRBIJI

Dimitrije Glišić[1*], Ljubiša Veljović[1], Jelena Maksimović Zorić[1], Branislav Kureljušić[1], Milan Ninković[1], Bojan Milovanović[1], Vesna Milićević[1]

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

Kratak sadržaj

Respiratorni korona virus svinja je uzročnik enzootskog respiratornog oboljenja svinja koje se manifestuje blagim kliničkim simptomima, ali učestvuje i u etiopatogenezi kompleksa respiratornog oboljenja svinja. Prvi put je otkriven u Belgiji 1984. kao delecioni-mutant virusa transmisivnog gastroenteritisa. Virusi su antigenski vrlo slični, usled čega unakrsno reaguju u serološkim reakcijama. U ovoj studiji, testirana su 276 uzorka krvnih seruma poreklom od svinja različitih proizvodnih kategorija komercijalnim ELISA testom koji omogućava razlikovanje infekcije respiratornim korona virusom od infekcije virusom transmisivnog gastroenteritisa. Kod 80,4% uzoraka, utvrđena je serokonverzija na korona virusnu infekciju. Od 222 pozitivna uzorka, 219 uzoraka (98,6%) je bilo pozitivno na specifična antitela protiv respiratornog korona virusa, dok su 3 (1,01%) uzorka bila pozitivna na antitela protiv virusa TGE. U zavisnosti od proizvodne kategorije, 97,7% prasadi, 83% krmača i 35% nazimica je bilo pozitivno na antitela protiv respiratornog korona virusa svinja. Kod 2,3% prasadi utvrđena su antitela protiv virusa transmisivnog gastroenteritisa. Uzimajući u obzir karakteristike ELISA testa, osetljivost i specifičnost, ovaj rezultat se može smatrati lažno pozitivnim, najverovatnije usled unakrsne reakcije antitela protiv respiratornog korona virusa i virusa transmisivnog gastroenteritisa. Pored toga, specifična antitela protiv virusa transmisivnog gastroenteritisa kod drugih kategorija svinja nisu ustanovljena.

Ključne reči: respiratorni korona virus svinja, virus transmisivnog gastroenteritisa svinja, seroprevalencija, Srbija

INTRODUCTION

Porcine respiratory coronavirus (PRCV) causes an enzootic, mild, respiratory disease in pigs. The first report of the virus was recorded after a prevalence study for transmissible gastroenteritis virus (TGEV) which revealed that over 60% of tested pigs had neutralising antibodies against TGEV without being vaccinated or developing the symptoms (Pensaert et al., 1986). It was reported that the virus that caused the infection was a mutant of TGEV, with a deletion (170-190 kDa) in the gene for spike (S) protein (Lin et al., 2015). Both PRCV and TGEV belong to the *Coronaviridae* family, and the *Alphacoronavirus* genus (International Committee on the Taxonomy of Viruses, ICTV.). Soon after the discovery of PRCV, it spread throughout the world suppressing TGEV in many regions (Whittaker, 2017). The TGEV causes gastrointestinal disease with a high affinity for the intestinal tract, and a high mortality rate among piglets. The deletion in the S gene resulted in a change in the spike protein of PRCV, thus inhibiting binding to the sialic acid and entering enterocytes (Turlewicz-Podbielska and Pomorska-Mól, 2021). The PRCV replicates in the lungs and causes a subclinical to mild infection of the respiratory tract, but induces a strong immunological response to the infection raising the levels of interferons and interleukins (Turlewicz-Podbielska and Pomorska-Mól, 2021; Keep et al., 2022). Since TGEV and PRCV are closely antigenically related, the antibodies against PRCV cross-react with the TGEV antigen thus protecting pigs against TGEV infection (Whittaker, 2017). Since its initial spread, PRCV has been reported as the dominant strain, with only a few sporadic outbreaks of TGE in Europe (Lőrincz et al., 2014)China (Zhenhui et al., 2015) and the USA (Chen et al., 2019). Coronaviruses have a high recombination rate (Whittaker, 2017), and there is a potential for a new mutant TGEV to arise, which could potentially break through the partial immunity acquired after PRCV infection. Recently there have been reports across Europe and Asia of novel swine enteric alphacoronavirus (SeACoV) (Pan et al., 2017) which represents a chimeric strain of TGEV genomic backbone and porcine epidemic diarrhoea virus (PDEV) spike protein (Akimkin et al., 2016; Boniotti et al., 2016).

There have been reports of difficulties in differentiating TGEV antibodies from PRCV antibodies, caused by the similarity in the antibody response to the viruses (Valkó et al., 2019). To circumvent this, various ELISA tests were created that would allow a quick assessment of serological status of the herd.

This study aimed to determine the seroprevalence of PRCV and TGEV infection in domestic swine on commercial farms in Central Serbia by testing different production categories of pigs for the presence of antibodies against PRCV and TGEV.

MATERIAL AND METHODS

Serum samples used in this study originated from Central Serbia, from commercial units, and were kept in the serum bank at the Serbian Institute of Veterinary Science. The total number of samples tested was 276. The samples were tested by blocking commercial ELISA test (INgezim Corona Diferencial 2.0, Ingenasa, Madrid, Spain), according to the manufacturer's instructions, and the optical density (OD) was measured at 450 nm with an ELISA reader (Multiscan, LabSystems). Based on the recommendations of the cut-off values by the manufacturer, the samples were considered positive or negative to PRCV or TGEV. The diagnostic sensitivity and specificity, according to the manufacturer, are 94% and 98.2% respectively (INgezim Corona Diferencial 2.0, 2022).

RESULTS AND DISCUSSION

Out of 276 tested samples, 222 (80.4%) samples were positive for coronavirus antibodies. For PRCV antibodies, 219 samples tested positive, which makes up 79.3% of the total samples and 98.6% of all positive samples. For TGEV antibodies, 3 samples were positive, which makes up 1.09% of the overall sample or 1.4% of all positive samples. Depending on the production category, 97.7% of piglets, 83% of sows, and 35% of gilts tested positive for porcine respiratory coronavirus antibodies (Table 1.).

Table 1. Table showing the number and percentage of positive and negative samples according to the production category.

Production category of pigs	Number of tested samples	Number of positive samples (%) for PRCV antibodies	Number of positive samples (%) for TGEV antibodies
Sow	88	73 (83%)	0
Piglet	128	125 (97.7%)	3 (2.3%)
Gilt	60	21 (35%)	0

Porcine respiratory coronavirus causes a subclinical disease in pigs, which in some cases results in fever, sneezing or mild coughing, and combined with other pathogens such as porcine respiratory and reproductive syndrome (PRRS) and swine influenza virus (SIV) can be a part of a post-weaning porcine

respiratory disease complex (PRDC) that causes a significant economic loss in swine industry (Brockmeier et al., 2002). However, strains 135 and 137 of PRCV are capable of producing similar pulmonary lesions to that of the swine influenza virus (Keep et al., 2022). Furthermore, a co-infection of PRCV and PRRS induces a disease with severe respiratory signs (Jung et al., 2009). Ever since the emergence of PRCV, the incidence of TGEV has decreased significantly through partial cross-protection by anti-PRCV antibodies. Nonetheless, there still might be a low TGEV circulation without a clinical manifestation usually explained by a high enough titre of antibodies against PRCV (Kim et al., 2000). Namely, only continuous reinfection allows for a high enough titre of antibodies for adequate protection against TGEV (Kim et al., 2000). Low seroprevalence of PRCV infection was recorded in wild boars in the region: 0.7% in Croatia (Roic et al., 2012), and 3% in Slovenia (Vengust et al., 2006). However, PRCV infection was not detected in wild boars in Serbia (Milicevic et al., 2016). On the contrary, a high seroprevalence of PRCV infection had been recorded in domestic swine in Slovenia at 65% (Vengust et al., 2006), while, in a study by Lőrincz et al. (2014) over 70% of tested gilts and 100% of tested sows seroconverted against PRCV in Hungary. These findings are in accordance with the results in this study where the overall seropositivity to PRCV infection was 79.3%, suggesting that there isn't a circulation of PRCV between the population of domestic swine and wild boars in Central Serbia. The reports regarding seroprevalence of TGE in neighbouring countries have been similar. Seroprevalence of TGE was 0.4% in Croatia (Roic et al., 2012; Brnić et al., 2020), and in wild boars in Slovenia TGE was not detected (Vengust et al., 2006). The results were similar in Serbia where TGEV was not detected either (Milicevic et al., 2010). In domestic swine, there was a single outbreak of TGE in the previous decade in Hungary (Lőrincz et al., 2014). In Serbia, there were reports of porcine epidemic diarrhoea virus (Prodanov-Radulović et al., 2017), but there were no reported outbreaks of TGE in domestic swine. In this study, 2.3% of piglets tested positive for TGE antibodies, which is similar to a study by Valkó et al., (2019) where a single serum positive for TGE antibodies was detected. The high-level serological cross-reactivity represents a hindrance in diagnosis since it allows for a false-positive result, especially at the individual pig level, which lowers the accuracy of blocking ELISA tests (Magtoto et al., 2019). In this study, coronavirus antibodies were detected in all tested production categories, with the highest seroprevalence in piglets, where all of the piglets tested positive for coronavirus antibodies, and 97.7% for antibodies against PRCV. Taking into account the characteristics of the ELISA test, its sensitivity and specificity, it can be concluded that 3 piglets that tested positive

for TGE antibodies were false-positive result, probably because of the cross-reaction between the PRCV antibodies and the TGEV. Following this, none of the tested sows or gilts had antibodies against TGEV, which would be expected if there were positive piglets. On the contrary, a high percentage of PRCV seropositive piglets is in accordance with the high percentage of seropositive sows, probably caused by their continuous reinfection. The other 65% of gilts were negative for antibodies against TGEV and PRCV coronavirus-antibodies, which could be connected to the waning of antibodies under the detection level of the ELISA kit. This could represent a risk since there is a report from 2014 by Lőrincz et al. (2014), which describes a re-emergence event of TGEV in piglets from primiparous gilts which had low levels of PRCV antibodies.

CONCLUSION

The seroprevalence of PRCV infection in domestic swine on commercial farms in Central Serbia is high (79.3%). Antibodies against PRCV were detected in all tested production categories of pigs, with the highest seroprevalence being in piglets (97.7%). The 2.3% of piglets tested positive for TGEV antibodies. Antibodies against TGEV were not found in either sows or gilts. Considering the characteristics of the ELISA test, coupled with a high degree of cross-reactivity between PRCV antibodies and TGEV and the lack of antibodies against TGEV in other tested production categories, this result can be considered a false positive.

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Author's Contribution:

DG and VLJ designed the study. JMZ carried out the serological tests, MN and BM contributed to the interpretation of the results and BK and VM DLJ supervised the findings of this work

Competing interest

The authors declare that they have no competing interests.

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Igor Stojanov, Principal Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)
Sandra Jakšić, Senior Research Fellow	Scientific Veterinary Institute "Novi Sad" (Serbia)

List of reviewers:

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