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NATURAL ANTIBIOTIC RESISTANCE GENES IN SOIL BACTERIA AND INFLUENCE OF ORGANIC FERTILISERS ON THEIR PREVALENCE AND HORIZONTAL TRANSFER

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Abstract

All natural antibiotics available to modern medicine are products of soil-dwelling bacteria and fungi. In addition, all resistance genes which are being detected in human pathogens existed in soil bacteria even before antibiotics were discovered and brought into use. However, the concentrations of natural antibiotics in soil are usually subinhibitory – insufficient for the selection of resistant subpopulations of microorganisms. The consumption of organic fertilisers for agricultural soil amendment increases proportionally to the consumers' growing demand for organically produced food. Manure originating from industrial pig, cattle and poultry farms is not only the source of nutrients which stimulate the vital functions of soil microorganisms, but also of antibiotics and bacteria harbouring various resistance mechanisms. The application of organic fertilizer leads to disruption of the natural balance between bacterial communities in the soil through several mechanisms, and influences the increase in the prevalence of resistance genes and promotes their horizontal transfer. Whether as-yet-unknown resistance genes in soil bacteria may pose threat to human health if transferred from commensal bacteria in the environment to pathogen species, or migrate to clinical settings via food chain or in some other possible route - remains an open question.

Key words: organic fertiliser, soil bacteria, antibiotic resistance genes

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GENI REZISTENCIJE NA ANTIBIOTIKE KOD BAKTERIJA U ZEMLJIŠTU I UTICAJ PRIMENE ORGANSKOG ĐUBRIVA NA NJIHOVU PREVALENCIJU I HORZONTALNI TRANSFER

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Kratak sadržaj

Svi prirodni antibiotici koji su na raspolaganju savremenoj medicini su produkti bakterija i glivica kojima je zemljište prirodno stanište, a svi geni rezistencije koji se danas ustanovljavaju kod humanih patogena postojali su kod bakterija u zemljištu i pre otkrića i upotrebe antibiotika. Međutim, koncentracije prirodnih antibiotika u zemljištu uobičajeno su na nivou subihnibitornih, a time i nedovoljne za selekciju rezistentnih subpopulacija mikroorganizama. Upotreba organskog đubriva za fertilizaciju poljoprivrednog zemljišta, povećava se srazmerno porastu zahteva potrošača za organski proizvednom hranom. Organsko đubrivo poreklom sa industrijalizovanih farmi svinja, goveda i živine, nije samo izvor nutrijenata koji podstiču životne funkcije mikroorganizama u zemljištu, već i antibiotika i bakterija sa raznovrsnim mehanizmima rezistencije. Dodavanje organskog đubriva preko više mehanizama remeti prirodnu ravnotežu zajednice bakterija u zemljištu, utiče na porast prevalencije gena rezistencije i podstiče njihov horizontalni transfer. Otvoreno je pitanje da li do sada neotkriveni geni rezistencije zemljišnih bakterija mogu biti nova pretnja ljudskom zdravlju ukoliko se prenesu sa komensalnih bakterija iz okruženja na patogene vrste, ili lancem ishrane i drugim mogućim putevima, migriraju u kliničke uslove.

Ključne reči: organsko đubrivo, bakterije u zemljištu, geni rezistencije na antibiotike

INTRODUCTION

Considered ecologically more acceptable than mineral, organic fertilisers have traditionally been used in agricultural areas throughout the world (Ding et al., 2014). The increasing consumer demands for organic products will very likely lead to the rise in their use in the future (Udikovic-Kolic et al., 2014).

Soil is fertilised mainly with manure or compost, derived from industrial, high-capacity poultry and pig farms. However, the sustainability of these branches of animal production has been based for decades on continual extensive use of antibiotics (Aarestrup, 2005; FAO, 2016). Tetracyclines, sulphonamides, aminoglycosides, β-lactams and macrolides are most frequently administered to animals and the majority of these, or some related, are in use in humans (Thiele-Bruhn, 2003; Allen et al., 2010). A dramatic development and spread of antibiotic resistance in bacteria rendered the maintenance of their efficacy a top priority in human healthcare, which can be achieved by their rational and justified use for therapeutic purposes only. Nonetheless, in food-producing animals antibiotics have increasingly been used in the absence of infection (for prophylaxis), and in many countries outside of the European Union, as growth promoters (Aarestrup, 2005). For instance, only in the US the sales and distribution of medically important antimicrobials approved for use in food-producing animals, in the period from 2009 to 2014 increased by 23% (FDA, 2015).

In poultry and pig production antibiotics are usually administered via food and/or water, which results in the fact that they are applied both to diseased and healthy animals. Orally administered antibiotics create favourable conditions for the selection, spread and persistence of antimicrobial-resistant bacteria in the digestive tract, including foodborne pathogens and other zoonotic bacteria (such as *Salmonella*, *Escherichia*, *Campylobacter* and *Enterococcus*). This effect concerns especially classes of antibiotics which are poorly or not at all absorbed from the gastrointestinal system, but are continually added to animal feed to stimulate growth. Antibiotics and their metabolic products, which may also be biologically active, are excreted from the body after a relatively short time of their action in the animal organisms (Thiele-Bruhn, 2003). For example, after oral application, 40-90% of sulphonamides consumed are excreted unchanged or as less active metabolites (Heuer and Smalla, 2007). Depending on their physical and chemical properties, antibiotics are eliminated predominantly via faeces or urine. Afterwards, they either do not undergo degradation, or degrade to a certain extent during manure processing (Heuer et al., 2010; Ambrožič Avguštin, 2012; Ding et al., 2014).

Manure provides soil microorganisms with nutrients which stimulate their vital functions, but is also a source of antibiotics and bacteria harbouring various mechanism of resistance (Heuer and Smalla, 2007; 2010). To assess the influence of organic fertilisers on the spread of resistance genes among soil bacteria is an especially difficult task. There is also an open question of potential transfer of resistance from soil bacteria to human opportunistic pathogens.

NATURAL ANTIBIOTIC RESISTANCE GENES IN SOIL BACTERIA

Real knowledge of the identity, natural diversity and patterns of distribution of antibiotics resistance genes (ARGs) in soil bacteria is limited, because it is virtually impossible to find a region without the influence of human activities (Riesenfeld et al., 2004; Allen et al., 2010; Forsberg et al., 2014; Wichmann et al., 2014). The period which preceded the beginning of chemotherapy (before 1936, when sulphonamides were first used) can be considered antibiotic-naive (Allen et al., 2010). Wide use of antibiotics in clinical and agricultural settings enormously contributed to the evolution and diversity of resistance genes in the environment. Antibiotic therapy began in 1941 when benzylpenicillin was first produced for clinical trials (EMEA, 1999). This was followed by the golden age of antibiotics, the period between the 1940s and the 1990s, when the majority of them, which are still available, were discovered and introduced into clinical practice (Nesme and Simonet, 2015). For centuries before the discovery of antibiotics, heavy metals were used to cure people. Metal contamination could have an important role in the maintenance od antibiotic resistance. These co-selection mechanisms include co-resistance: different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) (Baker-Austin et al., 2006).

It is especially difficult to conduct research on soil due to its physical and chemical heterogeneity and large genetic diversity, which is noticeable at distances of one metre, or even less (Allen et al., 2010; Nesme and Simonet, 2015). Additional hindrance results from the fact that sampling methods, storage time and conditions may strongly influence the results, and the absence of guidelines for resistance studies of environmental bacteria impede reaching the conclusions (Allen et al., 2010). Depending on the geographic location, a gram of soil contains from 10^3 to 10^6 different bacterial species, which varies with the methodology of investigations and the taxonomic units defined (Nesme and Simonet, 2015). Although the number of microorganisms per gram of soil is comparable with the human population on the earth, less than 1% can be iso-

lated in vitro on standard laboratory media (Allen et al., 2009; Wang and Yu, 2012; Schmieder and Edwards, 2012). Thus, the investigation into the diversity of resistance genes in soil bacteria requires the use of culture-independent methods (Schmieder and Edwards, 2012; Wang and Yu, 2012). The polymerase chain reaction (PCR) technique enables the detection of resistance genes which are already known or some closely related ones and, thus, has limited capability (Riesenfeld et al., 2004; Allen et al., 2009). Additional information has been obtained from functional metagenomics, which is based on the extraction and DNA cloning directly from environmental samples, such as soil (Riesenfeld et al., 2004). The analyses of metagenomic clones are performed with random sequencing or PCR amplification of target genes (Allen et al., 2009), and the expression of metagenomic DNA sequence in a heterologous, surrogate host (most frequently *Escherichia coli*) and activity–based screening (Demanèche et al., 2008; Allen et al., 2009, 2010; Udikovic-Kolic et al., 2014; Forsberg et al., 2014; Nesme and Simonet, 2015).

More than 80% of antibiotics available to modern medicine are produced by soil-dwelling bacteria and fungi or are their semi-synthetic pharmaceutical derivatives (D'Costa et al., 2006; Schmieder and Edwards, 2012). For example, about 30% to 50% soil actinomycetes of the genus *Streptomyces* can synthesise antibiotics (Thiele-Bruhn, 2003). The numbers of natural antibiotics, which are secondary metabolic products of soil bacteria, are far beyond those available to human and veterinary medicine. Given that actinomycetes alone synthesise approximately a hundred substances with antimicrobial activities (out of which only 30 has been purified and in use), it is clear why soil bacteria are a huge natural reservoir of resistance genes even in completely intact regions. ARGs, which are being detected nowadays in human pathogens, are common in soil community (which are designated as the soil resistome) and existed in the environment before the discovery and use of antibiotics in medicine (D'Costa et al., 2006; Allen et al., 2009; 2010; Knapp et al., 2011; FAO, 2016).

The first genes confering resistance to antibiotics were developed in antibiotic-producing bacterial species, with the aim of protection against self-inhibition (Baquero et al. 1998; D'Costa et al., 2006). To survive, bacteria living in the close vicinity of antibiotic producers had to develop defence mechanisms (Demanèche et al., 2008; FAO, 2016). For instance, soil is rich in microorganisms that produce β-lactam antibiotics, which is why resistance genes to a wide spectrum of β-lactam antibiotics, including third-generation cephalosporins, are naturally found in soil (Demanèche et al., 2008; Allen et al., 2009; Forsberg et al., 2014). The diversity of genes which code for enzymes β-lactamases in soil bacteria is wider than in clinical environments (Nesme and Simonet,

2015). Moreover, in bacteria from soil samples collected in Alaska, far from anthropogenic influence, 13 diverse β-lactamases belonging to all four structural classes of these enzymes were found, among which one was even bifunctional, wich in inusual in bacteria (Allen et al., 2009).

In agricultural and grassland soil samples 2895 ARGs were detected, which represent all the main resistance mechanisms to 18 types of antibiotics (Forsberg et al., 2014). In every cultivable isolate of actinomycetes originating from the soil collected in the woods, urban surroundings and agricultural areas the resistance to several natural antibiotics (from 7 to 8 on average), semisynthetic derivates and synthetic preparations was detected (D'Costa et al., 2006). Furthermore, some highly variable profiles of resistance, as well as resistotypes (antibiotic resistance profiles) unknown until then were discovered (D'Costa et al., 2006). From a 30,000-year-old Alaskan permafrost soil sample, DNA molecules containing *vanHAX* vancomycin resistance operon were extracted, whose expression in a heterologous host - *Escherichia coli* - results in a vancomycin-resistant phenotype (D'Costa et al., 2011).

The concentrations of natural antibiotics in soil are considered subinhibitory and, for this reason, insufficient to prompt the multiplication of species with resistance genes (Götz and Smalla, 1997). Thus, an interesting hypothesis has been proposed: antibiotics in the nature do not act as 'weapons' but those subminimum inhibitory concentrations modulate bacterial gene expression and play the role of signalling molecules (Davies et al., 2006; Allen et al., 2009). Indeed, multiple studies have confirmed the effects of subinhibitory concentrations of antibiotics on global changes in gene transcription (Davies et al., 2006). Moreover, the synthesis of β-lactamases as a response to the presence of β-lactams in soil is aimed at the intercellular signalisation, not unlike the enzymes in proteobacteria which hydrolyse acylhomoserine lactone signalling molecules (Allen et al., 2009). It has also been hypothesised that the plasmid-transferable resistance in the indigenous soil microflora in natural soil is a rare and virtually immeasurable event (Götz and Smalla, 1997). Soil is predominantly an oligotrophic environment in which bacteria have limited growth parameters and activity, including horizontal transfer of genetic material. Very little evidence exists for horizontal gene transfer of ARGs across soil communities (Forsberg et al., 2014). Amending soil with organic fertilisers influences the equilibrium in soil resistome and the horizontal transfer of mobile genetic elements containing ARGs.

EFFECTS OF ORGANIC FERTILISERS ON THE SOIL RESISTOME

A clear, strong connection between the use of antibiotics in animal farming and the increase in resistance genes in soil treated with manure was confirmed by the results of multiple investigations (Binh et al., 2008; Knap et al., 2010; 2011; Heuer et al., 2011; Zhu et al., 2013; Wichmann et al., 2014; Graham et al., 2016). The extensive research on resistance genes in DNA of soil samples collected from 1923 to 2010 and stored in the soil archive took place in Denmark, at Askov Experimental Station, when the emergence of β-lactam-resistance genes - bla_{TEM}, bla_{SHV}, bla_{OXA} and $bla_{CTX-M,}$ and class 1 integron (*int*1) - was monitored over time (Graham et al., 2016). Using qPCR it was proven that after 1940 the total levels of ARGs were significantly higher in soil treated with manure than in soil fertilised with inorganic fertilisers. The results revealed that the emergence of genes coding for β-lactam resistance found in manured soil coincided with the one in clinical bacterial isolates, as well as that the increase in *int*1 in soil was proportional to the usage of manure (Graham et al., 2016). Similarly, in soil samples from five locations collected in the Netherlands, from 1940 to 2008, the increase in the concentrations of genes coding for resistance towards tetracyclines and β-lactam antibiotics was detected: the levels of β-lactam ARG (bla_{TEM} and bla_{SHV}) increased by 15 fold in the monitored period (Knap et al., 2011).

In veterinary medicine, especially in poultry and pig farming, tetracyclines and sulphonamides are widely used. Extensive investigations confirmed that pig manure influences the increase in the tetracycline and sulphonamide resistance genes in soil bacteria (Schmitt et al., 2006; Heuer and Smalla, 2007). In bacteria isolated from soil which was not treated with manure, *tet*(T), *tet*(W) and *tet*(Z) resistance genes were detected (Schmitt et al., 2006). However, the treatment with pig manure resulted in direct transfer of tetracycline resistance genes *tet*(Y), *tet*(S), *tet*(C), *tet*(Q), and *tet*(H) and their appearance in soil bacteria (Schmitt et al., 2006). It was also experimentally proven that the use of manure originating from pigs treated with sulfadiazine (SDZ) leads to the increase in the total number of bacteria in soil and cultivable strains resistant to SDZ (Heuer and Smalla, 2007). Given that sulphonamide resistance is usually mediated by the *sul1*, *sul2* and *sul3* genes (Heuer et al., 2007; 2010), it is not surprising that in pig manure and manured soil samples a high prevalence of *sul1* resistance genes was detected, which are normally found in class 1 integrons (Heuer and Smalla, 2007). In addition, it was proven that SDZ can strongly influence the soil bacteria population: on the one hand it exerts a stimulating effect on the multiplication of species affiliated to the genera *Devo-*

sia, *Shinella*, *Stenotrophomonas*, *Clostridium*, *Peptostreptococcus*, *Leifsonia* and *Gemmatimonas* (among which there are human pathogens), but on the other, caused a decrease in the relative numbers of bacteria which are normally found in quality soils (*Pseudomonas*, *Lysobacter*, *Hydrogenophaga* and *Adhaeribacter)* (Ding et al., 2014). It has experimentally been proven that pig manure facilitates the horizontal transfer of IncQ pIE723 plasmide from donor *Escherichia coli* strains to recipient *Pseudomonas putida* UWC1 strains (Götz and Smalla, 1997). Plasmid transfer was detected even in non-manured soil, but the number of transconjugantss which had pIE723 in manured was a thousand times higher. Moreover, pig manure had a stimulating effect on the growth and survival of the recipient *P. putida* UWC1 in soil (Götz and Smalla, 1997).

China is the world's largest producer and consumer of antibiotics, which means that in its industrial pig farms all major classes of antibiotics are used as feed supplements or for therapeutic reasons. Thus, the annual swine manure production of 618 billion kilograms is highly likely to increase substantially the resistance gene concentrations in the environment (Zhu et al., 2013). In contrast to control soil samples, in soil treated with pig manure the genes coding for resistance to antibiotics critically important for human healthcare, such as macrolides, cephalosporins, aminoglycosides and tetracycline were detected. There was a 192-fold median increase in the top 63 ARGs, but also a maximum of 28,000-fold increase in one single gene (Zhu et al., 2013).

Although it is clear that the use of manure originating from animals treated with antibiotics contribute to the increase in the resistance towards antibiotics in soil bacteria, research revealed that a similar effect is exerted by the manure from animals which were untreated. A team led by Jo Handelsman, a microbiologist at Yale University in New Haven, Connecticut, investigated the influence of manure from cows which were free of antibiotics, and a nitrogen-based fertiliser, on the resistance of soil bacteria (Reardin, 2014). Two weeks after the treatment with manure significantly higher numbers of bacteria producing β-lactamases in soil were detected in comparison to those in soil treated with nitrogen-based fertilisers. By tracing genetic markers, it was discovered that these bacteria did not originate from the fertiliser but from soil: manure induced a bloom of resistant bacteria already present in soil, particularly of *Pseudomonas* species which is a human opportunistic pathogen.

Cows' manure is less explored in comparison to that of pigs and chickens, although it is commonly used in crop production. Only in the USA cows generate daily between 1.9 and 14.2 billion pounds of manure which is used to fertilize fields (Wichmann et al., 2014). Wichmann et al., (2014) applied functional metagenomics and identified 80 different antibiotic-resistance genes,

among which some chloramphenicol-resistance genes, unknown until then (coding for a novel clade of acetyltransferases), were specific to the cow microbiome, which are only distantly related to previously known genes. Udikovic-Kolic et al. (2014) claimed that soil treated with cow's manure originating from animals which had not been treated with antibiotics contain a higher abundance of β-lactam-resistant bacteria in comparison with the one treated with inorganic fertiliser. By means of identification of β-lactam-resistance gens with functional metagenomics and quantitative PCR analysis, it was proven that cows' manure influenced the enrichment of resident soil bacteria that harbour β-lactamases, such as *Pseudomonas* spp., *Janthinobacterium* sp. and *Psychrobacter pulmonis* (Udikovic-Kolic et al., 2014). It is assumed that the growth and multiplication of resistant soil bacteria are facilitated by either some nutritional component of the manure or by heavy metals (Knapp et al., 2011). Indeed, genes which make it possible for bacteria to resist widely used metals (copper, arsenic, zinc, silver and mercury), as well as those usually present in the environment (cadmium, lead, cobalt, nickel and tin) are frequently detected along with antimicrobial-resistance genes on mobile genetic elements (Summers, 2002; Knapp et al., 2011). Copper, arsenic and zinc, which are added to animal feed supplements, may also pose long-term pressure for antibiotic resistance (Zhu et al., 2013). Knapp et al. (2011) tested soil samples from the early phase of antibiotic era (between 1940 and 1970) and those collected in 2008 from fields containing sewage sludge amended with copper at 0, 50, 100 and 200 mg-copper/kg. In the extracted DNA materials the presence of genes coding resistance to tetracycline (*tet*), extended-spectrum beta-lactamases (*bla*) and erythromycin resistant methylases (*erm*) were detected. It was revealed that copper exerted the major influence on ARG abundances, and that the one of chromium and nickel were less strong (Knapp et al. 2011).

Bacteria are extremely capable of adjusting to unfavourable environmental conditions and in short time acquire or develop mechanisms which will enable their survival.Antibiotic residues which are excreted from animal organisms and reach agricultural land via organic fertilisers influence directly the resistance of soil bacteria. The selection of antibiotic-resistant bacteria strains is proportional to the concentration of antibiotics which they are exposed to and to the duration of this exposure. An antimicrobial agent can multiply the population of bacteria resistant to some other antibiotics, if their resistance genes are related to each other (Summers, 2002; Binh et al., 2008; Wang and Yu, 2012). If more than one antibiotic is present in the environment the resulting multiple pressure selectively influences those bacteria which use multiple or multipurpose mechanisms (Baquero et al., 1998).

Repeated exposure of bacteria to a certain antibiotic is of outstanding importance to the selection of resistant strains and horizontal transfer of resistance genes, which has clearly been proven in clinical practice. This is the reason why only few, but largely disseminated resistance genes can be found in clinical environment in comparison to soil (Nesme and Simonet, 2015). However, direct exposure of bacteria to antibiotics is only one of the factors collectively termed "selective pressure". In broader sense it comprises a set of factors which create favourable environment for the emergence of *de novo* mutations or the acquisition of properties which contribute to the survival and, what is of crucial importance, to the selection of resistant microorganism (Baquero et al., 1998). Although all mechanisms of resistance already exist in bacteria in the nature, the multiplication of resistant populations will occur only in multifactorial conditions which act as selective pressure.

POTENTIAL RISK FOR HUMAN HEALTH

Many bacterial ARG are located on mobile and horizontally transferable genetic elements such as plasmids, transposons or integrons (Allen et al., 2010). Their horizontal transfer enables efficacious spread of resistance among related and distant bacterial species, including both commensals and even species pathogenic to humans (Allen et al., 2010; Udikovic-Kolic et al., 2014). The exchange of ARG between soil bacteria and those of clinical importance is possible in both directions (Wang and Yu, 2012). Resistance genes in natural reservoirs (such as soil and ocean water) are the likely source of ARG in clinical environment due to various means of horizontal transfer (Nesme and Simonet, 2015).

Soil is the environment in which various bacterium species, including those important for public health, may acquire resistance determinants to known and possibly those classes of antibiotics which have yet to be synthesised (Demanèche et al., 2008). Little is known about natural reservoirs of resistance genes in the environment, as well as how much they can influence the resistance detected in clinical settings (Allen et al., 2009). Even currently unknown ARGs in soil bacteria may present a grave threat to human health if transferred to pathogen species and migrate into clinical environment. However, there still remains an open question: are all resistance genes which are being discovered nowadays in soil bacteria transferable to clinically important species, and how will this reflect on the efficacy of antibiotics in the future? There is considerable void in the understanding of mechanisms by which microorganisms acquire resistance to antibiotics, as well as on the interactions in microbial ecosystems which contribute to their transfer. The transfer of resistance genes between

bacteria in the soil is not as easy as it is between pathogenic species. The limited mobility of the soil resistome may explain why ARGs are rarely shared between soil and human pathogens (Forsberg et al., 2014).

Not only are vast quantities of antibiotics used in animal farming being deposited into soil, but are also resistant populations of gastrointestinal microbiota (including opportunistic human pathogens), both via organic fertilisers. From there bacteria carrying genes coding for resistance towards antibiotics may enter the food chain via contaminated crops or groundwater (Thiele-Bruhn, 2003; Wang and Yu, 2012; Udikovic-Kolic et al., 2014) and via vegetables which are consumed raw (Binh et al., 2008; Marti et al., 2013). Antibiotic use in intensive animal and genetically modified crop farming additionally increases the selection pressure and the risk from the distribution of resistance genes. The release of antibiotics and antibiotic-resistant bacteria into the environment poses a serious problem to the control of antimicrobial resistance, which is why animal health care has to be based on high hygiene and biosecurity, accompanied with prudent antibiotic deployment.

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INVESTIGATION OF PRESENCE OF METHICILLIN RESISTANT STAPHYLOCOCCI IN STUDENTS OF THE FACULTY OF VETERINARY MEDICINE AT THE UNIVERSITY OF BELGRADE

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Abstract

Resistance to methicillin in staphylococci is considered to be one of the most dangerous forms of bacterial resistances to antibiotics. Methicillin-resistant staphylococci (MRS) are zoonotic agents which cause local and systemic infections in humans and animals, often with a fatal outcome due to the absence of adequate antibiotic therapy. People colonized with strains of MRS are asymptomatic carriers and reservoirs of these strains in human populations. The aim of this research was to determine the prevalence of strains of MRS among clinically healthy students of the Faculty of Veterinary Medicine in Belgrade. The study was conducted on 100 volunteers: 62 males and 38 females. Given that staphylococci are expected to be found in the highest percentage in the nose and on the armpit skin, the swabs were taken from these regions of each person. Blood agar was innoculated immediately on taking the swabs After the incubation and isolation, the staphylococci were identified to species level. Their susceptibility to methicillin was tested in a disk-diffusion test with cefoxitin. All strains which were found to be resistant to cefoxitin were investigated for the presence of *mecA* gene with PCR. Staphylococci were isolated in 146 out of the 200 swabs taken: there were 79 nose swabs and 67 axillar swabs positive for these bacteria. Seventeen isolates were resistant to cefoxitin and the presence of the *mecA* gene was confirmed in seven, four of which were taken from the nose and three from the axillary region. The results of this research show that, being 6%, the prevalence of *mecA*-positive staphylococci in the

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population of clinically healthy students of veterinary medicine is significant. The percentage of methicillin-resistant staphylococci was higher in nose than in the axillar region of the students.

Key words: staphylococci, methicillin resistance, prevalence, asymptomatic carriers

PRISUSTVO SOJEVA STAFILOKOKA REZISTENTNIH NA METICILIN KOD STUDENATA FAKULTETA VETERINARSKE MEDICINE UNIVERZITETA U BEOGRADU

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Kratak sadržaj

Rezistencija na meticilin kod stafilokoka smatra se jednim od najopasnijih oblika rezistencije bakterija na antibiotike. Sojevi meticilin rezistentnih stafilokoka (MRS) pripadaju zoonotskim agensima i uzročnici su lokalnih i sistemskih infekcija kod ljudi i životinja, često sa fatalnim ishodom zbog nedostatka efikasne terapije. Ljudi kolonizovani sojevima MRS su asimptomatski nosioci i predstavljaju rezervoare ovih sojeva u humanoj populaciji. Cilj ovog istraživanja je bio da se utvrdi prevalencija izolata MRS kod klinički zdravih studenata Fakulteta veterinarske medicine u Beogradu. U istraživanje je bilo uključeno 100 studenata - dobrovoljaca, 62 muškog i 38 ženskog pola. Brisevi su uzimani iz nosa i pazuha zato što su stafilokoke u najvećem procentu zastupljene u tim regijama. Brisevi su odmah zasejavani na krvni agar. Nakon inkubacije i izolacije, izvršena je identifikacija stafilokoka do vrste. Ispitivanje osetljivosti stafilokoka na meticilin izvedeno je primenom disk difuzione metode sa cefoksitinom. Svi sojevi rezistentni na cefoksitin ispitani su na prisustvo *mecA* gena metodom lančane reakcije polimeraze (PCR). Od ukupno 200 uzetih briseva izolovano je 146 izolata stafilokoka, 79 iz briseva nosa i 67 iz briseva pazušne regije. Kod 17 izolata ustanovljena je rezistencija na cefoksitin, a kod 7 je utvrđeno prisus-

tvo *mecA* gena. Četiri *mecA* pozitivna izolata su poticala iz briseva nosa, a 3 sa kože pazušne regije. Utvrđena prevalencija meticilin-rezistentnih stafilokoka kod klinički zdravih studenata Fakulteta veterinarske medicine od 6%, procenjena je kao značajna. Veća učestalost stafilokoka rezistentnih na meticilin ustanovljena je na sluzokoži nosa u odnosu na kožu pazuha.

Ključne reči: stafilokoke, rezistencija na meticilin, prevalencija, asimptomatski nosioci

INTRODUCTION

The resistance to methicillin was first detected in *Staphylococcus aureus* in Great Britain in 1961 (Ašanin et al., 2012; Ćirković et al., 2015; Kluytmans et al., 1997). This type of resistance emerged only two years after methicillin, a semisynthetic penicillin preparation resistant to an inducible enzyme - penicillinase, was introduced into clinical practice. The same year the term MRSA (*methicillin-resistant Staphylococcus aureus*) was coined, which is still in use, although methicillin is not used for the detection of this type of resistance any more. The most frequent mechanism of resistance to methicillin is the acquisition of *mec*A gene (2.1 kb), which is part of the mobile genetic element of the staphylococcal cassette chromosome *mec* (SCC*mec*) (Oliveira et al., 2002; Schito, 2006, De Brito et al., 2015). *Mec*A gene codes for the synthesis of the new penicillin-binding protein, PBP 2a (78 kD), which has low affinity towards methicillin (Oliveira et al., 2002). The production of PBP 2a results in the resistance of staphylococci towards all beta-lactam antibiotics (penicillins, beta-lactams with beta-lactamase inhibitors, cephems excluding cephalosporins which are active against MRSA strains - ceftaroline and ceftobiprole - and carbapenems) (CLSI, 2016; EUCAST, 2016). Besides *mec*A genes, staphylococcal cassette chromosome carry many resistance genes to other antibiotics, which is why methicillin-resistant staphylococci (MRS) are considered multidrug-resistant pathogens that cause local and systemic infections in humans and animals with frequent fatal outcome due to the absence of efficacious antibiotics. In 2011 in MRSA strains isolated from cattle and humans another form of *mec*A gene, *mec*A_{LGA251} homologue (García-Álvarez et al., 2011), named *mec*C gene (Ito et al., 2012) was detected, which was impossible to be done earlier with the protocols for the detection of *mec*A genes. This discovery has raised a question of relevancy of data about the prevalence of MRS on the earth, since it has been clear that there must be more than it was thought.

Although the application of strict control measures in certain countries of the EU, such as Denmark and Sweden, has led to the decrease in the incidence

of infections caused by MRSA in humans to less than 1%, it is estimated that the global epidemiological situation concerning the emergence and spread of MRSA strains in the 20th century has dramatically worsened. Thus the resistance of staphylococci to methicillin is considered to be one of the most dangerous and most widespread resistances to antibiotics of all (David et al., 2010). Besides well-known categories, HA-MRSA (*healthcare-associated* MRSA) and CA-MRSA (*community-associated* MRSA), there is a third one - LA-MRSA (*livestock-associated* MRSA). The existence of asymptomatic carriers is of utmost importance in the epidemiology of MRS. These are people and animal which are not diseased, but are colonised by MRS strains, which means that these bacteria are part of the microbiota of the skin and mucous membranes. Asymptomatic MRS carriers are sources of infection for people who are most at risk of developing disease. Given that these asymptomatic carriers are clinically healthy people, it is difficult to identify them and impossible to treat by any means. Asymptomatic carriers may be colonised by any of the listed MRSAs (HA-MRSA, CA-MRSA and LA-MRSA). Due to the increasing number of reports concerning the transmission from animals to people, MRSA strains have been considered zoonotic infective agents and are in many countries governed by legislation, which precisely define procedures of their detection, prevention of emergence and spread, and how to combat infections. Special attention was paid to the MRSA strain which belongs to the clonal complex 398 (CC398), known by its jargon name - 'the pig clone'. Clone MRSA CC398 colonises pigs, which almost never become ill and as asymptomatic carriers are source of human infection (Lewis et al., 2008). It is thought that CC 398 strain has spread through farm workers and their family members to people in cities and can now be found in hospitals in the EU, in intensive-care units, where it causes 15% of the MRSA infections.

MATERIAL AND METHODS

The research was conducted on a hundred students of the Faculty of Veterinary Medicine in Belgrade. There were 62 males and 38 females aged from 19 to 31 years. Swabs were taken from students of all years, from the 1st to the 6th, as well as from interns. From each student two swabs were taken: one from the nose and the second from the skin of the axillary region. In addition, each person was asked to fill in the questionnaire connected with the research. The questions were divided into five groups. The first group dealt with the student's pet (if they had any). The second group of questions included anamnestic data on the previous use of antibiotics, and the third one information

about hospitalisation and visits to the hospital. The last two question groups concerned contact with farm animals (cattle and/or pigs) which are potential carriers of LA-MRSA.

Blood agar (Columbia agar with 5% sheep blood, bioMérieux, France) plates were inoculated for the isolation of staphylococci immediately after the swabs were taken and the petri dishes were incubated for 18-24 hours at 37°C. Commercial tests, ID32 STAPH (bioMérieux, France) and BBL Crystal Gram-Positive ID Kit (Becton Dickinson, USA) were used for the identification of the isolates.

Phenotypic research on the resistance to methicillin was performed by disc diffusion method on Mueller Hinton agar (bioMérieux, France) using antimicrobial-susceptibility test discs of cefoxitin (30 μg) (Becton Dickinson, USA). The results were interpreted according to the guidelines proposed by EUCAST (EUCAST, 2016). The inoculum density of the strains tested was approximately 1-2x10^8 CFU/mL, which is equivalent to 0.5 McFarland standard.

All isolates resistant to cefoxitin were tested for the presence of *mecA* and *mecC* genes using the PCR technique. *Staphylococcus spp.* DNA extraction was performed following the protocol proposed by the European Union's reference laboratory for antimicrobial resistance - EU Reference Laboratory-Antimicrobial resistance, Faculty of Veterinary Medicine, Lisbon, Portugal.

According to the protocol for the detection of *mecA* gene (Isenberg, 2004), the length of PCR product was 533 bp. The sequence of the primers (Invitrogen, USA) for the amplification of 533 bp region of *mec*A gene was the following: primer 1 (5'-AAA ATC GAT GGT AAA GGT TGG C-3') and primer 2 (5'-AGT TCT GCA GTA CCG GAT TTG C-3') each in final concentration of 0.25 μM. All deoxyribonucleoside triphosphates (Thermo Scientific) were used in concentration of 200 μM. In addition, the following reagents were used: PCR buffer 1x, 1.5 mM $MgCl_2$ and 5 U TaqDNA polymerase (Thermo Scientific).

The procedure started by initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94° C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, and, final extension at 72°C for 5 minutes in the thermocycler (Eppendorf, Germany). The After electrophoresis in 1.5% agarose gel with ethidium bromide in 1x TBE buffer at 100V, PCR products were visualised on the UV transilluminator (Vilber Lourmat, Germany). Two reference strains were used for quality control: *Staphylococcus aureus* ATCC 43300 as the positive control, and as the negative one *Staphylococcus aureus* ATCC 25923.

Isolates which were negative for the presence of *mecA* genes were assessed

for the presence of *mecC* gene using the protocol described by Garcia-Alvarez et al. in 2011. The primers produced by Invitrogen (USA), with the following sequences: FW 5' –TCACCAGGTTCAAC(Y)CAAAA -3' and RW 5' –CCTGAATC(W)GCTAATAATATTTC -3' were used. The length of the targeted PCR products was 356 bp. The *mecC*- positive *S. aureus* strain was used as a positive control, obtained from courtesy of the Department of microbiology of the Veterinary University in Vienna (Austria).

RESULTS

Staphylococci were isolated from 146 out of 200 swabs (79 isolates from the nose and 67 from the axilla). Using the disc diffusion method, resistance to cefoxitin was detected in 17 isolates, i.e. in 12 nose and 5 axillary swabs. PCR technique detected *mec*A gene in 7 isolates, which were identified as *S. epidermidis* (n=4), *S. aureus* (n=2) and *S. haemolyticus* (n=1). Four *mec*A-positive isolates were from the nose and three from the armpit. Six out of seven *mecA*-positive isolates originated from males. Five MRS isolates was identified in 3rd-year students and one in a 1st-year and a 4th-year student.

The diameters of the growth inhibition zone around the cefoxitin discs in the disc diffusion test, which were the criteria for the suspicion that the isolates tested were methicillin-resistant, are presented in Table 1. In addition, in Table 1 the results of the biochemical identification of the isolates and the molecular (PCR) research. In one student, methicillin-resistant *S. epidermidis* was found both in the nose and in the axillary region. The prevalence of MRS isolates detected in the students of the Faculty of Veterinary Medicine was 6% (Figure 1).

Concerning ten isolates, the diameter of growth inhibition zone around the cefoxitin disc was less than 25 mm for coagulase-negative staphylococci and less than 22 mm for *S. aureus*, which is why they were, in accordance with the recommendations of EUCAST, considered resistant to methicillin. However, with the PCR technique in those isolates the presence of *mec*A and *mec*C genes was not detected.

Table 1. Species of staphylococci, their origin, results of cefoxitin disk diffusion test and presence of methicillin resistance genes by PCR

Isolate number	Species	Origin	Growth inhibition zone diameter (mm) cefoxitin disk (30 µg)	Resistance genes	
				*mec*A	*mec*C
1	*S. vitulinus*	nose	15	-	-
2	*S. aureus*	nose	18	-	-
3	*S. haemolyticus*	nose	20	+	NT
4	*S. aureus*	nose	17	-	-
5	*S. aureus*	axillary region	20	-	-
6	*S. aureus*	nose	14	-	-
7	*S. aureus*	nose	16	-	-
8	*S. aureus*	nose	20	-	-
9	*S. epidermidis*	nose	19	-	-
10	*S. epidermidis*	axillary region	20	+	NT
11	*S. epidermidis*	nose	23	+	NT
12	*S. epidermidis*	axillary region	17	+	NT
13	*S. epidermidis*	axillary region	18	+	NT
14	*S. aureus*	nose	19	+	NT
15	*S. aureus*	nose	20	+	NT
16	*S. aureus*	nose	20	-	-
17	*S. haemolyticus*	axillary region	23	-	-

*NT- not tested for *mec*C

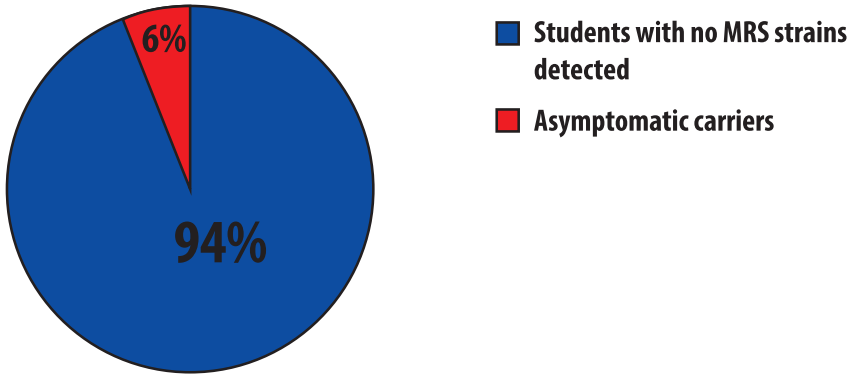

Figure 1. The percentages of asymptomatic carriers of methicillin-resistant staphylococci (MRS) among students of Belgrade University, Faculty of Veterinary Medicine

DISCUSSION

The prevalence of staphylococci resistant to methicillin can vary significantly among various studies and may result from genuine differences in the prevalence of MRSA in the populations tested (Ćirković et al., 2015). The complete control of the presence of certain clones of MRS in the people of the whole country is virtually impossible. However, monitoring the presence of MRS in certain populations may be the first step in the battle against the high prevalence of methicillin-resistant staphylococci. This research was based on that principle, with the aim of detecting the number of asymptomatic carriers of MRS strains in a small population of the students of the Faculty of Veterinary Medicine in Belgrade. From the investigation conducted on 100 students, the data on the prevalence of staphylococci resistant to methicillin in our nearest surrounding can be obtained.

The nasal mucous membrane is considered the primary location colonised by MRS strains (Rohr et al., 2004), which has been confirmed in our study. MRS may colonise other locations on the human body, despite not having been found on the nasal mucosa. In research performed in England, it was discovered that the colonisation of the pharynx with MRSs (72/635, or 11.3%) occurs more frequently than that of the nose (59/635, 9.3%) or other body parts (Bignardi et al., 2009). Some authors consider that there are three types of asymptomatic carriers of CA-MRSA in a population (Kluytmans et al., 1997). According to these, 20% of asymptomatic carriers are colonised incessantly, whilst in 60% the presence of MRSA happens periodically. The rest of the asymptomatic carriers, 20%, are rarely colonised with MRS. The research done by Lim et al. (2006) suggests that persistent asymptomatic MRS carriers who have these in the nose are protected from the colonisation with new *S. aureus* strains.

Statistically, MRSs were detected in 6% of the students of the Faculty of Veterinary Medicine in Belgrade. This is significantly lower prevalence in comparison to the countries with high prevalence of MRS, such as in India, where it has been detected to be 41% (Joshi et al., 2013). The highest prevalence of MRS was detected in hospital environments, which was confirmed by multiple studies. For example, in a hospital in Portugal 54 strains of *S. aureus* were isolated and in 14 (25.9%) the *mec*A gene was detected (Espadinha et al., 2013). Out of the 1,195 patients of the Saint Louis hospital in USA, 31.8% were colonised with MRS (Fritz et al., 2009).

The investigation completed by researchers in Serbia (Ćirković et al., 2013) revealed a low prevalence of MRSA strains: it was 0.37% in 533 second-, third-

and fourth-year students of the School of Medicine in Belgrade. It is to be underlined that in this research coagulase-negative staphylococci (CoNS) were not taken into consideration. However, our investigation was performed on fewer people but the prevalence of MRSA was 2%. Due to direct contacts with hospital patients, higher-year students of the School of Medicine were at considerably higher risk of being colonised by MRSA strains. By contrast, according to the survey, students of the Faculty of Veterinary medicine had no risky contacts, thus it remains unclear what is the reason of higher prevalence of MRSA strains in this population compared to medical students.

Investigation into the presence of MRSA strains in the nose of healthy students of the Medical Faculty in Czech Republic detected as many as 32% 1st-year and 30% 5th-year students asymptomatically colonised with MRSA (Holý et al., 2015).

Similar research to ours was undertaken in Mexico, when nasal and pharyngeal swabs were collected from 21-year old clinically healthy volunteers (Hamdan-Partida et al., 2013). None of them had been hospitalised before. Out of 1,039 staphylococci isolated, 131 (12.61%) were methicillin-resistant. Unlike in our research, in which the majority of MRS were isolated from the nose, in the one conducted by Hamdan-Partida et al. (2013) the numbers of MRS isolates from the nose and pharynx were roughly equal.

According to the survey, 5 out of the 6 examined people from whom MRS were isolated, had immediate contact with farm animals (cattle and swine) and domestic pets (dogs and cats). None of them came into either permanent or periodic contact with people who work at hospitals or some other healthcare facilities. One person had contact neither with pets nor with farm animals. As stated in the questionnaires, none of the persons with MRS had taken any antibiotics or had visited hospitals in the previous six months. Three persons harbouring MRS were in direct contact with family members or friends who had taken antibiotics in the recent past. Thus, it is confirmed that all people are susceptible to MRS (Mišić, 2013).

More than 50% isolates which proved to be resistant to methicillin in the disc diffusion test were not *mec*A-positive, which points to the unreliability of the disc diffusion method in the investigation into the resistance of staphylococci towards methicillin.

CONCLUSIONS

The diameter of growth inhibition zone in the disc diffusion method may serve only as a tool for arising suspicion that there is resistance to methicillin

in routine diagnostic. The prevalence of MRS in clinically healthy students of the Faculty of Veterinary Medicine, which was 6%, was considered to be significant. The incidence of methicillin-resistant staphylococci was higher on the nasal mucous membrane than in the axillary region.

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GENETIC MECHANISMS OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS* (MRSA) AND ITS CAPACITY OF TRANSMISSION: A BRIEF REVIEW

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Abstract

Methicillin-resistant *Staphylococcus aureus* is one of the most important human pathogens. These bacteria have the ability to colonize animals and cross species barriers. Three major groups of MRSA strains have emerged. Healthcare associated MRSA and community acquired MRSA strains have potential to spread worldwide and often persist in hospitals and communities as clonal strains. The livestock-associated MRSA has been isolated from healthy human carriers but also from infected patients all around the world. Molecular typing of staphylococcal cassette chromosome *mec* (SCC*mec*), multilocus sequence typing in combination with pulse field gel electrophoresis and *spa* typing are most frequently used for genetic characterization of MRSA strains. The community-acquired MRSA strains are capable of producing Panton Valentine leukocidin (PVL) cytotoxin which is their major virulence determinant. MRSA strains possess a number of virulence factors that are common in other bacteria and it is still not entirely explained which virulence factors or mechanisms of their regulation are important for the pathogenic potential, persistence in the environment or the ability to cause detrimental infection in patients. Recently, as a contribution to the progress of molecular biology, peculiar mechanisms of genetic regulation of virulence genes have been discovered and their role in pathogenesis of infection and epidemiology of MRSA has been studied.

Key words: MRSA, pigs, humans, epidemiology, *mecA*, PVL

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GENETIČKI MEHANIZMI REZISTENCIJE NA METICILIN KOD *STAPHYLOCOCCUS AUREUS* (MRSA) I NJIHOVO PRENOŠENJE: KRATAK PREGLED

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Kratak sadržaj

Staphylococcus aureus koji poseduje rezistenciju na meticilin, jedan je od najvažnijih humanih patogena. Ovi mikroorganizmi imaju sposobnost da koloniziraju životinje i da prelaze barijeru između vrsta. Do sada su utvrđene tri glavne grupe MRSA. MRSA sojevi koji su bolničkog ili vanbolničkog porekla raširenи su u celom svetu i često perzistiraju klonalno. MRSA poreklom od životinja su takođe nađeni i kod ljudi koji su latentno inficirani i kod inficiranih pacijenta u celom svetu. Molekularna tipizacija hromozomske kasete *mec* (SCC*mec*), sekvenciranje većeg broja genetičkih lokusa u kombinaciji sa elektroforezom u pulsnom polju, kao i *spa* tipizacija, najčešće se koriste za genetičku karakterizaciju MRSA. Vanbolnički MRSA sojevi iz zajednice imaju sposobnost da produkuju Panton Valentin leukocidin (PVL) citotoksin koji je ujedno i najvažnija determinanta njihove virulencije. MRSA poseduju mnogobrojne faktore virulencije koje se nalaze i kod drugih bakterija i još uvek nije u potpunosti objašnjeno koji su geni virulencije ili mehanizmi njihove regulacije važni za patogeni potencijal, perzistiranje u životnoj sredini ili u nekim slučajevima, za nastanak po život opasnih infekcija ljudi. Paralelno sa razvojem molekularne biologije, naučnici otkrivaju specifične mehanizme genetske regulacije faktora virulencije i istraživanje njihove uloge u patogenezi i epidemiologiji infekcija koje izazivaju MRSA sojevi.

Ključne reči: MRSA, svinje, ljudi, epidemiologija, *mecA*, PVL

Introduction

Methicillin is semisynthetic penicillin which was discovered in 1959 and it was used for the therapy of infections caused by *Staphylococcus aureus* which are resistant to penicillin and extended spectrum beta-lactam antibiotics. However, soon after methicillin had been introduced into clinical practice, resistance occurred and healthcare-associated MRSA (HA-MRSA) became one of the most important nosocomial pathogens worldwide (Gordon and Lowy, 2008). The methicillin resistance gene *mecA* that encodes a penicillin-binding protein (PBP2A) is integrated in staphylococcal cassette chromosome *mec* (SCC*mec*) locus. The mobile genetic element SCC*mec* is composed of the terminal inverted and direct repeats and the junkyard region (J). Major components of the cassette are *mecA* gene complex and its regulatory elements include gene encoding recombinases (*ccr*) which are involved in the integration or excision of the cassette into the chromosome and subsequent intra and interspecies transmission. The cassette is classified into types I, II, III, IV, V (based on *mec* and *scc* genes sequences) and subtypes (based on differences determined in junkyard region). In hospital acquired MRSA (HA- MRSA) this large SCC*mec* cassette is represented by types I, II and III. Community associated infections with methicillin resistant *S. aureus* (CA- MRSA) strains also became a major threat to the public health. The CA-MRSA has a smaller SCC*mec* cassette type IV and V and a smaller number of the resistance genes are incorporated inside cassette (Zhang et al., 2005).

Genetic analysis of MRSA strains, especially of the SCC*mec* cassette, is important from the epidemiological standpoint. Polymerase chain reaction or multiplex PCR are used as traditional typing methods for the characterization of the structural types of *mec* elements (Oliveira and Lencastre, 2002). The multilocus sequence typing (MLST) has also become a prestigious method for genetic analysis of the entire genome of MRSA strains and has helped in providing the newest nomenclature of MRSA (Zhang et al., 2005). The identification of the genes encoding exotoxins, present additional tool in the analysis of the MRSA isolates. Panton Valentine leukocidin genes (*lukS-PV* and *lukF-PV)*, which encode cytotoxin are of particular interest. PVL cytotoxin causes leukocytes destruction and tissue necrosis. The PVL genes are most often found in CA- MRSA strains and much less frequently in HA-MRSA. The γ hemolysin variant and Lekocidin E-D gene, as well as other genes encoding exotoxins, were detected evenly in HA and CA-MRSA strains, while *sec* and *sek* genes were found only in CA-MRSA strains in a cohort study of patients with MRSA infection in Minnesota in the year 2000 (Naimi et al., 2003).

Important discriminatory tool for MRSA is single-locus DNA sequencing of the *spa* gene (encoding A protein) (Frenay et al., 1994, Frenay et al., 1996). The *spa* gene is represented by a number of repeats that are susceptible to spontaneous mutations, loss or insertion or repeat exchange. A software program was applied for the first time for the *spa* typing by Harmsen et al. (2003) who have studied *spa* types of the MRSA isolates from a single hospital at the Würzburg University Clinic, Germany. Only single unrepeated isolates (first isolate from a patient) were used for genotyping during the study period. Software has been designed to use internet database for the automatic recognition of the *spa* type after entering the sequence data. This method is not as expensive as MLST and it is not as time consuming as PFGE. However, in order to determine genetic differences in MRSA isolates the best choice is to combine the *spa* typing with MLST and PFGE. Particular *spa* types were found to be associated with the specific MLST clones in regard to resistotype and colony morphology on blood agar. Hence, bacteriological and genetic methods combined are preferable for the distinguishing of the MRSA epidemic clones (Harmsen et al., 2003).

LIVESTOCK-ASSOCIATED MRSA (LA-MRSA)

Livestock-associated MRSA human infection was reported in 2004 in the Netherlands for the first time. A case study included a young girl and other family members who were involved in pig farming as well as two patients, and a nurse. All isolates from the outbreak were untypeable by PFGE using *Sma*I restriction enzyme. However, a single random amplified polymorphic DNA analysis and the single *spa* type (Spa-type 108) have shown that pig associated MRSA had caused the infection of humans (Voss et al., 2003). This was a first report of a transmission from animals to humans which was immediately followed by a number of reports about the infection of humans with livestock-associated MRSA (LA-MRSA) all around the world (Burns et al., 2014, Chuang et al., 2015, van Cleef et al., 2011, Fang et al., 2014, Golding et al., 2010, Huang et al., 2014., Patchanee et al., 2014). The majority of the LA-MRSA isolates belonged to the clonal lineage ST 398. Other specific features included the lack of PVL toxin and the absence of the *Sma*I restriction site. Several *spy* types were found in LA-MRSA strains which are also showing peculiar genetic differences and ability to spread among humans causing serious health disorders (Huijsdens et al., 2006). People living close to farms are more often exposed to LA-MRSA and may become silent carriers. However, LA-MRSA infection in patients that had not had direct contact with animals was also reported, im-

plicating different transmission pathways. Healthcare facilities may become an important place where infection may occur through contact with objects, contaminated foods and humans. Air borne transmission in places highly populated with pigs, also present an additional risk for spreading of MRSA in the environment (Deiters et al., 2015).

TWO WAY ADAPTATION OF MRSA

Over the years MRSA has shown capacity to transfer from humans to cattle and to other animal species and has become the most prevalent in pig herds. Soon after the initial establishment in animals, MRSA has found the way back to humans. However, MRSA is constantly changing and its different invasion capacity and pathogenicity mechanisms are recognized. Much work has been done to compare various MRSA strains of human and livestock origin utilizing different scientific approaches including phenotypic and genotypic experimental designs.

PATHOGENESIS AND EPIDEMIOLOGY OF MRSA

It was established that MRSA originate from MSSA strains after successful receipt of the SCC*mec* element, early after methicillin was introduced to clinical practice. MRSA has many virulence factors which are common to other bacteria. When inducing clinical infection, MRSA recruits specific genetic elements to enable adherence to the epithelial and endothelial cells. These strains have profound capacity for biofilm formation and evasion of immune responses of the host. Initial colonization ability is enabled by the early expression of the so called "microbial surface components recognizing adhesive matrix molecules"- MSCRAMMs, while toxins facilitate their further spread. Due to their specific pathogenic properties, it can cause recurrent infections. The HA-MRSA strains are often clonal and widespread all around the world. They become established in the hospital environment due to the number of virulence factors and multidrug resistant phenotype. However, the occurrence of MRSA infections with fatal outcome was recorded in the year 1990, in humans without healthcare contact or other risk factors for MRSA. Two well established clones of CA-MRSA in the USA are USA400 and USA300 (SCCmecIV) which can cause fatal necrotizing pneumonia or skin infections in patients. Infection with USA400 and USA300 was also identified in individuals with no history of hospitalization (Gordon and Lowy, 2008). Therefore, CA-MRSA became widespread in hospitals and crossed roads with the HA-MRSA strains.

In epidemiological studies several key points are important in determining the type of MRSA strains: the onset of the infection, recent or longer admission to hospital, prior infections, genetic characterization and clinical symptoms. Data collected from a single patient history or from an outbreak are important in epidemiological studies and have to be considered for establishing antibiotic therapy (David and Daum, 2010).

INVADING CAPACITIES OF MRSA *IN VITRO*

MRSA strains have different invading capacity *in vitro*. It was shown that LA-MRSA decreased potential of adherence to epithelial and endothelial cells with no apparent difference between the hosts (human or pig) comparing to HA and CA-MRSA strains. However, the specific *spa* type (the *spa* type t108) showed increased adhesive response comparing to *spa* types t011 and t034. Binding capacity to human and bovine plasma fibronectin was less efficient in LA-MRSA comparing to HA and CA MRSA strains, except for the *spa* type t108, which had a better binding ability comparing to other LA-MRSA strains. Invasiveness in embryonic kidney cells was more prominent in HA and CA-MRSA except for LA-MRSA *spa* type t108 which showed even higher invasiveness compared to HA and CA-MRSA. The *spa* type t108 was also less effectively phagocytosed by human polymorphonuclear leukocytes comparing to other LA-MRSA *spa* types and also comparing to some CA-MRSA strains. The evasion of this type of immune response may be driven by fibrin clots formation to which bacteria becomes encased and to which it binds, but clear differences between the molecular mechanisms were not identified in MRSA strains. In addition, LA-MRSA strains are forming capsules that protect MRSA from opsonization by a macrophage. This property is not related only to the *spa* type t108, rather it is a common feature among various *spa* types in human and animal MRSA strains. Other immune evasion mechanisms that have been studied so far often produced conflicting results or could not be adequately extrapolated on various MRSA isolates. However, LA-MRSA is found to exhibit strong toxic effect on epithelial cells by releasing eukaryotic lactate-dehydrogenase (LDH) enzyme. The upregulation of genes *hlb* and *hla* (encoding β-hemolysin) is also pronounced in LA- MRSA strain and it is comparable to the alpha-hemolysin producer USA300-*LAC*. Therefore, LA-MRSA has a clear pathogenic potential for humans quite similar to HA and CA-MRSA and presents an important pathogen all around the globe (Ballhausen et al., 2014).

CONCLUSION

MRSA is constantly changing and over decades has developed new features essential for their successful adaptation to new environments. Studies which aim to identify major genetic elements in MRSA and mechanism of their regulation, contribute in development of new or improved drugs which are important to reduce infection of humans. Agricultural use of antibiotics has to be minimized in the future which will in part "close the door" for future development of new antimicrobial resistance mechanism in bacteria.

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INFLUENCE OF *Saccharomyces cerevisiae* (Actisaf SC 47®) AS FEED ADITIVE IN GESTATION OR LACTATION DIETS ON SOWS AND NURSING PIGLETS HEALTH AND PERFORMANCE

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Abstract

The aim of this study was to investigate the effect of sows gestating or lactating diets supplemented with a commercial probiotic preparation (live yeast culture - *Saccharomyces cerevisiae*, "Actisaf Sc47®") on their health status, as well as the health status and productivity of their piglets during lactation. A total of 120 sows were divided into three groups: first (G, n=40) and second (L, n=40) group was fed diets with probiotic during pregnancy (G) or lactation (L), respectively. The third group (C, n = 40) was the control, which was fed without probiotic. Uterus and/or the udder diseases were manifested in the smaller ($p<0.01$) proportion in treated group (G=7.5%, L=12.5%) compared to control group (22.5%). The incidence of piglets diarrhea was lower ($p<0.05$) in the treated litters (12.5%) compared to the control litters (27.5 %). The average weaned piglets per litter (p/l) and weaning litter weight (lw) (G=11.6 p/l and 103.6 kg/lw; L=11.1 p/l and 102.8 kg/lw, C=10 p/l and 79 kg/lw) were higher ($p<0.01$ and $p<0.05$, respectively) in treated, compared to the control sows. These results show that the use of probiotic significantly improves the health status of lactating sows and piglets, as well as the piglets productivity within lactation.

Keywords: probiotics, diets, supplementation, performance, sows, piglets.

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UTICAJ DODAVANJA Saccharomyces cerevisiae (Actisaf SC 47®) U HRANU ZA KRMAČE TOKOM PERIODA GESTACIJE I LAKTACIJE NA ZDRAVLJE I PRODUKTIVNE PERFORMANSE PRASADI

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Kratak sadržaj

Cilj ovog rada je da se ispita uticaj dodatog komercijalnog probiotika (kulture živih kvasaca - *Saccharomyces cerevisiae*, "Actisaf Sc47™") u hranu za krmače u periodu gestacije i laktacije, na njihov zdravstveni status, kao i na zdravstveni status i produktivnost njihovih prasadi tokom perioda laktacije. Ukupno 120 krmača je podeljeno u tri grupe: prva (G, n=40) i druga (L, n=40) grupa koje su hranjene hranom sa dodatim probiotikom tokom gestacije (G) i laktacije (L). Treću grupu (n=40) su činile krmače hranjene hranom bez dodatka probiotika. Oboljenja uterusa i/ili vimena su manje ($p<0,01$) pokazivale tretirane grupe (G=7,5%, L=12,5%) u poređenju sa grupom kontrolnih krmača (22,5%). Incidenca pojave dijareja kod prasadi je niža ($p<0,05$) u tretiranoj grupi (12,5%) u odnosu na Prasad krmača iz kontrolne grupe (27,5 %). Prosečan broj odbijene prasadi po leglu (p/l) kao i težina legla kod odbijanja (tl) (G=11,6 p/l i 103,6 kg tl; L=11,1 p/l i 102,8 kg tl; C=10 p/l i 79 kg tl) su bili veći ($p<0,01$ i $p<0,05$, istim redom) u ogledним grupama u poređenju sa kontrolnom grupom krmača. Dobijeni rezultati pokazuju da se upotrebom probiotskih preparata značajno poboljšava zdravstveni status krmača u laktaciji i prasadi na sisi, kao i prasadi tokom perioda laktacije.

Ključne reči: probiotici, ishrana, performance, krmače, prasad

INTRODUCTION

Under the intensive production conditions, sows are exposed to numerous chronically stresogenic factors (Hyun et al., 1998; Sutherland et al., 2006), which reduce their immunity (Kick et al., 2012) and increase susceptibility to various infectious agents (Sutherland et al., 2006). In addition, the long-term application of conventional antimicrobial drugs for prevention or treatment of infectious diseases, result in significantly increase of infectious agent resistance to these antimicrobial preparations (Cromwell et al., 2002; Pugh, 2002; Le Coz, 2012). Reduced immunity and increased resistance of microorganisms to antimicrobial agents result in the increase of numerous infectious diseases, and consequently, reduce sows reproductive performances (Yeske, 2007; Stančić et al., 2010).

Nowadays, the problem of lower sow reproductive performance, as a result of reduced immunity and increased infective agents resistance to conventional antimicrobial preparation, is frequently attempt to solve by using probiotic preparations as natural immunomodulators (Blecha, 2001; Gallois et al., 2008; Zvekić et al., 2012; Apić and Zvekić, 2013). Live yeast culture (Gallois et al., 2009; Trckova et al., 2014) or their bioactive products (Kogan and Kocher, 2007; Shen et al., 2011) are one of the most active natural immunomodulator added to feed in order to prevent infectious diseases of the udder and the uterus, as well as to increase the reproductive performance of sows and their litters. However, according to previous studies, the effectiveness of replacing conventional antimicrobial agents with probiotics for the prevention and treatment of infectious diseases, as well as their impact on the sows reproductive performance, are not entirely consistent (Zvekić et al., 2012; Gallois et al., 2009; Trckova et al., 2014; Apić and Zvekić, 2013). The results of Serbian authors (Gagrčin et al., 2002; Stančić et al., 2012), demonstrated that in more than 50% of pig farms in R. of Serbia, there is a problem of udder and/or uterus infectious diseases (mastitis-metritis-agalactia syndrome; MMA - syndrome), resulting in significantly reduced sows reproductive performance.

Therefore, the aim of this study was to investigate the effect of adding one commercial probiotic preparation (live culture of *Saccharomyces cerevisiae*) in gestating or lactating diets on health status of sows and their litters, as well on productive performance of piglets within lactation.

MATERIALS AND METHODS

Farm and sows management

The experiment was carried out at one Serbian commercial pig farm, with about 1,200 Swedish Landrace and Large White sows in the reproductive herd. A total of 120 experimental sows (between the first and the sixth parity) were divided into three separate groups, immediately after artificial insemination. The first group (n=40 sows) was fed with probiotic supplemented diets during gestation (G group), the second group (n=40 sows) was fed with probiotic supplemented diets only within lactation period (L group), and the third control group (n=40 sows) was fed only with basic diets, without probiotics (C group). The sows in each experimental group were equalized according to body condition, parity and health. Average lactation period was 33 days. Duration of lactation period and parity was not significantly different ($p<0.05$) between the experimental group of sows (Table 1).

Table 1. Sows parity and duration of lactation ($\overline{X}$ ± SD)

Parameters	Sows feeding diets with the probiotic in:		Control group, without probiotic (C)
	Gestation (G)	Lactation (L)	
Number of sows	40	40	40
Parity (n)	$3,4^{a}$ ±1,48 (1-6)	$3,3^{a}$ ±1,39 (1-6)	$3,4^{a}$ ±1,38 (1-6)
Average lactation (days)	$33,0^{a}$±1,60 (30-35)	$33,3^{a}$±1,06 (31-35)	$33,2^{a}$±1,20 (31-35)

Minimal and maximal values are in parenthesis.

Values with different superscripts significantly differ: ^{ABC} ($p<0,01$); ^{abc}($p<0,05$).

The pregnant sows were housed in group pens (10 sows per group) and equalized by age, body condition and the stage of pregnancy. Between 7 and 10 days before the scheduled date of farrowing, the sows were moved into the farrowing house with individual pens, where they stayed with their litters during lactation.

Experimental sows diets

Standard feed for gestating and lactating sows were used as complete concentrate diet (produced by Veterinary Institute, "Subotica", Serbia). Composition of basic diets are given in Table 1. These diets were supplemented with 600g per ton of commercial probiotic preparation "Actisaf Sc47®", which contains live cells of *Saccharomyces cerevisiae strain CNCM I-4407* (Société Industrielle Lesaffre, Lesaffre Feed Additives, Marcq-en-Baroeul, France), to the basic feed for sows in gestation or lactation. During the first half of gestation, all sows received 3.2 kg of complete basic diet per day, and during the second half of gestation, 3.5 kg per day. Water was available ad libitum for pregnant and lactating sows.

Table 1. Composition of sows basic diets

Components	Gestating sows	Lactating sows
Crude protein (%)	13	16
Metabolic energy (MJ/kg)	11,5	11,8
Crude celulose (%)	9	7
Ca (%)	0,75-1	0,75-1
P (%)	0,55	0,55

Estrus detection, artificial insemination and pregnancy diagnosis

The estrus detection was performed twice daily by direct contact with the sexually mature teaser boar, starting on the first day after weaning. The semen was collected by hand-gloved method, from the boars of proven fertility, using phantom. The double artificial insemination (AI) was performed in the sows with estrus detected within first 7 days after weaning. The sows were first AI a few hours after standing estrus detection, and second time about 24 hours later. Freshly diluted insemination doses were used (dose volume of 100 mL, with about 4×10^9 progressively motile sperm). The insemination doses were kept in a thermo box at +17°C, and were used not more than 12 hours after collection.

The detection of possible return to estrus (i.e. first repeated estrus, rebreeding) start at day 14 after the first post-weaning AI. The diagnosis of pregnancy was recorded based on the absence of repeated estrus manifestation, as well as on the basis of a positive pregnancy testing results, using the "pulse- echo" ultrasound device. The ultrasound examination was performed 30 and 40 days after the last AI.

Lactating sows management

Gestating, lactating and the control sows and their litters were housed in a farrowing house with individual pens. First 3 days after farrowing, the sow rectal temperature was measured twice daily (according to the usual farm clinical practice, elevated rectal temperature was considered as ≥39.3°C). Water and adequate diets for each group of sows was available *ad libitum*. Group C was fed with basic diet, and group L and group G were fed with basic diet supplemented with "Actisaf Sc47®". Sows with clinically manifestation of uterine and/or udder disease, were treated by standard classical antimicrobial procedure.

In the case of occurrence of the uterine disease, the following signs were recorded: elevated rectal temperature, uterine discharge, no appetite. For udder disease (mastitis) recognizable clinical symptoms were: elevated rectal temperature, udder edema, hyperemia and pain, sternal position, hypo- or agalactia and no appetite.

Piglets were heated by floor heater and electric lamp (150W). Ten days after farrowing, until to weaning, piglets has received complete concentrated diets for nursing piglets (produced by Veterinary Institute "Subotica", R. of Serbia).

Data recorded

For sows the following data were recorded: sows rectal temperature within first 3 days after farrowing, clinically manifestation of uterine and/or mammary gland diseases after farrowing. Litter size and litter weight at farrowing, diarrhea, preweaning piglets mortality, litter size and litter weight at weaning were recorded.

Statistical analysis

The evaluation of phenotypic parameters of the research results was done by the "Statistic 12" software package according to the average, minimum and maximum values and standard deviation of the experimental results.

T-test was used to test the difference between the arithmetic means of the results and $p<0.05$ or lower was considered as a significant difference.

RESULTS

The experimental groups (G and L group) and control group (c group) were set up in such a way that between them there is no a statistically signifi-

cant difference ($p<0.05$) in the duration of lactation period and sows parity, as shown in Table 2.

Table 2. Sows parity and duration of lactation ($\bar{x}$ ± SD)

Parameters	Sows feeding diets with the probiotic in:		Control group, without probiotic (C)
	Gestation (G)	Lactation (L)	
Number of sows	40	40	40
Parity (n)	3.4^{a} ±1.48 (1-6)	3.3^{a} ±1.39 (1-6)	3.4^{a} ±1.38 (1-6)
Average lactation (days)	33.0^{a}±1.60 (30-35)	33.3^{a}±1.06 (31-35)	33.2^{a}±1.20 (31-35)

Minimal and maximal values are in parenthesis.
Values with different superscripts significantly differ: ^{ABC} ($p<0.01$); ^{abc}($p<0.05$).

In the first three days after farrowing a significantly higher proportion of control sows (22.5%) had elevated rectal temperatures (≥39.3°C) compared with those fed with diets supplemented with probiotic in gestation (7.5%) or lactation (12.5%). The results are given in Table 3.

Table 3. Sows health status within lactation ($\bar{x}$ ± SD)

Parameters Gestation (G)		Sows diets with the probiotic in:		Control group, without probiotic (C)
		Lactation (L)		
Number of sows		40	40	40
Sows with elevated rectal temperature[1]	n	3	5	9
	%	7.5^{A} ±1.68	12.5^{a} ±1.87	22.5^{Bb} ±2.43
Aver. elevated rectal temp. (°C)		39.6	39.9	39.8
Clinically manifestation of uterine and/or mammary gland diseases				
Metritis + hypogalactia	n	0	0	2
	%	0,0	0,0	5.0
Metritis + agalactia	n	0	1	0
	%	0.0	2.5	0,0
Mastitis + hypogalactia	n	3	2	2
	%	7.5	5.0	5.0

Parameters Gestation (G)		Sows diets with the probiotic in:		Control group, without probiotic (C)
		Lactation (L)		
Mastitis + agalactia	n	0	0	1
	%	0,0	0,0	2.5
MMA - syndrome	n	0	2	4
	%	0,0	5.0	10.0
Total sick sows	n	3	5	9
	%	7.5^{A} ±1.68	12.5^{a} ±1.87	22.5^{Bb} ±2.43

Minimal and maximal values in parenthesis; [1] Elevated rectal temperature: ≥39.3°C. Values with different superscripts significantly differ: ^{ABC} ($p<0.01$); ^{abc} ($p<0.05$).

In the sows with elevated rectal temperature, metritis with hypogalactia or agalactia, mastitis with hypogalactia or agalactia, or mastitis-metritis-agalactia syndrome (MMA) was noted mainly within the first week after farrowing (Table 3).

The average number of live born piglets per litter were 12.22 in the G-group, 11.53 in the L-group, and 11.42 piglets in the control group. These values were significantly ($p<0.05$) higher in the G-group in comparison with the control and L group of sows. However, average number of live born piglets per litter was not significantly different ($p>0.05$) between L and C group of sows (Table 4).

Table 4. Litter parameters from farrowing to weaning ($\overline{x}$ ± SD)

Parameters	Sows diets with the probiotic in:		Control group, without probiotic (C)
	Gestation (G)	Lactation (L)	
Number of litters	40	40	40
Total piglets born (n)	516	492	487
Total live born piglets (n)	489	461	457
Average stillborn piglets per litter (n)	0,67	0,77	0,75
Average live born piglets per litter (n)	12.22^{a} ±1.88 (8-16)	11.53^{b} ±2.17 (9-15)	11.42^{b} ±2.48 (7-16)
Average live born piglet weight (kg)	1.32 (1.0-1.72)	1.39 (1.0-1.65)	1.44 (1.2-1.80)

Parameters		Sows diets with the probiotic in:		Control group, without probiotic (C)
		Gestation (G)	Lactation (L)	
Average live born litter weight (kg)		16.07^{a} ±2.26 (12-21)	16.43^{a} ±2.17 (13-21)	16.47^{a} ±2.14 (7-21)
Litters with diarrhea	n	$5±0.33^{a}$	$5±0,33^{a}$	$11±0,45^{b}$
	%	12.5	12.5	27.5
Total piglets weaned (n)		466	446	400
Average weaned piglets per litter (n)		11.65^{A}±2.09 (7-16)	11.15^{a}±1.76 (7-14)	10.0^{Bb}±2.50 (4-16)
Total preweaning piglets mortality	n	23^{a}±2.32	15^{a}±2,26	57^{B}±2.92
	%	4.9	3,4	14.2
Average litter weight at weaning (kg)		103.6^{A}±15.66 (51-128)	102.8^{A}±13.53 (63-103)	79.1^{B}±16.9 (34-102)

Minimal and maximal values in parenthesis.
Values with different superscripts significantly differ: ^{ABC} ($p<0.01$); ^{abc} ($p<0.05$).

The average weight of a live born piglets per litter, in both treatment groups (G = 16.4 kg; L = 16.1 kg) was significantly higher ($p<0.01$) than in the control group (13.5 kg). Significantly higher average weaned piglets per litter were estimated in G-group (11.65 piglets, $p<0.01$), and in L-group sows (11.15 piglets, $p<0.05$) than in the control group of sows (10.0 piglets). Diarrhea in suckling piglets was manifested in 12.5% litters in both probiotic treated groups (G and L group), which is significantly lower ($p<0.05$) than in the untreated (control, C group) sows (27.5%). Preweaning piglet mortality was approximately 10% lower in the probiotic-treated sows (G = 4.9%; L = 3.4%) compared to the control sows (C = 14.2%). The average weaned litter weight was significantly higher ($p<0.01$) in both probiotic treated groups (G = 103.6 kg; L = 102.8 kg) in comparison with the control group of sows (79.1 kg) (Table 4).

DISCUSSION

Studies carried out on a large farm, representative for Serbian intensive pig production, shows that the average farrowing number per sow was 3.5 with the average 2.1 annual farrowing index. The average farrowing rate was 78.9%, and the average number of live born piglets per litter was 10.9. Total sows culling rate was 38.4% per year. About 42% of total culled sows were culled due to the health problems. According to Stančić et al. (2012) and Maletić et al. (2012) the diseases of the uterus and/or udder were primary reasons for culling 30,4% of the total sows culled due to the health problems in the Serbian pig farms. These authors also found permanent efficacy decreasment of conventional antimicrobial drugs used for treatment of uterus and/or udder diseases in sows, as well as for treatment of infectious diarrhea in newborn piglets, primarily due to increased resistance of the infectious agents to the number of antimicrobials. Similar problems related to increasing microbial resistance to conventional antimicrobial preparations have been shown by other authors (Wray and Gananou, 2000; McEwen and Fedorka-Cray, 2002). Therefore, the aim of this study was to solve this problem in lactating sows and their litters, by addition of commercial natural probiotic preparations in the diets of gestating or lactating sows, under Serbian intensive pig production conditions.

The results obtained in the present study indicate that feeding sows during pregnancy or lactation period by standard complete diets supplemented with probiotic *Saccharomyces cerevisiae CNCM I-4407 (Actisaf Sc47)* significantly improve their health status, (7.5% in G and 12.5% in L group of sows with clinical manifestation of uterine and/or mammary gland diseases, compared with 22.5% in the control group), as well as the health status of their piglets (12.5% litters with diarrhea in probiotic treated group in comparation with 27.5% litters with diarrhea in the control sows), and preweaning piglet mortality (4.9% in G-group, 3.4% in L-group and 14.2% in control group of sows). In addition, the average number of weaned piglets per litter (G = 11.65; L = 11.15) and the average litter weight at weaning (G = 103.6 kg; L= 102.8 kg) were significantly higher in the sows feed with probiotics in comparison with the control sows (79.1 kg).

Periparturient uterine and/or udder infectious diseases in the sows and coliform diarrhea in the newborn piglets are the main health factors that significantly reduce weaned piglets production (Yeske, 2007; Trckova et al., 2014; Shen et al., 2011; Böhmer et al., 2006; Kim et al., 2008).

In recent years, natural probiotics are used as substitutes for traditional antimicrobial preparations in animal production (Gallois et al., 2008; Giang,

2010; Bass, et al., 2012). Namely, it has been shown that microbial resistance to conventional antibiotics can be avoided by the application of probiotics (McEwen and Fedorka-Cray, 2002; Williams, 2010). Consequently, this prevents the appearance of residual antibiotics in the feed of animal origin used in human nutrition, and their harmful impact on the health of the human population (Wray and Gananou, 2000; Marshall and Stuart, 2011).

Although not clearly consistent, most researches show that the use of probiotic preparations (natural immunomodulators), containing live yeast, in the diets of pregnant and lactating sows, can significantly reduce puerperal uterus and/or mammary gland diseases (Giang at al., 2010; Kogan at al., 2007), newborn piglet infectious diarrhea (Gallois et al., 2009; Kim et al., 2008; Bass et al., 2012) and increase the preweaning piglet performance (Shen et al., 2011; Apić and Zvekić, 2013; Bass et al., 2012).

In the present study, the significant decrease of postpartal uterus and/or udder infectious diseases in the sows fed with diets supplemented with probiotics during gestation or lactation, may be the result of probiotics ability to enhance the sows natural immunity, as effect of Glucans + Mannan Oligosaccharide (Kogan and Kocher, 2007; Böhmer et al., 2006; Salmon, 2012). On the other hand, it has been shown that live yeast or their bioactive product mannan oligosaccharides can stimulate maternal immunoglobulin (Ig) production and their increasing presence in colostrum and milk (Gallois et al., 2009). Health protection of newborn piglets solely depends on these Ig (Zanello et al., 2012). Consequently, significant higher preweaning piglets mortality in the litters of postpartal sick sows, particulary in the control sows, obtained in the present study, can be primarily due to increasing incidence of diarrhea (Blecha, 2001), as a result of significant reduced or totally absent of milk production and/or Ig in colostrum and milk (Giang, 2010).

CONCLUSION

Live yeast probiotic supplementation in gestation diets significantly decrease the occurrence of postpartal uterine and/or udder diseases (7.5%) and increase the average number of live born piglets per liter (12.2), compared to sows fed diets supplemented with probiotic within lactation period (uterine and/or udder diseases 12.5%, and 11.5 live born piglets per litter), as well as with the control sows (uterine and/or udder diseases 22.5%, and 11.4 live born piglets per litter). Utilization of *Saccharomyces cerevisiae* live culture in the diets for pregnant or lactating sows, significantly improves their health status and the health status of their piglets within lactation in Serbian intensive

pig production conditions. In addition, litter productive parameters (average number of weaned piglets per litter and litter weight at weaning) were significantly higher in sows treated with probiotic than in untreated (control) sows.

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DIROFILARIOSIS AND ANGIOSTRONGILOSIS IN PET AND HUNTING DOGS IN NOVI SAD, VOJVODINA, SERBIA

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Abstract

The aim of this study is to update the data on the prevalence of *Dirofilaria immitis* (*D. immitis*) and *Dirofilaria repens* (*D. repens*) infections in pet dogs, to report a preliminary result of the prevalence of *Angiostrongylus vasorum* (*A. vasorum*) in hunting dogs, and to assess the presence of concurrent infection with *D. immitis* and *A. vasorum* in hunting and pet dogs in Novi Sad. The methods used to estimate the prevalence of dirofilaria infections were modified Knott test and detection of antigen of *D. immitis*. The prevalence of *A. vasorum* was determined using Baermann fecal technique and detection of *A. vasorum* antigen. Concurrent infection with *D. immitis* and *A. vasorum* was assessed only by detection of antigens of each parasite. Overall prevalence values for *D. immitis* and *D. repens* were 18.95% (24/143) and 16.32% (27/143), respectively. The prevalence of *A. vasorum* in hunting dogs was 1.96% (1/51). Concurrent infection with *D. immitis* and *A. vasorum* did not exist in examined hunting and pet dogs. Further studies with larger number of examined dogs and samples from other region of the country are needed to determine the prevalence of these parasites.

Key words: *Dirofilaria spp*., *A. vasorum*, prevalence, dogs.

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DIROFILARIOZA I ANGIOSTRONGILOZA KOD PASA KUĆNIH LJUBIMACA I LOVAČKIH PASA U NOVOM SADU, VOJVODINA, SRBIJA

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Kratak sadržaj

Cilj ovog istraživanja je da pruži nove podatke o prevalenciji *D. immitis* i *D. repens* kod pasa kućnih ljubimaca u Novom Sadu, da objavi preliminarne rezultate prevalencije *A. vasorum* kod lovačkih pasa u Novom Sadu, i da proceni da li postoji istovremena infekcija sa oba parazita kod lovačkih pasa i pasa kućnih ljubimaca u Novom Sadu. Za procenu prevalencije infekcije dirofilarijama korišćeni su modifikovani Knotov test i detekcija antigena *D. immitis*. Prevalencija *A. vasorum* je ispitivana kod lovačkih pasa na osnovu Bermanovog testa i detekcijom antigena parazita. Postojanje istovremene infekcije sa *D. immitis* i *A. vasorum* kod lovačkih i pasa kućnih ljubimaca je proveravano samo na osnovu detektovanja antigena oba parazita. Ukupna prevalencija infekcije *D. immitis* iznosila je 18,95% (24/143), dok je za *D. repens* prevalencija bila 16,32% (27/143). Prevalencija *A. vasorum* kod lovačkih pasa je bila 1,96% (1/51). Nije utvrđeno postojanje istovremene infekcije sa *D. immitis* i *A. vasorum* u ispitivanoj populaciji lovačkih i pasa kućnih ljubimaca. Dalja ispitivanja sa većim brojem pasa i uzoraka od pasa iz različitih delova zemlje su potrebna kako bi se odredila prevalencija za ove parazite.

Ključne reči: *Dirofilaria spp.*, *A. vasorum*, prevalencija, psi.

INTRODUCTION

Heartworm diseases caused by *Dirofilaria immitis (D. immitis)* and angiostrongylosis caused by French heartworm *Angiostrongylus vasorum (A. vasorum)* are canine parasitic diseases affecting mainly the respiratory and cardiovascular system. Both parasites are lungworms having the same primary site of residence - pulmonary arteries, where they can induce severe pathologic alterations. On the other hand, intermediate hosts of these parasites are

different, thus defining their specific seasonal characteristics. Mosquitoes as intermediate hosts for *Dirofilaria spp*. determine transmission period of the diseases. In case of *A. vasorum,* a wide range of terrestrial and aquatic gastropods act as obligatory intermediate hosts. Mild and wet climate is suitable for rapid multiplication of these intervertebrate hosts. Both parasites mostly cause chronic diseases with severe clinical complications such as pulmonary thromboembolism, right-sided heart failure and caval syndrome associated with *D. immitis* infections, whereas verminous pneumonia, right-sided heart failure and bleeding tendencies are associated with *A. vasorum*. In addition, the diseases are different in their zoonotic potential. *D. immitis*, capable to form pulmonary nodules in humans, is considered to have a zoonotic potential, while *A. vasorum* is not a zoonotic agent (Ware, 2011; Morgan and Show, 2010). While canine cardiopulmonary dirofilariosis and pulmonary angiostrongylosis are diseases of obvious clinical importance, *D. repens* infection, also known as subcutaneous dirofilariosis, is less clinically important in dogs (Scott and Vaughn, 1987). Due to the importance of *D. repens* in humans, it is upon veterinary profession to deal with this agent as well.

Previous study on the prevalence of dirofilariosis in pet dogs in Novi Sad has shown the increase of *D. immitis* infection and decrease of infection with *D. repens* (Spasojević Kosić et al., 2012, 2014) as compared with first reports on the prevalence of *D. repens* infection (Tasić et al., 2008) and mixed infection with both parasites in dogs (Spasojević Kosić et al., 2014). Such significant prevalence rates make the diseases highly important from both epizootical and clinical point of view. Clinical importance of heartworm disease in dogs and zoonotic potential of *Dirofilaria* spp. prompted us to monitor the prevalence of both parasites among dogs and report data periodically.

Fecal examination technique or sera analysis have been used for studying the prevalence for *A. vasorum* in dogs also in some surrounding countries including Greece, Hungary, Bulgaria (Papazahariadou et al., 2007; Schnyder et al., 2015; Pantchev et al., 2015). Couple of years ago, the first case of *A. vasorum* was reported in Posavac hound in Serbia. Moreover, Serbia is abundant with terrestrial and aquatic gastropods proved to be either natural or experimental host for *A. vasorum*, while climate in Vojvodina offers suitable conditions for their survival (Simin et al., 2014). From the epizootical point of view, this finding is important, yet not sufficient; thus, further studies are needed to estimate the prevalence of canine pulmonary angiostrongylosis among dogs in Serbia. In regions where this disease is endemic, the true prevalence of the disease is probably underestimated, because most diagnoses of angiostrongylosis are made when infection results in clinical signs (Morgan et al. 2005; Koch and

Willesen, 2009). To our knowledge, this is the first report on the prevalence of *A. vasorum* in dogs in Serbia. Due to the fact that both heartworm diseases and French heartworm can manifest as subclinical conditions (Savić et al., 2012; Simin et al., 2014) or with respiratory signs such as cough and dyspnea, in this study, we investigated the existence of the concurrent infection in some asymptomatic hunting dogs and in pet dogs with clinical signs. Hence, parasitic infestations of respiratory and cardiovascular system should be considered by a clinician when a differential diagnosis list is made for patients with respiratory and/or cardiovascular system signs.

MATERIAL AND METHODS

In the period from 2010 to 2016, pet dogs from Novi Sad were tested for dirofilaria infections. The research included 190 privately owned pet dogs. At the moment of testing, the dogs were at least 7 months old, exposed minimally to one mosquito season (in Serbia it is from April to October), and without history of treatment with macrocyclic lactones. All animals were clinically examined and blood samples were taken from all dogs to the purpose of parasitological examination. The parasitological examination consisted of wet blood smears, modified Knott test and antigen testing. Techniques for detecting circulating microfilariae included microscopic examination of fresh blood smears and modified Knott test. Detection and enumeration of circulating microfilariae (mf) of both *D. immitis* and *D. repens* were carried out using modified Knott test (Bazzochi et al., 2008). Morphological characteristics of microfilariae such as length, width, cephalic and caudal ends, were assessed in order to differentiate microfilariae of two *Dirofilaria* species (Genchi et al., 2007). Detection of circulating *D. immitis* antigens was carried out using commercial kit (SNAP Heartworm RT Test, IDEXX Veterinary Diagnostics) according to manufacturer's instruction.

In order to detect infestation with *A. vasorum* in hunting dogs, Baermann fecal examination method and antigen detection (Angio Detect Test, IDEXX Laboratories) were used. For the detection of the *A. vasorum* larvae, modified Baermann test was performed (Zajac and Conboy, 2006). A total of 51 hunting dogs were examined for *A. vasorum* infestation by both Baermann fecal examination and antigen detection. Fecal examinations by Baerman test in dogs were done first. The sera of these dogs were frozen and later tested for *A. vasorum* antigen.

The number of hunting dogs examined for concurrent infections with heartworm and French heartworm was 37, and the analyses were done by anti-

gen detections for both parasites (SNAP Heartworm RT Test, Idexx Veterinary Diagnostics and Angio Detect Test, Idexx Laboratories). Twelve pet dogs with respiratory clinical signs (mainly cough and dyspnea) were also evaluated for the infestation with both *D. immitis* and *A.vasorum* using antigen detection for each parasite.

RESULTS AND DISCUSSION

In this study as well as in our previous studies (Spasojević Kosić et al., 2012, 2014), we have been applying well established and recognized methods in the diagnosing of dirofilariosis (American Heartworm Society Canine Guidelines, 2014, ESCCAP 2012). Our main criteria for testing a dog included it's classification into the susceptible population with respect to age, exposure to mosquitoes and lack of prophylactic treatment. The number of tested dogs varied throughout the time period, and we observed increase in number of dogs with clinical signs among the susceptible population. Clinical signs observed in dogs included cough, dyspnea, fatigue, cachexia, weakness, syncope, skin nodules, lameness, ascites, neurological signs, with cough and skin nodules being the most common ones. The number of dogs infested with *D. repens* was either higher or equal to the number of dogs infested with *D. immitis*. The exceptions in view of numbers of infested dogs were recorded in 2013, 2015 and 2016, when we observed more dogs infested with *D. immitis* and dogs infested with both *Dirofilaria spp*. We started with diagnosing concurrent prevalence of infection with both *Dirofilaria spp*. among pet dogs in 2013, and for the entire study period the infection was confirmed in 11 dogs (table 1).

Table 1. Prevalence of infection with *Dirofilaria spp*. as single or mixed infection in pet dogs during the study period

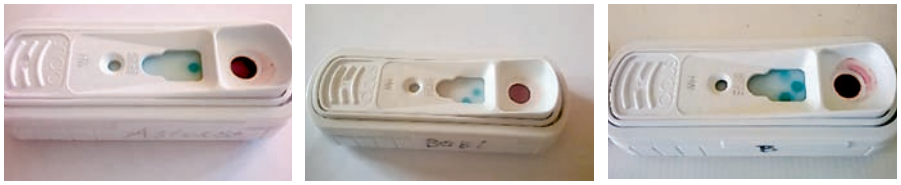
Year	Number of dogs	Prevalence *D. repens*	Prevalence *D. immitis*	Prevalence Mixed infection
2010	39	10 .26% (4/39)	5 .13% (2/39)	0
2011	16	12 .5% (2/16)	12 .5% (2/16)	0
2012	26	15 .38% (4/26)	11 .54% (3/26)	0
2013	39	12 .82% (5/39)	15 .38% (6/39)	20 .51% (8/39)
2014	26	15 .38% (4/26)	7 .69% (2/26)	3 .84% (1/26)
2015	24	4 .17% (1/24)	12 .5% (3/24)	0
2016	20	0	35% (7/20)	10% (2/20)
2010 -2016	190	10 .53% (20/190)	13 .16% (25/190)	5 .79% (11/190)

In order to compare the prevalence reported in this study with the prevalence of dirofilariosis in the previous studies, it is necessary to take into consideration the methods used for the diagnosing of *D. immitis* and *D repens* infection. Having in mind this fact, we can compare our new results with our previously published results, and with the study of Tasić et al. (2008). In this study, we observed an overall prevalence for *D. repens* of 16.32% (31/190), which is lower than that from our previous study (18.88%, 27/143 dogs) (Spasojević Kosić et al., 2014) and lower than that from the first report of *D. repens* in dogs in Novi Sad (Tasić et al., 2008). The prevalence of *D. immitis* in dogs is 18.95% (36/190), which is higher than that reported in our previous study (16.78%, 24/143 dogs). The prevalence of *D. immitis* is particularly increased in this year, which could be explained by the fact that the majority of dogs examined in this year were those with respiratory clinical signs. For the period 2009-2013, the prevalence of dirofilariosis in Vojvodina was reported by Savić et al. (Savić et al., 2014.); however, in this study, the differentiation of microfilariae has not been done and no conclusion can be made on the prevalence of *D. immitis* and *D. repens*. In the study of Savić et al. (2015), the prevalence of dirofilariosis, being 15.29%, was established for the period of 2 years of study, and in 92.3% of positive samples, *D. immitis* were determined by PCR.

In order to diagnose *A. vasorum*, Baermann fecal examination technique was used because the test is useful when larvae are being shed. In hunting dogs examined by this test, *A. vasorum* larvae have been detected in one dog (Simin et al., 2014) making the prevalence for *A. vasorum* of 1.96%. Prevalence of 1.1% was found in Greece based on fecal examination of 281 samples (Papazahariadou et al., 2007), while the prevalence determined in 1247 dogs from Hungary ranged from 1.36% to 2.73% (depending on sera analyses) (Schnyder et al., 2015). In 167 sera of dogs from Bulgaria, no positive findings were recorded (Pantchev et al., 2015). Overall estimated prevalence in this region of Europe in not so high, but the number of examined dogs was higher as compared with our study population. Having in mind that some dogs might not be shedding larvae at the moment of examination, we wanted to increase a detection of infestation with *A. vasorum* by using the detection of the parasite's antigen. However, the antigen of *A. vasorum* was confirmed in none of these animals, not even in the dog in which the larvae of the parasite were detected. The possible explanation for this result could be the formation of antigen-antibody complexes, which inhibit detection of antigen in a sample as it has been previously shown with *D. immitis* detection (Matsumura et al., 1986; Brunner et al., 1988) and with *A. vasorum* detection by rapid device (Schnyder et al., 2014).

In hunting dogs examined for antigen of both parasites, only low level of

heartworm antigen was detected in two dogs, indicating the prevalence of *D. immitis* in examined hunting dogs of 5.40% (2/37). Concurrent infection with *D. immitis* and *A. vasorum* was established in neither hunting nor pet dogs. In Portugal, where the aforementioned study was conducted, concurrent infection with *D. immitis* and *A. vasorum* was found in one dog (Alho et al., 2014).



A B C

Fig 1. SNAP Heartworm RT Test: (A) negative (one dot) and positive findings: (B) two dots (low level) and (C) three dots (high antigen level)

Fig 2. Angio Detect test – showing negative findings (one line) in several pet dogs

Further studies are needed to determine the actual prevalence of *A. vasorum* in dogs in Serbia. Studies like these, aimed at emphasizing the need for specific diagnosis and prevention in dogs, could be useful for clinical practice. With regard to proven heartworm disease and *A. vasorum* finding in Serbia, use of specific diagnostic procedures for detection of these two parasites in dogs with signs indicating respiratory disease is essential. This approach would provide reliable results of clinical studies on dogs.

CONCLUSION

This study revealed the prevalence rates of 13.16% and 10.53% for single infections with *D. immitis* and *D. repens,* respectively, whereas the prevalence for mixed infections with both *Dirofilaria* species in pet dogs in Novi Sad was

5.79%. Prevalence of *D. immitis* in hunting dogs was 5.40%. According to the results of Baermann fecal examination method, the prevalence of *A. vasorum* in hunting dogs from Novi Sad was 1.96%. Concurrent infection with *D. immitis* and *A. vasorum* was established in neither hunting nor pet dogs in Novi Sad, Vojvodina, Serbia.

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PCR DETECTION OF GENITAL INFECTIONS IN BULL SEMEN FROM DIFFERENT REGIONS OF UKRAINE, 2013 - 2016

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Abstract

The most important infectious diseases that affect fertility of the bull via semen are reviewed in this article. The article describes the results of a study of cryopreserved semen samples from different regions of Ukraine for the period 2013 to 2016. The strategy of regular testing of semen donors under official veterinary supervision has been adopted by governments worldwide as a means of avoiding the spread of pathogens and reducing excessive contamination of semen by ubiquitous bacteria and viruses. During this period, four hundred fifty (n=450) bull semen samples from 10 farms across Ukraine have been tested, PCR diagnostics was performed by the standard method developed in NSC «IECVM». According to the PCR analysis of cryopreserved semen from the bull-sires, 19 samples (6%) from Kharkiv region contained genetic material of *Mycoplasma spp.*, 2 samples from Poltava region (3.5%) were positive for BoHV-1, and 2 samples from Cherkasy region (4.5%) contained genetic material of *Chlamydia spp*. Based on the obtained results we can conclude that it is necessary to permanently perform PCR screening of bulls semen.

Key words: genital infections, *Mycoplasma,* BoHV-1, *Chlamydia,* semen, bulls, Ukraine

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PCR DETEKCIJA GENITALNIH INFEKCIJA U SEMENU BIKOVA IZ RAZLIČITIH REGIONA UKRAJINE, 2013 - 2016

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Kratak sadržaj

Najznačajnije infektivne bolesti koje utiču na plodnost bikova, a prenose se semenom, su opisane u ovom radu. Ovaj rad prikazuje rezultate istraživanja na zamrznutim uzorcima semena iz različitih regiona Ukrajine u periodu od 2013. do 2016. godine. Redovno testiranje semena bikova donora pod službenim veterinarskim nadzorom prihvaćeno je od strane vlada širom sveta kao način da se izbegne širenje uzročnika oboljenja i smanji kontaminacija semena ubikvitarnim bakterijama i virusima. Tokom ovog perioda testirano je četristopedeset (n=450) uzoraka semena bikova sa 10 farmi širom Ukrajine, a PCR dijagnostika je izvršena standardnim metodom ravijenim u NNC „IEKVM". Rezultati ispitivanja zamrznutih uzoraka semena priplodnih bikova pokazuju da su u 19 uzoraka (6%) iz Harkovske oblasti dokazani geni *Mycoplasma* spp., u dva uzorka iz Poltavske oblasti (3,5%) su dokazani geni BoHV-1, dok su dva uzorka iz Čerkaške oblasti (4,5%) bila pozitivna na *Chlamydia* vrste. Na osnovu ovih rezultata možemo zaključiti da je neophodno permanentno sprovoditi PCR skrining semena bikova na prisustvo patogenih uzročnika.

Ključne reči: polno prenosive infekcije, *Mycoplasma,* BoHV-1, *Chlamydia,* seme, bikovi, Ukrajina

INTRODUCTION

The main goal of artificial insemination (AI) in cattle is to achieve genetic improvement. However, transmission of infectious diseases by semen (sexual transmission, ST) constitutes a risk that must be avoided. The semen used for

AI must, therefore, be free of infectious agents (Wentink et al., 2000). The bull semen also plays a key role in reproductive excrete in cow herds, acting as a source of infectious diseases. The regular testing of semen donors under official veterinary supervision has been adopted by governments worldwide as a means of avoiding the spread of pathogens and reducing excessive contamination of semen by ubiquitous bacteria and viruses (Eaglesome et al., 1997).

Abortion among dairy cows is one of the sources of substantial economic losses in cattle industry. Although the risk of abortion depends on several factors (i.e., genetic abnormalities, heat stress, toxic agents), infectious agents are likely to be one of the most important risk factors associated with abortions. A variety of sexually transmitted infectious agents has been reported to cause bovine abortion throughout the world (Tramuta et al., 2011). Hence, one of the main goals of the present communication is to review major pathogens associated with ST in cattle.

Infectious bovine rhinotracheitis *(IBR)*. The infection with *Bovineherpesvirus*, leading to balanoposthitis in bulls and infectious pustular vulvovaginitis (IPV) in cows, has been recognized for several decades. It is a complex, baffling and changing disease, giving rise to much controversy, with prophylaxis proving difficult to devise and implement. The etiological agent, *Bovine herpesvirus 1* (BoHV-1) is a double-stranded DNA (dsDNA) virus and is a member of subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. The virus is not very resistant, and it is transmitted directly by respiratory, venereal or bucco-genital routes or indirectly via contaminated hands of a farmer or a semen collector. The infection can occur in both males and females without obvious clinical signs, or can be accompanied by non-specific inflammation, granulation tissue, vesicles and ulceration (which may be due to secondary infection with non-specific microorganisms) (Parez et al., 1985).

Bovine viral diarrhea (BVD). *Bovine viral diarrhea virus* (BVDV) is a *pestivirus* from the family *Flaviviridae* (Becher and Thiel, 2011), capable of causing serious clinical disease in cattle. The virus is divided into two genotypes (BVDV-1 and BVDV-2) on the basis of antigenic and genetic differences (Vilcek et al., 2005). Infection with BVDV is known to have a significant financial impact (Houe, 1999), stemming primarily from the reproductive and immunosuppressive effects of acute infection. The most common economic losses resulting from BVDV infection are associated with failure in fertilization, abortion, congenital malformation, stillbirth or birth of persistently infected (PI) progeny (Grahn et al., 1984; Rüfenachtet al., 2001).

Chlamydial infections. Recognised for some time as the cause of epidemic abortion in cows in the USA, *Chlamydia psittaci* in Europe is associated with

sporadic abortion (at 3 to 7 months) due to necrotic placentitis and a direct effect on the fetus (producing liver lesions). Infertility associated with vaginitis and endometritis was also described.

Chlamydiae are obligate intracellular, gram-negative bacteria with atypical developmental life cycle. During the last ten years, four *Chlamydia* species have been isolated from humans and from different domestic and wild animals. Infection with chlamydial bacteria, specifically *C. pecorum*, *C. abortus*, and *C. psittaci*, are common in cattle. An infection with chlamydia may cause sub- or infertility. Infected bulls may suffer from vesiculitis, or may not be affected at all (Storz et al., 1968).

Mycoplasmoses. Different species of Mycoplasmas can affect bovines causing several diseases. Mycoplasmas can cause clinical, subclinical or chronic intramammary infection affecting cattle of all ages and at any stage of lactation (Tamiozzo et al., 2014). The numerous mycoplasmas involved in diseases of cattle create a complex situation with regard to lesions and the causal agent. Species commonly associated with genital tract infection in Europe are *Mycoplasma bovigenitalium, Mycoplasma bovis, Acholeplasma laidlawii* and *Ureaplasma spp.* Organisms of the genera *Mycoplasma*, *Acholeplasma* and *Ureaplasma* have been isolated from the distal part of both male and female bovine genital tract. *Mycoplasma bovis*, *M. bovigenitalium* and *U. diversum* appear to be the most important pathogens of the genital tract, involved in diseases such as decreased sperm motility (*M. bovigenitalium*), seminal vesiculitis, epididymitis (*M. bovis* and *M. bovigenitalium*), endometritis (*M. bovis*), granular vulvovaginitis, infertility and abortion (*U. diversum*) (Fish et al., 1985).

Solving the problem of animal pathology is the key to successful management of industrial livestock in Ukraine (Stegniy et. al., 2015). Most common infectious diseases transmitted via bull semen in Ukraine include infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVDV), chlamydioses, and mycoplasmoses. Hence, it is important to focus on the control of genetic resources in breeding work in order to effectively break the epizootic chain of the diseases.

MATERIALS AND METHODS

Monitoring studies for ST infections were conducted from 2013 to 2016. During this time, four hundred fifty (n=450) bull semen samples from 10 farms across Ukraine have been tested. PCR diagnostics was performed by the standard method developed in National Scientific Center "Institute for Experimental and Clinical Veterinary Medicine" (NSC «IECVM») (Stegniy et. al., 2015).

Isolation of total DNA was performed using a commercial kit for extraction of nucleic acids "DNA Sorb-B", produced by "Central Research Institute of Epidemiology" (Russian Federation). Reverse transcription was performed using a set of reagents RT-Core production company IsoGene (Russian Federation).

The amplification reaction was performed using commercial set «PCR-Core» manufactured by IsoGene systems and primers (Table 1).

Table 1. Primers positive control samples and the size of amplicon generated for IBR, BVDV, and *Chlamydia*

	Primers	PCT	Product size (bp)
IBR	BOHVF_ R	SL	202
BVDV	BVDVF_R	Oregon	267
Chlamydia spp.	CHLAMF_R	V.Olexandrivka\11	237
1. PCT - positive control template 2.SL - DNA formalin inactivated strain of IBR 3.V.Olexandrivka\11 - DNA boiling inactivated *Chl. psittaci* 4.Oregon - formalin inactivated strain of cattle BVDV.			

Identification of the BoHV-1, BVDV, *Chlamydia* and *Mycoplasma spp.* genetic material was performed according to standard amplification protocols.

RESULTS AND DISCUSSIONS

The analysis of research samples of cryopreserved semen from the bull-sires for PCR revealed that genetic material of *Mycoplasma spp* was contained in 19 samples (Fig. 1) (6%), which came from farms in Kharkiv region, genetic material of BoHV-1 pathogen was detected in 2 (3.5%) positive samples from Poltava region, and 2 samples (4.5%) containing genetic material of the pathogen *Chlamydia spp* were obtained from Cherkasy region.

Table 2. Results of the study of bull semen in PCR 2013 – 2016 (n = 450)

Region	Research samples	The positive samples							
		BoHV-1		BVDV		*Chlamydia* spp.		*Mycoplasma* spp.	
		s	%	s	%	s	%	s	%
Kharkiv region	332	-	-	-	-	-	-	19	6
Poltava region	58	2	3.5	-	-	-	-	-	-
Zhytomyr region	13	-	-	-	-	-	-	-	-
Cherkasy region	44	-	-	-	-	2	4.5	-	-
Dnipropetrovsk region	3	-	-	-	-	-	-	-	-
s - sample									

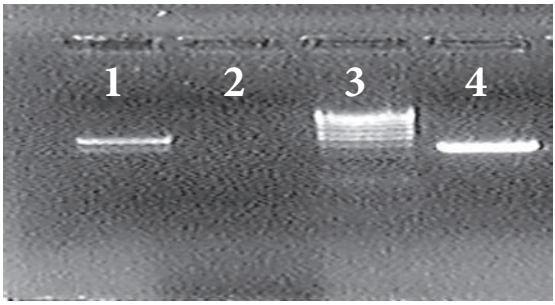

Figure 1. Results for of bull semen PCR detection on gel, positive sample of genetic material of *Mycoplasma spp*. Track number 1 – positive sample, lane number 3 – 100 base pair (bp) DNA ladder plus, lane number 4 – positive control.

We compared current research results of our study regarding frozen bull semen monitoring (period 2013-2016) with the results for period of 2010-2013. Significant reduction of contamination of semen samples with viruses, chlamydia and mycoplasmosis has been established (Fig. 2).

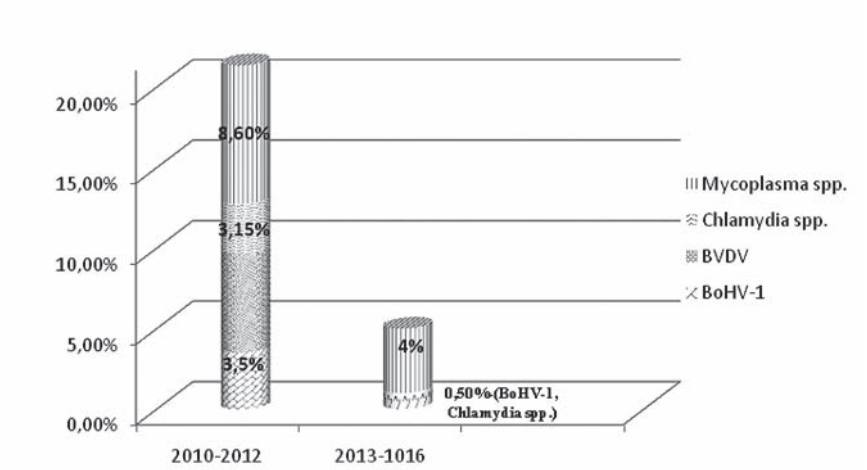

Figure 2. Comparison of PCR screening studies for the period 2010-2012 with the research conducted in the period 2013-2016.

CONCLUSIONS

According to the results obtained in this study, 19 samples (6%) from Kharkiv region contained genetic material of *Mycoplasma spp.* in frozen bulls semen samples, 2 samples from Poltava region (3.5%) were positive for BoHV-1, and 2 samples from Cherkasy region (4.5%) contained genetic material of *Chlamydia spp*.

Based on the monitoring results we can conclude that regular PCR screening of bulls' semen for the most common genital infections is positively affected by the dynamics of the testing rate. At present, there are no uniform requirements for contamination monitoring of sperm for the presence of viruses and Chlamydia. There are national requirements for the evaluation of semen contamination operating in Canada and the United States. Further work is therefore important to improve the system for control ofcontamination of cattle genetic resources.

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Case report UDK 619:636.8

OCCURRENCE OF THELAZIA CALLIPAEDA IN CATS - CASE REPORT

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Abstract

Thelazia callipaeda is a vector-borne zoonotic nematode, a parasite of the conjunctival sac in domestic and wild carnivores (dogs, cats, foxes and wolves) as well as in humans. Over the last decade, the infection with that particular Spirudida in dogs and cats has increased in many European countries, including the Balkans. During the last few years, the infection with this parasite in dogs and foxes has also been detected in Serbia. The first cases of cat infection were detected during 2015 in Belgrade and later in other parts of Serbia. In this paper we present a case report of cat infection with *T. callipaeda*. Adult nematodes were retrieved from the conjunctival sacs of cats during control at the local veterinary ambulance. In total, we extracted 17 adult worms, 11 females and 6 males.

Key words: cats, Serbia, *Thelazia callipaeda*

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POJAVA THELAZIA CALLIPAEDA KOD MAČAKA – PRIKAZ SLUČAJA

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Kratak sadržaj

Thelazia callipaeda je vektorski prenosiva zoonotska nematoda, koja parazitira u konjunktivalnoj kesi domaćih i divljih mesojeda (psi, mačke, lisice i vukovi) i ljudi. Tokom poslednje decenije, infekcija sa tim spirudidama sve češće se javlja kod pasa i mačaka u brojnim evropskim zemljama, uključujući i područje Balkana. U poslednjih nekoliko godina, u Srbiji je takođe ustanovljena infekcija sa ovim parazitima kod pasa i lisica. Prvi slučajevi infekcije kod mačaka su ustanovljeni tokom 2015. godine u Beogradu, a kasnije i u drugim delovima zemlje. U ovom radu predstavljamo prikaz slučaja infekcije *T.callipeda* kod mačke. Odrasle nematode su izvađene iz konjuktivalnih kesa mačaka tokom kontrole u lokalnoj veterinarskoj ambulanti. Ukupno smo izvadili 17 odraslih crva, 11 ženki i 6 mužjaka.

Ključne reči: mačke, Srbija, *Thelazia callipaeda*

INTRODUCTION

Thelazia callipaeda (Spirurida, Thelaziidae), is a nematode parasite attacking the conjunctival sac of domestic and wild carnivores and humans (Anderson, 2000; Otranto et al, 2005; Rossi et al, 1989). The infection is caused by an adult and larval stages of parasites. Parasites are also known as the "oriental eyeworm", due to the fact that it prevalently occurs in the Far East and in the countries of the former Soviet Union (Khabarovsk Krai) (Otranto et al., 2004). The presence of the worms in the infected hosts may induce clinical signs of various severity, ranging from mild (e.g., conjunctivitis, epiphora and ocular discharge) to severe (e.g. keratitis, corneal ulcers and blindness).

In Europe, the first autochthonous infection was reported in Italy in 1989 (Lia et al., 2000; Rossi et al., 1989). During the past few decades thelaziosis

spread from northern Italy to numerous European countries (e.g. France, Germany, Switzerland, Spain, Portugal) including the Balkan countries (Croatia, Romania, Bosnia and Herzegovina) (Dorchies et al., 2007; Hodžić et al., 2014; Malacrida et al., 2008; Miró et al., 2011; Otranto et al., 2003; Rodrigues et al., 2012). The infection was detected in dogs, cats, foxes and brown hares. Human ocular infections caused by *T. callipaeda* were detected in endemic areas of Italy, France and Spain (Otranto et al., 2008; Otranto et al., 2013).

The first autochthonous cases of infection by *T. callipaeda* in Serbia in dogs were detected in 2012 and in foxes in 2015 (Gajić et al., 2014; Pavlović et al., 2016). The parasites were, in most cases, encountered in animals of the Canidae family, and also in cats (family Felidae) (Maia et al., 2014; Motta et al., 2014; Soares et al., 2013). For that reason, we present a clinical case of thelaziosis in cats.

MATERIAL AND METHODS

In 2016, we observed ocular changes in the left eye of a one year old female cat – mildly increased lacrimation, exudative conjunctivitis and epiphora. The patient is very territorial half-breed, uncastrated three year old domestic male cat. *T. calliapdea* was diagnosed during the treatment after the fight with the other cat. There are other cats and dogs in the patient's habitat, but none of the pet's owners has reported changes of the animal's health status. During observation we established the presence of worms in conjunctival sac. Mechanical removal of worms was performed by washing the eye with sterile 98 physiological saline solution (NaCl 0.9%) recovering a total of seventeen worms. The collected nematodes were preserved in 70% ethanol solution and sent to the laboratory of parasitology of NIVS, Belgrade. For the purpose of further examination, parasites were cleared in lactophenol and nematodes were identified by its morphometric characteristics described by Skrjabin et al. (Skrjabin et al., 1967). We measured the body length and the maximal width of adult parasites, the number and the position of postcloacal papillae and the spicule length in males, as well as the position of the vulva in females.

RESULTS AND DISCUSION

During our examination we collected 17 samples of adult *T. callipaeda* - 11 females and 6 males. All parasites were located in the left eye of the cat. Male worms ranged from 10.17-13.26 mm in length, and 327-432 μm in width. Female worms ranged from 14.31-17.39 mm in length, and 399-423 μm in width. All

male worms had five pairs of postcloacal papillae on the ventral side of the body. The distance from the position of the cloaca to the end of the tail ranged from 69-81 μm. Right spicule was shorter and its length ranged from 141-150 μm. Left spicule was much longer, ranging from 1.433-1.757 mm. In female worms, vulva was situated anterior to the oesophago-intestinal junction, and the distance between the vulva and buccal extremity ranged from 557-639 μm in length.

Figure 1.
Caudal end Thelazia male

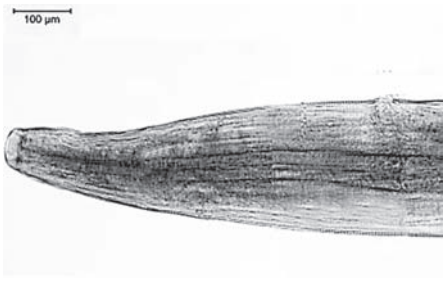

Figure 2.
Thelazia buccal end

Thelazia callipaeda (Spirurida, Thelaziidae) is an arthropod-borne disease. The expansion of this nematode is related to the occurrence of its vector, non-biting dipteran insect – fruit fly *Phortica variegata* (Drosophilidae, Steganinae). Adult female worms produce first-stage larvae that are ingested by the *P. variegata* that feeds on the lacrimal secretions of the vertebrate hosts (Otranto et al., 2006). It has been noticed that the disease occurs seasonally and primarily in rural areas, where there is a close contact between vector and domestic animals. While still in a vector, larvae develops into the infectious third - stage larvae, which takes 14-21 days and as such larvae may be transferred to the host where they develop into the adult form in the eye cavity during the period of 35 days. The parasite usually lives under the conjunctiva, where the adult females release first stage larvae into the lacrimal secretion (Otranto et al., 2005a; Otranto et al., 2005b). An increased number of cases of infection is usually reported in spring and summer, when the vector is active. Adult parasites remain viable for more than one year, which is crucial for the dynamics pattern of their parasitic abilities, consisting of two peaks of infection: one in the early summer (adult parasites that overwinter) and other in the late summer (adults developing from infectious stages laid by the vector in the early summer) (Otranto et al., 2003; Otranto et al., 2004).

Primary treatment for thelaziosis includes mechanical removal of worms. Additionally, it is recommended to treat infected animals with a macrocyclic lactone (e.g. moxidectin, milbemycin oxime), considering the fact that it can never be guaranteed that the mechanical removal of the worms was entirely successful (Bianciardi et al., 2005).

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PATHOLOGIC CHANGES IN SWANS INFECTED WITH HIGHLY PATHOGENIC AVIAN INFLUENZA (H5N8) VIRUS

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Abstract

Since early 2014 several outbreaks have occurred in Asia, Europe and North America involving novel highly pathogenic avian influenza subtype A(H5N8) viruses. This type of avian influenza virus have infected poultry and wild bird species. Global spread of these viruses was attributed to intracontinental and intercontinental movement of migratory birds. First case of Highly Pathogenic Avian Influenza (HPAI) H5N8 virus infection in Serbia was detected in mute swans (*Cygnus olor*) located in coastline of Danube River, in November 2016. During this outbreak, many sick or dead mute swans were found around habitats frequented by migratory birds. In this outbreak, swans appeared to be highly susceptible and represented the main reported affected species. Many sick mute swans showed neurologic symptoms, including torticollis, incoordination and ataxia. Here we report the results of post-mortem examinations of mute swans that were naturally infected and succumbed from avian influenza during the recent outbreak in Serbia. Examination was conducted on the carcasses of ten mute swans. The most significant pathologic lesions induced by HPAI H5N8 virus are necrosis in the pancreas, petechial hemorrhage in subepicardium and mesenteric adipose tissue. Characteristic, but not present in all infected swans was a congestion of the lungs.

Key words: Highly Pathogenic Avian Influenza, H5N8, swans, pathologic lesions

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PATOLOŠKE PROMENE KOD LABUDOVA INFICIRANIH VISOKO PATOGENIM SOJEM VIRUSA AVIJARNE INFLUENCE (H5N8)

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Kratak sadržaj

Od 2014 godine nekoliko epizootija izazvanih visoko patogenim virusom avijarne influence podtipa H5N8, izbilo je u Aziji, Evropi i severnoj Americi. Ovaj tip virusa influence uzrokovao je infekcije domaće živine i divljih ptica. Globalnom širenju virusa doprinele su intrakontinentalne i interkontinentalne migracije ptica. Prvi slučaj visoko patogene avijarne influence podtipa H5N8 u Srbiji detektovan je kod labudova (*Cygnus olor*), lociranih na rukavcu Dunava (područje Koviljskog-Petrovaradinskog rita) u novembru 2016.godine. Tokom ove epizootije, veći broj bolesnih i uginulih labudova pronađen je oko staništa migratornih ptica. Pokazalo se da su labudovi tokom epizootije bili visoko osetljiva i najviše pogođena vrsta ptica virusom influence podtipa H5N8. Oboleli labudovi su ispoljavali neurološke simptome, uključujući tortikolis, inkoordinaciju i ataksiju. U ovom radu su predstavljeni rezultati postmortalnog pregleda labudova inficiranih virusom influence. Ispitivanje je rađeno na leševima deset uginulih labudova. Najdominantnije patološke promene izazvane virusom influence podtipa H5N8 bile su nekroze pankreasa, petehijalna krvarenja u subepikardijumu i masnom tkivu mezenterijuma. Kod većine uginulih labudova bila je prisutna kongestija pluća.

Ključne reči: visoko patogena avijarna influenca, H5N8, labudovi, patološke promene

INTRODUCTION

All avian influenza (AI) viruses belong to the Influenza virus A genus of the Orthomyxoviridae family and are negative-strand, segmented RNA viruses (Capua and Alexander, 2004). Avian influenza is a highly pathogenic infectious

disease of poultry and other avian species. This disease is also recognized as a natural infection and disease of human and other mammals (Yin et al., 2013). Influenza A viruses are classified into subtypes based on two surface proteins, the hemagglutinin (H) and neuraminidase (N). Currently, the influenza virus has been subtyped into 18 hemagglutinin (H1-18) and 11 neuraminidase (N1-11) (Tong et al., 2013). Influenza A viruses infecting birds can be divided into two distinct groups on the basis of their ability to cause disease. The very virulent viruses cause highly pathogenic avian influenza (HPAI), which may result in mortality as high as 100%. These viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. All other viruses cause a much milder disease (low pathogenicity avian influenza - LPAI) (Capua and Alexander, 2004). The World Organization for Animal Health (OIE, 2012) has categorized the HPAI virus as a notifiable disease, due to its virulence and ability to transmit from animal to animal. Furthermore, avian influenza can be transmitted from birds to humans and can cause a severe clinical condition or have an effect of humans and animal health but it can also affect the poultry industry and exportation. Wild birds are a natural reservoir of type A influenza viruses, which generally cause asymptomatic infection. Swans, belonging to the order Anseriformes, are assumed to play a role like ducks and geese as a natural reservoir for influenza viruses (Teifke et al., 2007). Especially in Europe, swans proved to be the most frequently affected wild bird species (Terregino et al., 2006).

During November 2016, eight countries in Europe (Austria, Croatia, Denmark, Germany, Hungary, Netherlands, Poland and Switzerland) have reported numerous detections of H5N8 HPAI cases. These outbreaks have affected various wild bird species including Tufted Ducks (*Aythya fuligula*), Coots (*Fulica atra*), Common Pochard (*Aythya ferina*), gull species, curlews, wild geese and mute swans (*Cygnus olor*).

In Serbia, increased mortality among mute swans has been observed since the end of November 2016 along the shores of Danube armlet, which is part of Special Nature Reserve Koviljsko-Petrovaradinski Rit. This area is characterized by shallows and inlets of the Danube River, which are a temporary home for thousands of migratory and resident aquatic birds. Infected swans showed lethargy and signs of central nervous system involvement such as torticollis and other unusual positions of the head.

In November 30, in the laboratory for virology of the Scientific Veterinary Institute "Novi Sad", Serbia, the presence of the HPAI subtype H5N8 was confirmed in a swan carcass. The HPAI H5N8 virus presence were detected in organ samples of brain, lungs, spleen and intestine by molecular diagnostic

techniques (by real-time RT-PCR technique) and further confirmed by Singer sequencing. At that moment, our neighbouring states - Croatia and Hungary, also reported the occurrence of this virus in wild birds and domestic poultry to the OIE. The H5N8 avian influenza virus had never previously been detected in Serbia. This report of the outbreak in Serbia was the first official notification of HPAI since the epidemic in 2006. This first outbreak was immediately reported to the World Organisation for Animal Health after its confirmation.

MATERIAL AND METHODS

Ten juvenile adult swans analysed in this study were found dead at the coastline of Danube armlet, near Kovilj (Special Nature Reserve Koviljsko-Petrovaradinski Rit) in late November 2016. Swans carcass were in good post-mortem condition. All found ten HPAI H5N8 positive animals were necropsied. The necropsy was performed at the autopsy hall of the Scientific Veterinary Institute "Novi Sad"

RESULTS

Gross pathology

In general, all of ten swans were in good body condition, with sufficient body fat reserves. One swan was partially emaciated. Noticeably, all swans showed no external gross lesions. Few animals had diarrhoea and showed dark brown discoloured feathers around the cloaca. In three swans, bleeding from beak and nostrils was present. At necropsy, the predominant lesions in swans were multifocal, sharply demarcated necrosis in the pancreas (Fig. 1A). Petechiation in mesenteric adipose tissue were consistent finding (Fig. 1B). Characteristic but not present in all infected animals was a congestion of the lungs with alveolar and bronchiolar edema. Petechial haemorrhage in subepicardium (Fig. 2A) with scattered intramyocardial ecchymoses were present (Fig. 2B). In many cases, liver and spleen were moderately enlarged and congested. The mucoses of the small intestine and rectum were diffusely hyperaemic, in some cases haemorrhages were present in the small intestine. The lumen of small intestine was filled with bloody mucoid debris (Fig. 3A). Petechial haemorrhage was present in caecal tonsils of few infected animals. In two cases, haemorrhagic exudate was found in the lumen of oesophagus (Fig. 3B). Haemorrhage in muscles of the neck, intercostal and pectoral muscles were present in few cases (Fig. 3C). Blood vessels of the brain and meninges

were distinctly initiated (Fig. 3D). The frequencies of the occurrence of gross lesions in 10 infected swans are presented in Table 1.

Table 1. Frequency of occurrence of macroscopic lesions caused by HPAI (H5N8 subtype) infection in 10 mute swans (*Cygnus olor*):

Gross lesions	Number of affected swans (n=10)
Pancreas (Necrosis)	10
Heart (petechial haemorrhage in subepicardium)	10
Mesenteric adipose tissue (petechial haemorrhage)	10
Lungs (congestion and edema)	6
Liver (congestion, enlargement)	5
Spleen (congestion, enlargement)	4
Intestines (haemorrhages, mucoid exudate)	7
Oesophagus (haemorrhagic exudate)	2
Skeletal muscles (haemorrhages)	4
Brain (initiated blood vessels)	6

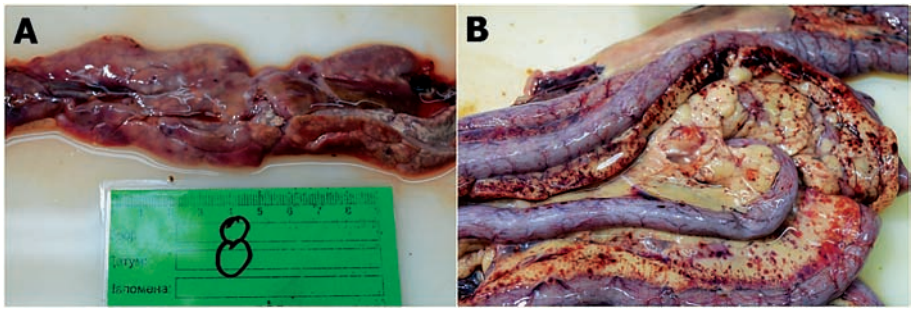

Figure 1: Gross pathology findings. (A) Multifocal areas of necrosis in the pancreas. (B) Petechiation in mesenteric adipose tissue.

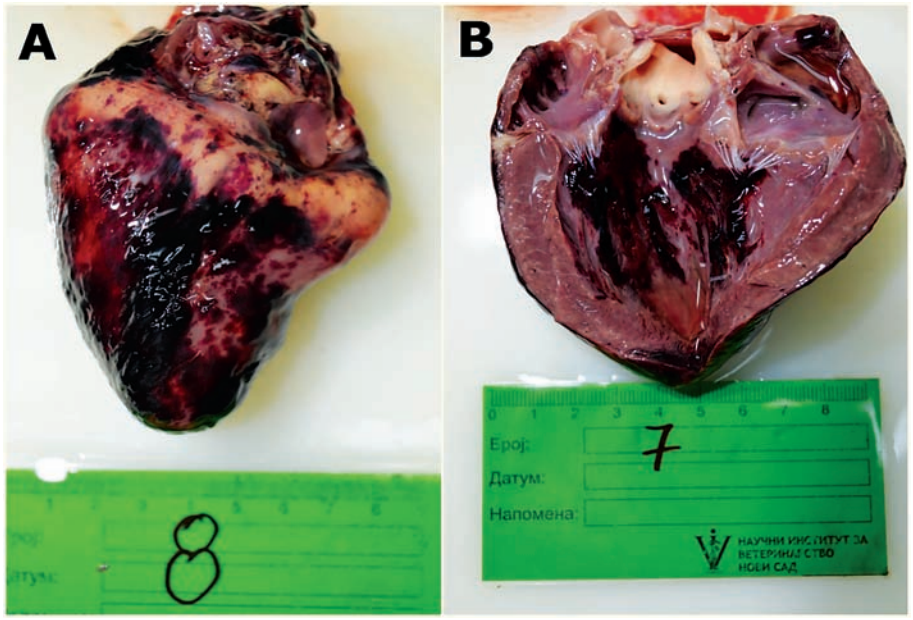

Figure 2: Gross pathology findings. (A) Petechial haemorrhage in subepicardium. (B) Intramyocardial ecchymoses.

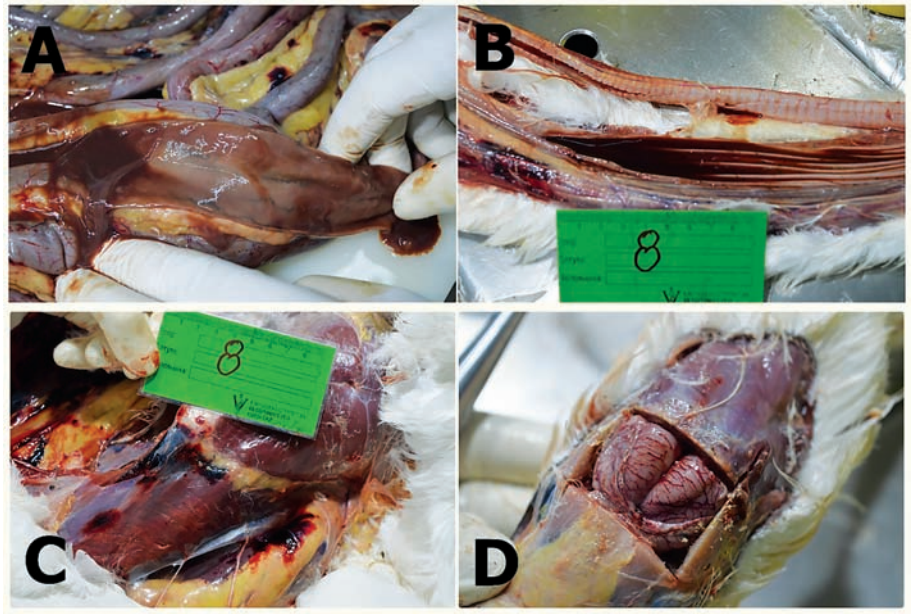

Figure 3: Gross pathology findings. (A) Bloody mucoid debris in small intestine. (B) Haemorrhagic exudate in the lumen of oesophagus. (C) Haemorrhage in muscles of the neck and pectoral muscles. (D) Initiated blood vessels of the brain.

DISCUSSION

Aquatic birds are considered to be the natural reservoir of low pathogenicity avian influenza viruses of subtypes H1–H16. In these birds, including ducks, they generally do not cause clinical signs (Hulse-Post et al., 2005). In contrast, highly pathogenic avian influenza (HPAI) viruses of certain H5 and H7 strains cause high death rates in poultry with substantial economic losses and are thought to be derived from low pathogenicity avian influenza viruses of wild bird origin. Mute swans are assumed to play a role as a natural, asymptomatically infected reservoir for influenza viruses (Teifke et al., 2007). The mute swan is a bird species with wandering or partially migratory behaviour in the middle Europe, (Nagy et al., 2006) however, during the winter season, many populations of swans move, so this possibility has been suggested as a factor that contributed to the spread of HPAI virus in Europe during 2016. The H5N8 avian influenza virus had never previously been detected in Serbia in the active surveillance of poultry and wild birds. During HPAI outbreak in Serbia in November 2016, the significant mortality attributable to HPAI virus infection has only been noticed in swans located in the area near Danube armlet. Many sick mute swans were found around this locality, showing neurologic symptoms, including torticollis, incoordination and ataxia. Likewise, neurologic signs were common finding among affected domestic ducks during HPAI outbreak in Hungary in 2015 (Bányai et al., 2016). Death swans were found in characteristic posture, consisting of head stocked under wings.

After necropsy, the distribution and character of lesions in these swans suggest an acute course of disease, and the lethal outcome is attributed to the systemic viral infection. Gross lesions in infected swans were independent of age, or sex of infected swans. The most frequent gross findings in all examined swans were multifocal necrosis in the pancreas and petechiation in mesenteric adipose tissue. Ecchymosis present within the myocardium was frequent finding and the same results were obtained from experimentally infected swans in Germany (Kalthoff et al., 2008). Petechiation in the epicardium was also frequent finding in naturally infected ducks (Nunez et al., 2015). In studies with experimentally infected waterfowl, it has been shown that HPAI (H5N1) infects the tubular epithelium in the kidneys (Teifke et al., 2007; Kwon et al., 2010) Also, during outbreak of highly pathogenic avian influenza H5N8 in a German Zoo, the most striking feature in infected storks were necrosis in kidneys (Globig et al., 2016). However, in our case, there was no evidence of gross lesions in the kidneys of infected swans. Haemorrhages in the subcutaneous tissue and the sternal serosa were relatively frequent finding in wild

birds infected with H5N1 avian influenza strain during outbreak in Serbia in 2006 (Vaskovic et al., 2011). This kind of lesions also were found in swans experimentally infected with avian influenza virus H5N1 strain (Kalthoff et al., 2008). In our study, lung congestion and edema were found in 6 swans. Similar findings, including marked congestion, edema, haemorrhage, and thrombosis was found in the alveolar capillaries of wild birds infected with H5N8 strain in South Korea (Kim et al., 2014). In experimentally infected mute swans with HPAI virus H5N1 ecchymoses were also present within the lungs and in the peritracheal connective tissue (Kalthoff et al., 2008). Contrary to this findings, post-mortem examination of ducks naturally infected with H5N8 show numerous white nodules in lungs and air sacs (Nunez et al., 2015).

CONCLUSION

The results reported herein suggests that the swans died and became moribund due to necrotizing pancreatitis and neurological disorders caused by H5N8 HPAI virus infection. Considering that neurologic signs were common among affected mute swans, it seems that neurotropism of the H5N8 virus may be the factor contributing to death in these swans.

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