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ICHTHYOPHTHIRIOSIS – CAUSE OF SIGNIFICANT LOSSES OF CARP FINGERLINGS

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Abstract

Ichthyophthiriosis is widespread parasitic disease of fishes caused by a ciliated protozoan, *Ichthyophthirius multifilis*. This parasite is capable of affecting most of species of freshwater fishes, including all cyprinids. The parasites usually can be found on skin and gill in the form of white prominent spots 0.1-1 mm in diameter, which looks as if the fish was sprinkled with grits. Within these tissues, infections cause localized lymphocyte infiltration, focal necrosis and varying degrees of epithelial proliferation. Investigations were carrieed out during a last 10 years, while monitoring the health condition of carp fish. Diagnosis of ichthyophthiriosis was performed by clinical and microscopic examination. Disease was present in carps throughout their life, but the most susceptible were young categories. The parasites were present on the skin and gills earlier in 10-day-old fingerlings. The outbreaks are most common in spring, after overwintering when water temperatures increase and also does the parasite replication rate. High stock density, water quality and poor condition contribute to illness. Since the disease causes significant losses of carp fingerlings it is necessary to perform its adequate controls and therapy. In order to prevent the disease it is need to to rear young fish separately from other fish categories, prevent weed fishes from entering the ponds and employ hygienic and prophylactic measures. All technological measures which can improve the condition of fish are most effective against ichthyophthiriosis. It is important to add lime into the pond from time to time. Effective chemical treatments for *I.*

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multifiliis include copper sulfate, potassium permanganate, malachite green, salt and formalin. Malachite green is a teratogen, and prohibited for use, while copper sulfate, potassium permanganate and formalin are also under currently reviewing for the use as parasiticides in food fish. So that, non-iodized salt is the only permitted and safe therapeutic for the moment.

Key words: *Ichthyophthirius multifilis,* carp fingerlings, losess, preventive measurements

IHTIOFTIRIOZA – UZROK ZNAČAJNIH GUBITAKA KOD MLADUNACA ŠARANA

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Kratak sadržaj

Ihtioftirioza je široko rasprostranjeno parazitsko obolenje izazvano protozoom, trepljašem *Ichthyophthirius multifilis.* Ovaj parazit može dovesti do infekcije kod skoro svih slatkovodne vrsta riba, uključujući i sve ciprinidne vrste. Parazit se najčešće nalazi na koži i škrgama, u vidu belih uzdignutih tačkica veličine 0.1-1 mm, pri čemu riba izgleda kao da je posuta grizom. U zahvaćenom tkivu dolazi do stvaranja lokalizovanih infiltracija limfocitima, fokalne nekroze i različitog stepena epitelne proliferacije. Istraživanja su sprovedena tokom poslednjih 10 godina, u sklopu praćenja zdravstvenog stanja šaranskih riba. Dijagnoza ihtioftirioze je postavljena na osnovu kliničkog i mikroskopskog pregleda. Obolenje je bilo prisutno kod svih starosnih kategorija šarana, dok su najosetljiviji bili mladunci. Najranije je ovaj parazit bio prisutan na koži i škrgama kod deset dana starih mladunaca šarana. Pojava obolenja najčešća je tokom proleća nakon prezimljavanja ribe kada dolazi do povećanja temperature a takođe i ubrzanog razmnožavanja parazita, odnosno razvojnih oblika. Vielika gustina nasada,

slab kvalitet vode i loši uslovi držanja itekako doprinose pojavi ovog obolenja. Imajući u vidu da *I. multifilis* dovodi do značajnih gubitaka šaranske mlađi veoma je važna adekvatna kontrola i terapija ovog obolenja. U cilju preveniranja bolesti neophodno je gajiti mladunce odvjeno od drugih kategorija riba, sprečavati ulazak korovske ribe i održavati opšte higijenske i profilaktičke mere. Sve tehnološke mere koje imaju za cilj poboljšanje kondicije riba takođe su efikasne u preventivi ihtioftirioze. S vremena na vreme u ribnjake treba dodavati i kreč. U efikasna terapeutska sredstva za *I. multifilis* spadaju bakar sulfat, kalijum permanganat, malahit zeleno, so i formalin. Malahit zeleno je teratogen i zabranjen za upotrebu, dok su bakar sulfat, kalijum permanganat i formalin pod trenutnim nadzorom da li bi smeli da se koriste kao antiparazitici kod riba namenjenih za konzum. Za sada je nejonizovana so jedino dozvoljeno i sigurno terapeutsko sredstvo.

Ključne reči: *Ichthyophthirius multifilis,* mladunci šarana, gubici, preventivne mere

INTRODUCTION

Ichthyophthiriosis, also known as ich or white spot disease is a cosmopolitan parasitosis of fishes (Nigrelli et al., 1976; Valtonen and Keränen, 1981). Infections have been reported from all regions where fishes are cultured, from the Equator to the Arctic Circle (Rinramaki-Kinnanen and Valtonen, 1997; Valtonen et al., 1994). *I. multifiliis* is believed to have originated as a parasite of carp (Hoffman, 1999). Ich appears to parasitize all freshwater fishes. There are no records of species with complete natural resistance (Ventura and Paperna, 1985). *Ichthyophthirius multifilis* is the largest known parasitic protozoan found on fishes. Adult organisms are oval to round and measure 0.3 to 1.0 mm in size and can be visible by the naked eye. The adult is uniformly ciliated and contains a horseshoe-shaped nucleus which can be seen in older individuals. Parasite has a simple life cycle consisting of three developmental stages each of which is ciliated: the infective form (theront), the host associated form (trophont) and the encysted environmental form (tomont) (Hines and Spira, 1973). The motile theront (30 x 60 μm in size) has limited energy reserves and remains infectious for approximately three days (Dickerson and Dawe, 1995). It penetrates under the epithelium, feeds on cells and tissue fluids and transforms into the next stadium - trophont (Ewing and Kocan, 1986). When fully developed, trophonts abandon the host and transforms into tomonts. The life cycle of *I. multifiliis* is influenced by water temperature. A single round of replication occurs in 4-5 days at water temperatures of 20-24°C (Ewing et al., 1986). The

parasite cannot survive in water temperature greater than 30°C. At colder temperatures (<10°C) parasite development is slowed. Epizootics usually occur during spring and summer months when warmer water temperatures increase parasite replication rate and reduced levels of dissolved oxygen cause stress in fish populations (Maki, 2002). Other factors influencing the severity of infection include stocking density, water quality, and susceptibility of various fish species (Johnson, 1993). Lesions associated with *I. multifiliis* infection have been well characterized. The classic sign of infection is presence of small white spots on the skin or gills 0.1-1 mm in diameter. Prior to the appearance of white spots, fish may show signs of irritation, flashing, weakness, loss of appetite, and decreased activity. If the parasite is only present on the gills, white spots will not be seen at all, but fish will die in large numbers (Francis-Floyd and Reed, 1991). In these fish, gills will be pale and very swollen. The diagnosis is established by light microscopy, when the parasites are found in fresh skin and gill samples (Francis-Floyd and Reed, 2011). Thomits are continuously moving, while the trophonts are located cysts. In order to prevent the disease it is need to to rear young fish separately from other fish categories, prevent weed fishes from entering the ponds and employ hygienic and prophylactic measures (Ćirković and Novakov, 2013). The most used chemical treatments for *I. multifiliis* include copper sulfate, potassium permanganate, malachite green, salt and formalin (Johnson, 1993). Commercial aquaculture operations are limited to using only those compounds approved for food fish and are hampered by the cost of treating large volumes of water for extended periods of time. The goal of this paper is to do a survey of ichthyophthiriosis in carp fish pond, of Serbia and to indicate the importance of the disease, as well as to perform and recommend the most appropriate preventive and therapeutic measures.

MATERIALS AND METHODS

Diagnostics and investigations were conducted during last 10 years, while monitoring the health condition of carp fish. Over a 10-year period more than thousands of samples were examined for the presence of *Ichthyophthirius multifilis*. The examined fishes originated from 18 common carp fish ponds in Serbia. Fish were collected with a net and suspected fish were transported alive to the laboratory where they were processed.

Samples for microscopic examination were taken from tissue of gill arch, the body surface and a tail fin. We removed several white spots from an infected fish, then mount them on a microscope slide with a few drops of water

and a cover glass. The mature parasite is the large (200 to 800 μm) ciliated trophonts which were easily seen in unstained wet mounts (× 10–40 magnification). The trophont of *Ichthyophthirius multifilis* has a distinctive horseshoe-shaped nucleus, which is a pathognomonic sign of infection. In early or very heavy infections, theronts were also present.

RESULTS AND DISCUSSION

During the investigations(study) *Ichthyophthirius multifilis* (Figure 1) was present in all carp categories, but the most susceptible were carp fingerlings where in some ponds mortality ranged up to 90%. The parasites were present on the skin and gills in 10-day-old carp fingerlings earlier. The outbreaks were most common in spring, after overwintering when water temperatures increased as well as parasite replication rate, but the fish immunity is still weaker. Also, the disease was present in ponds with high stock density, lower water quality and poor condition which contributed to the illness.

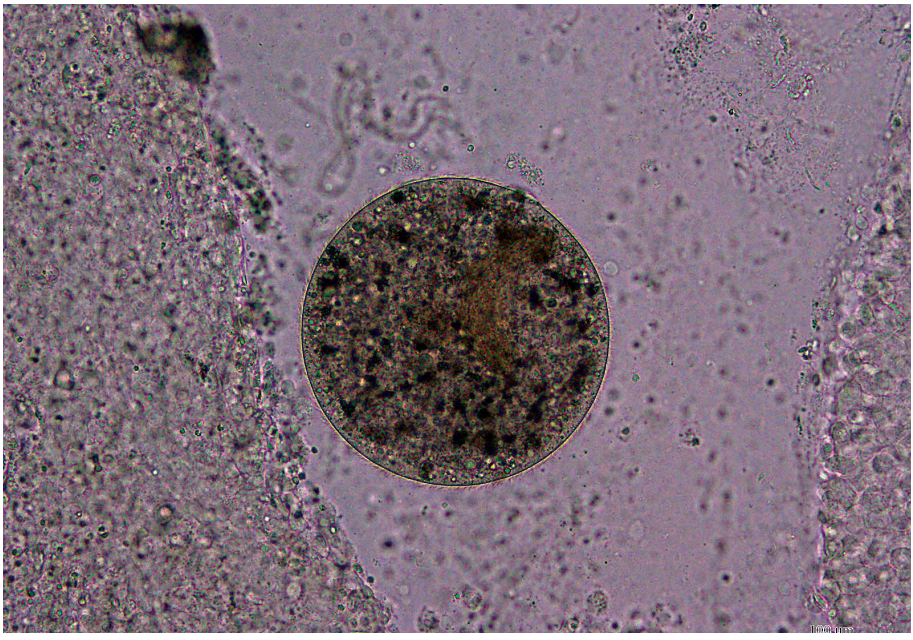

Figure 1. *Ichthyophthirius multifilis* trophont with characteristic horseshoe-shaped nucleus. (Bar =100 μm).

At the beginning of the disease there were no visible changes in the fish behaviour, but as the infection intensifies the fish were agitated, moving quickly to the water surface, swiming in circles and were less reactive to external stimuli. Fish infected with *I. multifiliis* demonstrated an aberrant behavior called "flashing" (i.e., darting or making quick movements against objects in their environment). This behavior may be seen early in infection before parasites are visible on the exterior surface of the fish. On the skin there were necroses in the form of white spots 0.1-1 mm in diameter, which looks as if the fish was sprinkled with grits (Figure 2).

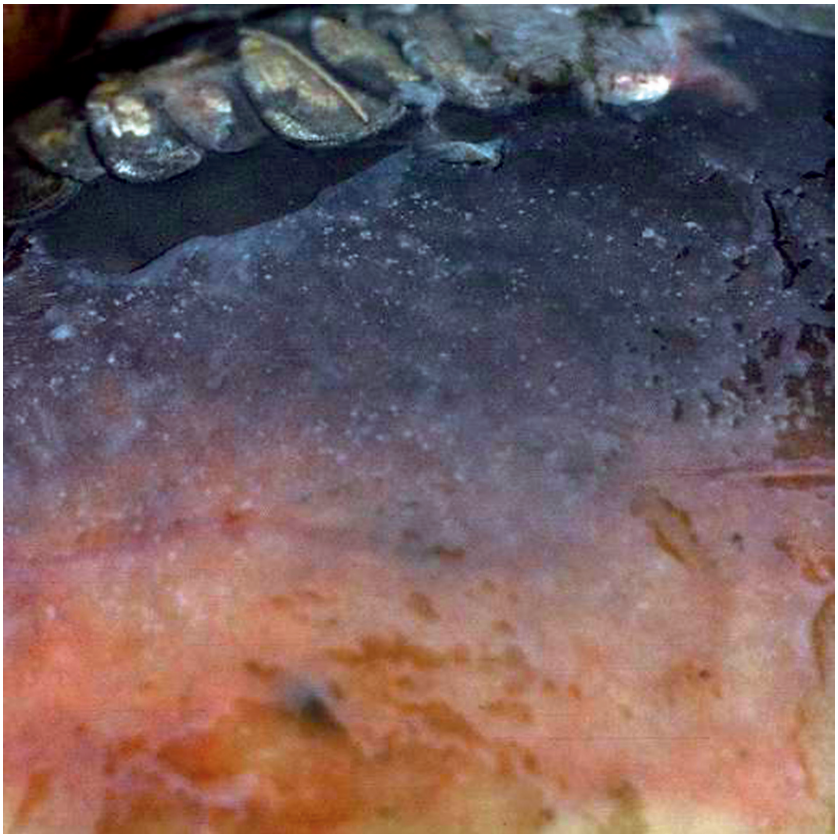

Figure 2. Ichthyophthiriosis in carp with characteristic skin lesions.

Intense infections resulted in death, when fish died in large numbers, especially when parasites were present on the gills, which were pale and very swollen, so asphyxia was the cause of the death. Lesions made by parasites may

become infected with bacteria and especially fungi of which saprolegnia being the most frequent one (Figure 4).

Figure 3. Ichthyophthiriosis in a carp-secondary infection with fungi of *Saprolegnia* species

Ichthyophthiriosis is highly important disease because is one of the most common diseases in freshwater fishes and can cause mortality rate up to 100% (Meyer, 1974; Maki, 2002; Ćirković and Novakov, 2013). Mild infections may resolve without treatment, but in closed systems multiple rounds of replication usually result in heavy parasite loads and high mortality. Infected fish often die due to impaired respiration and disrupted osmoregulation (Hines and Spira, 1974). Disease control and prevention in an intensive fish production is the most important and depend upon an integrated management program. The basic is to prevent the fish from the exposure of the parasites, identify the disease if it occurs and perform treatment of infected fishes.

In order to prevent the disease it is necessary to rear young fish separately from other fish categories and prevent weed fishes from entering the ponds. This is because fish which contracted the disease are the source of the infection

as well as some weed fish species. Also all hygienic and prophylactic measures are necessary combatting this disease. All technological measures which can improve the condition of fish (nutrition, reduction of stress, good water quality) are effective against ichthyophthiriosis. It is also important to add lime into the pond from time to time. Effective chemical treatments for *I. multifiliis* include copper sulfate, potassium permanganate, malachite green, salt and formalin (Francis-Floyd and Reed, 2011; Johnson, 1993). Malachite green is a teratogen, and prohibited for use. Copper sulfate must be taken into consideration since copper sulphate is toxic at low pH and can diminish the oxygen content in water and lead to lethality. If the alkality is less than 50 ppm the application of copper sulphate is not advisable (Watson and Yanong, 2011). Copper sulfat, potassium permanganate and formalin are also under currently reviewing for the use as parasitic ides in food fish. So that, Non-iodized salt is the only permitted and safe therapeutic for use. Salt can be used to control white spot disease in small volumes of water. (i.e., tanks or vats). Fish can be dipped in a 3% (30,000 mg/l) solution for thirty seconds to several minutes, or they can be treated in a prolonged bath at a lower concentration (0.05% = 500 mg/l) (Francis-Floyd and Reed, 2011).

CONCLUSION

Ichthyophthiriosis is one of the most common diseases in freshwater fishes. White spot disease were present on the skin and gills in 10-day-old carp fingerlings earlier, and caused mortality rate up to 90%. The outbreaks were most common in spring and connected with high stock density, lower water quality and poor condition. Characteristic white spots 0.1-1 mm in diameter, were present on the skin of infectede fish. Separate breading of carp fry from older fish categories, disable the entrance of wild fish, technological measures (optimal density, feeding, reduction of stress, good water quality) and lime addition are necessary for combatting this disease. Using of non-iodized salt is also effective in the control of the disease.

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LITERATURE

1. Ćirković M., Novakov N.: Parazitske bolesti ciprinidnih riba, Naučni institut za veterinarstvo Novi Sad, Elman štampa, 2013.
2. Dickerson W., Dawe L.: *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (Phylum *Ciliophora*), pp. 181-221. *In* P.T.K. Woo (ed.) Fish Diseases and Disorders. Wallingford, UK, CAB International, 1995.
3. Ewing S., Kocan M.: *Ichthyophthirius multifiliis* (Cilophora) developement in gill epithelium, *J Protozool*, 33, 369-374. 1986.
4. Ewing S., Lynn E., Ewing A.: Critical periods in development of *Ichthyophthirius multifiliis* (*Ciliophora*) populations. *J Protozool*, 33, 3, 388-391, 1986.
5. Francis-Floyd R., Reed P.: *Ichthyophthirius multifiliis* (White Spot) Infections in Fish, Institute of Food and Agricultural Sciences, University of Florida, UF/IFAS Extension publications, 2011.
6. Hines S., Spira T.: *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp, *Cyprinus carpio* L. I. Course of infection. *J. Fish Biol,* 5, 385-392, 1973.
7. Hines S., Spira T.: *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp *Cyprinus carpio* L., IV. Physiological dysfunction. *J Fish Biol*, 6, 365-371, 1974.
8. Hoffman G.: Parasites of North American Fishes. Ithaca, New York, Comstock Publishing Associates, 1999.
9. Johnson M.: The veterinary approach to channel catfish. pp. 249-270. *In* L. Brown (ed.), Aquaculture for Veterinarians. Tarrytown, NY, USA, Pergamon Press, Inc., 1993.
10. Maki J.L.: *Ichthyophthirius multifiliis* Infection and Elements of Mucosal Immunity in the Channel Catfish, (*Ictalurus punctatus*), Doctoral thesis, The University of Georgia, pp 136. (in English), 2002.
11. Meyer P.: Parasites of freshwater fishes. Part 2. *Protozoa* 3, *Ichthyophthirius multifiliis.* Washington, DC Fish DiseaseLeaflet No. 2, United States department of the Interior, U.S.F.W.S. 1974.
12. Nigrelli F., Pokorny S. Ruggieri D.: Notes on *Ichthyophthirius multifilis*, a ciliate parasitic on freshwater fishes, with some remarks on possible physiological races and species. *Transactions of the American Microscopical Soceity*, 95, 607–613, 1976.
13. Rinramaki-Kinnanen P., Valtonen T.: Epizootiology of protozoans in farmed salmonids at northern latitudes. *Intl J Parasitol.* 27, 10, 89-99, 1997.
14. Valtonen T., Keränen A.: Ichthyophthiriasis of Atlantic salmon, *Salmo salar* L. at the Montta Hatchery in northern Finland in 1978–1979. *Journal of Fish Diseases*, 4, 405–411, 1981.

15. Valtonen T., Koskivaara M.: Relationship between the parasites of some wild and culture fishes in two lakes and a fish farm in central Finland. *Intl J Parasitol*, 24, 1,109-118, 1994.
16. Watson C., Yanong R.P.E.: Use of Copper in Freshwater Aquaculture and Farm Ponds, Institute of Food and Agricultural Sciences, University of Florida, UF/IFAS Extension publications, 2011.

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INVESTIGATIONS ON THE RESISTANCE OF COMMENSAL SWINE *ESCHERICHIA COLI* TO SOME AMINOGLYCOSIDES-AMINOCYCLITOLS

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Abstract

The aim of this study was to describe the prevalence of antibiotic resistance to some aminoglycosides, streptomycin, spectinomycin and gentamicin and three aminoglycoside- resistance genes in *Escherichia coli* isolated from feces and lagoon manure in six swine farms in Republic of Bulgaria. A total of 274 *E. coli* isolates from 270 fecal samples and twelve samples from lagoon manure were tested by disk diffusion method to determine resistance patterns to 11 antimicrobial agents. Aminoglycosides resistance also was determined by E-test, agar dilution method, PCR and qPCR. The highest resistance observed to streptomycin (70.0%) and spectinomycin (65.5%). Multi-resistance patterns in studied *E. coli* strains showed that the resistance to streptomycin/spectinomycin was most frequently seen together with resistance to ampicillin, tetracycline, and sulfonamides (39.6%). The *E. coli* isolates resistant to streptomycin, spectinomycin were examined for the presence of *strA/strB, aadA1* genes, and resistant isolates to gentamicin were evaluated for the presence of the *aacC1* gene. The most common gene determining resistance to aminoglycosides was *aadA1* which was found in 54.0% of swine isolates and lagoon manure isolates followed by *straA/strB* genes (32.3%). The *aacC1*gene was not identified in *E. coli* isolates resistant to gentamicin.

Keywords: aminoglycoside resistance, commensal *Escherichia coli*, pigs, lagoon manure

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ISPITIVANJE REZISTENCIJE KOMENSALNE *ESCHERICHIA COLI* KOD SVINJA NA NEKE AMINOGLIKOZIDE-AMINOCIKLITOLE

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Kratki sadržaj

Cilj ovog istraživanja je prikaz prevalencije rezistentnosti na antibiotike i to neke aminoglikozide, streptomicin, spektinomicin i gentamicin kao i tri gena *Escherichia coli* koji uslovljavaju rezistenciju na aminoglikozide , izolovanih iz fecesa i osoke iz sabirnih bazena na 6 farmi svinja u Republici Bugarskoj. Ispitano je 274 izolata *E. coli* dobijenih iz 270 uzoraka fecesa i 12 uzoraka osoke. Uzorci su testirani metodom disk difuzije u cilju odredjivanja modela rezistencije na 11 antimikrobnih agenasa. Rezistencija na aminoglikozide je takodje odredjivana primenom E-testa, metode dilucije agara, PCR i qPCR. Najveći stepen rezistencije ustanovljen je za streptomicin (70.0%) i spektinomicin (65.5%). Modeli višestruke rezistencije uočeni kod sojeva *E. coli* pokazali su da se rezistencija na streptomicin/spektinomicin najčešće javlja udružena sa rezistencijom na ampicilin, tetraciklin i sulfonamide (39.6%). Izolati *E. coli* rezistentni na streptomicin i spektinomicin ispitani su na prisustvo gena *strA/strB, aadA1*, a izolati rezistentni na gentamicin na prisustvo gena *aacC1*. Ustanovljeno je da je rezistencija na aminoglikozide najčešće bila uslovljena genom *aadA1* koji je detektovan kod 54.0% izolata svinja i izolata osoke iz sabirnih bazena, a nakon njega slede geni *straA/strB* (32.3%). Ben *aacC1* nije identifikovan kod izolata *E. coli* rezistentnih na gentamicin.

Ključne reči: rezistencija na aminoglikozide, komensalna *Escherichia coli*, svinje, osoka iz sabirnih bazena

INTRODUCTION

The concept of EMA (2014) on the use of aminoglycosides in livestock and companion animals in the EU, development of resistance and public health risks is grounded on data about the increasing resistance to aminoglycosides in animal and human bacterial isolates. Data of EMA/ESVAC (2013) demonstra-

te a widespread use of aminoglycosides in some animal species in particular (large and small ruminants, swine, horses, and pets) for treatment of septicaemic states, gastrointestinal infections, respiratory and urogenital infections. Some authors discussed the potential of resistant *E. coli* from domestic animals as a reservoir for the spread of resistance to aminoglycoside antibiotics among human population (Chaslus-Dancla et al., 1991; Jonson et al., 1994, 1995). The primary mechanism of aminoglycoside resistance is the production of aminoglycoside modifying enzymes. Three genetic determinants are associated to the expression of resistance to streptomycin in enterobacteria: *ant(3″)-Ia* (synonym *aadA*) coding for the production of adenyltransferase ANT (3″)-I, modifying streptomycin and spectinomycin, *aph(3″) –Ib* (synonym *strA*), determining the production of the phosphoryltransferase APH (3″)-I, modifying streptomycin and *aph(6)-Id* (synonym *strB*), responsible for the production of phosphoryltransferase APH (6)-I, that also modifies streptomycin (Heinzel et al., 1988; Hollingshead and Vapnek, 1985; Scholz et al., 1989). During the last years, reports about a new type of aminoglycoside resistance in animal bacterial isolates related to the prevalence of 16S rRNA methylases and the respective high levels of resistance are increasing (Gonzalez-Zorn et al., 2005; Chen et al., 2007; Liu et al., 2008; Du et al., 2009; Davis et al., 2010; Hopkins et al., 2010; Deng et al., 2011). From modifying enzymes coding for resistance to gentamicin in E. coli strains from livestock, adenyltransferase ACC(3) – IV whose production is coded by the *aac3-IV* gene and that determines a combined resistance to gentamicin and apramycin, is of special interest. There are also data, although limited, on the prevalence of *aacC1* and *aac3-II* genes in domestic animals (Guerra et al., 2003; Sãenz et al., 2004). These genes determine the production of acetyltransferases ACC (3)- I and AAC (3)-II, distinguished with their phenotype profile, which for the latter gene includes also resistance to tobramycin apart to gentamicin (Vaculenko and Mobashery, 2003).

MATERIAL AND METHODS

Sample collection

Between January 2013 and September 2014, 282 faecal swab samples were collected from different age groups of pigs (suckling, weaned, finisher) and lagoon manure from 6 farrow-to-finish farms. Faecal swabs were transported in Stuart Transport Medium (BD, USA) at low temperature within 18-24 hours.

Culturing and identification of E. coli isolates

Swabs were cultured on McConkey agar (Emapol, Poland) at 37 °C for 24

hours. Lactose-positive colonies were subcultured onto TSI agar (BD, USA) and submitted to preliminary biochemical typing via citrate utilisation, methyl red, Vogues Proskauer and indole production tests. The identification of strains was performed with kits for non-fermenting and enteric bacteria (BD, USA) and the semi-automated identification Crystal BBL system.

Determination of the sensitivity of E. coli isolates to antibiotics

The sensitivity of *E. coli* isolates to 11 chemotherapeutics was evaluated by the disk diffusion method as per CLSI, using Muller-Hinton agar (Emapol, Poland) and antibiotic disks (Emapol, Poland), loaded as followed: ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), cephalotin (30 μg), ceftazidime (10 μg), cefotaxime (30 μg), gentamicin (10 μg), streptomycin (10 μg), spectinomycin (25 μg), tetracycline (30 μg), ciprofloxacin (5 μg), sulfamethoxazole (25 μg). The reference strain *Escherichia coli* ATTC 25922 was used for the control of disk diffusion method and MIC method.

The streptomycin MIC was determined in the agar dilution test and Muller-Hinton agar (Emapol, Poland), by preparation of doubling dilutions of streptomycin (Sigma-Aldrich) within 0.01-256 μg/mL. MIC for gentamicin was defined by E test strips (AB Biodisk, Solna Sweden). MICs were interpreted according to epidemiological criteria (EUCAST, www.eucast.org).

Determination of resistance genes to aminoglycoside in commensal E. coli

DNA extraction: For DNA extraction, 24-hour cultures incubated at 37°C, respectively 3-4 colonies on McConkey agar were suspended in 100 μl sterile distilled water free of inhibitors for molecular diagnostics (Qiagen). The DNA extraction kit DNeasy Blood Tissue Kit (Qiagen) was used.

Detection of resistance genes: The presence of resistance genes to aminoglycoside antibiotics, *aadA1* was detected by qPCR and *strA/strB* by PCR. The primers sequences for *strA/strB* were strA–F ATGGTGGACCCTAAAACTCT and strB-R CGTCTAGGATCGAGACAAAG (Kozak et al., 2009). PCR assays in 25 μl final volume contained 12.5 μl Taq PCR Master mix (Qiagen) and 3 μl DNA template. The PCR reaction for *strA/strB* consisted of an initial activation step at 94°C for 10 min, followed by 30 cycles of DNA denaturation at 94 °C for 30 s, primer annealing at 63°C for 1 min, and primer extension at 72°C for 30 s, and final extension for 10 min at 72°C. All reactions were carried out in Eppendorf gradient thermal cycler. Ten μL aliquots of PCR products were analyzed by gel electrophoresis with 1.5% agarose gel (Peqlab, Germany). Gels were stained with ethidium bromide at concentration 10 μg/mL and visualized by UV transillumination. A 100 bp DNA ladder plus (Qiagen) was used as marker. As positive control strain *E. coli* 94.4 was used, provided by Mrs. J. Mazurec from

the Department of Molecular Biology, Faculty of Biological Sciences, University of Zielona Góra, Poland. Negative controls were PCR mixtures with the addition of water in place of template DNA.

To determine the *aadA1*gene, Microbial DNA qPCR Assay, *aadA1* (Qiagen) was used. qPCR amplification was done with Stratagene Mx3000P instrument. The thermocycler protocol consisted of: initial PCR activation 1 cycle of 10 min at 95°C, and 40 cycles of 2-step cycling – denaturation - 15 sec 95°C, annealing and extension 2 min 60°C. The results were interpreted according to manufacturer's instructions (negative control signal at CT>35 and CT= 22±2 for positive control).

RESULTS

Number of isolates: The total number of *E. coli* isolates from examined faecal swabs obtained from the different age categories and from lagoon manure at studied farms were 274.

Prevalence of antibiotic resistance by disk-diffusion method

Tables 1, 2 and 3 present results from the phenotype analysis of resistance of *E. coli* isolates from the 6 surveyed farms to 11 chemotherapeutics. With respect to aminoglycosides, the highest resistance percentages to streptomycin and spectinomycin (93.2% and 91.0 % respectively) were observed in finisher pigs. Higher resistance to gentamicin (15.7%) was established in *E. coli* isolates from weaned pigs than in finisher (7.9%) or neonatal pigs (4.5%).

Table1. Prevalence of antibiotic resistance in *E. coli* strains from suckling pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates %						
	Farm I n=15	Farm II n=14	Farm III n=15	Farm IV n=15	Farm V n=15	Farm VI n=15	Total n=89
Ampicillin	1 (6.7)	3 (21.4)	6 (40.0)*	3 (20.0)	2 (13.3)	2 (13.3)	17 (19.0.)
Amoxicillin/ clavulanic acid	0	0	0	0	0	0	0
Cephalotin	0	1 (7.1)	3 (20.0)	0	0	0	4 (4.5)

Antibiotic	Resistant isolates %						
	Farm I n=15	Farm II n=14	Farm III n=15	Farm IV n=15	Farm V n=15	Farm VI n=15	Total n=89
Ceftazidime	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0
Gentamicin	0	1 (7.1)	3 (20.0)	0	0	0	4 (4.5)
Strep-tomycin	1 (6.7)	3 (21.4)	9 (60.0)**	3 (20.0)	4 (26.6)	2 (13.3)	22 (24.7)
Specti-nomycin	0	2 (14.3)	7 (46.6)*	2 (13.3)	4 (26.6)	0	15 (16.8)
Tetracycline	3 (20.0)	9 (64.3)**	12 (80.0)***	10 (66.6)**	5 (33.3)	8 (53.3)*	47 (52.8)
Cipro-floxacin	0	1 (7.1)	1 (6.7)	0	0	0	2 (2.2)
Sulfamet-hoxazole	2 (13.3)	3 (21.4)	5 (33.3)	3 (20.0)	3 (20.0)	2 (13.3)	18 (20.2)

Legend: p≤0.05 (*); p≤ 0.01 (**); p≤ 0.001 (***)

Data about the resistance of *E. coli* isolates to aminoglycosides in groups of suckling pigs showed the highest resistance to streptomycin, spectinomycin and gentamicin (60%, 46.6%, and 20% respectively) at farm 3.

Isolates resistant to streptomycin and spectinomycin were the most prevalent (100%) among weaned pigs from farms 3 and 5, followed by the occurrence of resistance to aminoglycosides in 86.6% of isolates from farms 4 and 6 and in 85.7% of isolates from farms 1 and 2. The percentage of isolates from weaned pigs resistant to gentamicin was the highest at farm 4 (7.8%).

Table 2. Prevalence of antibiotic resistance in *E. coli* strains from weaned pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates %						
	Farm I n=14	Farm II n=15	Farm III n=15	Farm IV n=15	Farm V n=15	Farm VI n=15	Total n=89
Ampicillin	3 (21.4)	10 (66.6)**	15 (100)***	14 (93.3)***	12 (13.3)***	6 (40.0)	60 (67.4)
Amoxicillin/ clavulanic acid	0	0	2 (13.3)	0	0	0	2 (2.2)
Cephalotin	0	4 (26.6)	12 (80.0)***	10 (66.6)***	2 (13.3)	4 (26.6)	32 (35.9)
Ceftazidime	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0
Gentamicin	0	3 (20.0)	4 (26.6)	7 (46.6)	0	0	14 (15.7)
Streptomycin	12 (85.7)	12 (85.7)	15 (100)*	13 (86.6)	15 (100)*	13 (86.6)	80 (89.8)
Spectinomycin	12 (85.7)	12 (85.7)	15 (100)*	13 (86.6)	15 (100)*	13 (86.6)	80 (89.8)
Tetracycline	10 (71.4)	15 (100)**	15 (100)**	11 (73.3)	14 (93.3)**	11 (73.3)	76 (85.3)
Ciprofloxacin	0	2 (13.3)	3 (20.0)	0	1 (6.7)	0	6 (6.7)
Sulfamethoxazole	5 (35.7)	14 (93.3)***	15 (100)***	11 (73.3)*	15 (100)***	12 (80.0)**	72 (80.8)

Legend: p≤0.05 (*); p≤ 0.01 (**); p≤ 0.001 (***)

The highest resistance percentage to streptomycin and spectinomycin (100%) was demonstrated in *E. coli* isolates from finisher pigs at farms 2, 3, 4 and 5. With respect to gentamicin, the highest resistance (28.5%) was found out among isolates from farm 3.

Table 3. Prevalence of antibiotic resistance in *E. coli* strains from finishers pigs from 6 farrow-to finish farms

Antibiotic	Resistant isolates %						
	Farm I n=15	Farm II n=15	Farm III n=14	Farm IV n=14	Farm V n=15	Farm VI n=15	Total n=88
Ampicillin	1 (6.7)	3 (20.0)	4 (28.5)	3 (21.4)	3 (20.0)	4 (26.6)	18 (20.3)
Amoxi-cillin/ cla-vulanic acid	0	0	0	0	0	0	0
Cephalotin	0	3 (20.0)	6 (42.8)**	2 (14.2)	3 (20.0)	1 (6.7)	15 (17.0)
Ceftazidime	0	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0	0
Gentamicin	0	2 (13.3)	4 (28.5)	1 (7.1)	0	0	7 (7.9)
Strep-tomycin	10 (66.6)	15 (100)***	14 (100)***	14 (100)***	15 (100)***	14 (93.3)*	82 (93.2)
Specti-nomycin	9 (60.0)	15 (100)***	14 (100)***	13 (92.8)	15 (100)***	14 (93.3)*	80 (91.0)
Tetracycline	9 (60.0)	14 (93.3)*	14 (100)***	12 (85.7)	10 (66.6)	10 (66.6)	69 (78.2)
Cipro-floxacin	0	1 (6.7)	2 (14.2)	0	1 (6.7)	0	4 (4.5)
Sulfamet-hoxazole	4 (26.6)	8 (53.3)	13 (92.8)***	8 (57.1)	6 (40.0)	6 (40.0)	45 (51.1)

Legend: p≤0.05 (*); p≤ 0.01 (**); p≤ 0.001 (***)

Among multiresistant isolates, the highest prevalence of 23.6% was that of the phenotype profile including ampicillin, streptomycin, spectinomycin and tetracycline, following by the profiles of resistance to ampicillin, streptomycin, spectinomycin and sulfamethoxazole (9.7%) and to ampicillin, cephalotin, gentamicin, streptomycin and tetracycline (6.3%).

Phenotypic analysis of MIC concentrations for streptomycin and gentamicin

Table 4 presents the cumulative MIC percentages to streptomycin and gentamicin. The MIC90 of isolates to streptomycin was 16 μg/mL, whereas to gentamicin MIC90 was 1 μg/mL.

Table 4. Distribution of MICs among commensal *E. coli* (n=274) isolated from pigs and lagoon manure

Antibiotic	Cumulative (%) MIC in μg/mL												
	0.06	0.125	0.250	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	128	≥256
Strep-tomycin			4.8	10.1	25.7	27.0	28.7	30.0	82.8	97.8	100		
Gentamicin	58.3	82.7	85.3	88.3	98.5	98.9	98.9	100					

Occurrence of resistance determinants

Table 5 presents the prevalence of resistance genes *strA/strB* and *aadA1* among *E. coli* isolates from different categories of pigs and manure lagoons resistant to streptomycin and spectinomycin. The highest occurrence (54.0%) was that of *aadA1* among isolates resistant to streptomycin and spectinomycin, whereas 32.3% of isolates were positive for *strA/strB*. The combination of *aadA1* and *strA/strB* genes was determined in 3.1% of strains. The analysis of data on the distribution of resistance genes among the different age categories, the highest prevalence of *aadA1* (23.4%) was observed in finisher pigs, while *strA/strB* genes were the most frequently encountered among weaned pigs. Isolates form suckling pigs also showed a higher prevalence of *aadA1* (7.8%). Higher prevalence of *aadA1* (2.1%) was established in *E. coli* isolates from manure lagoons as compared to *strA/strB* positive strains (1.0%). The combination of *aadA1* and *strA/strB* was observed in 3.2% of resistant strains; similar were percentages (1.5%, 1.0%) among isolates from weaned and finisher pigs. The same combination of resistance genes was not found out in isolates from suckling pigs.

None of E. coli strains resistant to gentamicin has exhibited the *aacC1* gene.

Table 5. Occurrence of resistance genes determined among commensal *E. coli* (n= 274) from pigs and lagoon manure

Occurrence [n (%)]						
Genotype	Suckling pigs	Weaned pigs	Finishers	Manure lagoon	Total	95%CL
Resistance to streptomycin (%)	22 (24.7)***	80 (89.8)***	82 (93.2.)***	8 (2.9)	192 (70)	60.1÷79.0
aadA1	15 (7.8)	40 (20.8)	45 (23.4)	4 (2.1)	104 (54.0)	46.9÷60.9
strA/strB	3 (1.5)	30 (15.6)	27 (14.1)	2 (1.0)	62 (32.3)	25.9÷39.0
aadA1+strA/strB	-	3 (1.5)	2 (1.0)	1 (0.5)	6 (3.1)	1.1÷6.0

Legend: p≤0.05 (*); p≤0. 01(**); p≤0.001 (***)

DISCUSSION

Molecular characteristics of the commonest co-resistant phenotypes in commensal *E. coli* isolates from domestic animals are related to the presence of *bla TEM-1* coding resistance to ampicillin, *aadA1* and strA/*strB* determining streptomycin resistance, *tet (A)* and *tet (B)* in tetracycline-resistant strains, *sul1* in sulfamethoxazole-resistant and *dfrA1* – in trimethoprim-resistant isolates. The resistance to gentamicin among commensal poultry and swine *E. coli* isolates is outlined with an ascending trend (Szmolka et al., 2013). The authors presented data from the Hungarian Antimicrobial Monitoring System, showing that the resistance to gentamicin among commensal porcine *E. coli* isolates kept the usual low levels throughout the monitoring period from 2004 to 2008. In their view, the prevalence of *strA*, *strB*, and *aadA1* genes among porcine *E. coli* strains was within the ranges 30%-60%, 60%-100%, and 1-30% respectively. Sundin et al. (1996) commented the wide spread of *strA/strB* genes in the environment and the relationship between their spread and exchange in the different ecological niches, plants, livestock animal species and humans. Sandvang et al. (2000) and Jakobsen et al. (2007) also discussed the incidence of *ant (2$^{//}$) –I*, *aac (3)-IIa*, and *aac(3)-IVa* among gentamicin-resistant *E. coli*

strains from pigs and other domestic animals. Guerra et al. (2003) presented the prevalence of *aadA1* (61%) and *strA/strB* (59%) resistance genes in commensal *E. coli* from poultry, swine and cattle. Mazurec et al. (2013) detected the presence of *aadA1* in 35.0% of streptomycin-resistant commensal porcine *E. coli* isolates.

Data about the resistance to streptomycin among commensal *E. coli* isolates from pigs show a substantial variability. For instance Wasyl et al. (2007) reported the presence of resistance in 37.0% commensal porcine *E. coli* isolates while streptomycin resistance among isolates from weaned animals reported by Stannarius et al. (2009) was 60.6%. The results of Mazurec et al. (2013) with respect to streptomycin resistance showed that it was present in 88.3% of tested commensal *E. coli* strains from swine.

The prevalence of resistance to streptomycin (70.0%) and spectinomycin (65.5%) among commensal porcine *E. coli* isolates in the present study was comparable to the results of Stannarius et al. (2009) and Mazurec et al. (2013). It should be noted that according to our data, the resistance to gentamicin (12.4%) was considerably higher that reported by Szmolka et al. (2012).

The percentage of *strA/strB* positive streptomycin-resistant strains in this study (32.3%) was the same as results presented by Szmolka and Nagy (2013), yet the occurrence of the *aadA1* gene (54.0%) was higher.

CONCLUSION

The established resistance to streptomycin and spectinomycin to commensal *E. coli* strains from pigs was close to the highest percentages reported by different research teams from the EU member states. As the genetic resistance profile was concerned, the prevalence of the *aadA1* genes was incontestable among isolates from the different age categories of pigs and environmental strains (from manure lagoons). The lack of *aacC1* genes from the genetic profile of resistance to gentamicin was not an exception as could be seen from limited data on their prevalence among domestic animals and at present, the data for the occurrence of these genes in commensal porcine *E. coli* strains are predominantly from the Asia region.

LITERATURE

1. Chaslus-Dancla E., Pohl P., Meurisse M., Marin M., Lafont J.P.: High genetic homology between plasmids of human and animal origins conferring resistance to the aminoglycosides gentamicin and apramycin. *Antimicrob*

Agents Chemother, 35, 590-593, 1991.
2. Chen L., Chen Z.L., Liu J.H., Zeng Z.L., Ma J.Y., Jiang H.X.: Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. *J Antimicrob Chemother*, 59, 880-885, 2007.
3. Davis M.A., Baker K.N., Orfe L.H., Shah D.H., Besser T.E., Call D.R.: Discovery of a gene conferring multiple-aminoglycoside resistance in *Escherichia coli*. *Antimicrob Agents Chemother*, 54, 2666-2669, 2010.
4. Deng Y., He L., Chen S., Zheng Z., Zeng Z., Liu Y., Sun Y., Ma J., Chen Z., Liu J.H.: F33:A-B and F2; A-B; -plasmids mediate dissemination of *rmtB-bla* CTX-M-9 group genes and *rmtB-qepA* in *Enterobacteriaceae* isolates from pets in China. *Antimicrob Agents Chemother*, 55, 4926-4929, 2011.
5. Du X.D., Wu C.M., Liu H.B., Li X.S., Beier R.C., Xiao F., Qin S.S., Huang S.Y., Shen J.Z.: Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens. *J Antimicrob Chemother*, 64, 1328-1330, 2009.
6. EMA/ESVAC.: European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption. Sales of antimicrobial agents in 25 EU/EEA countries in 2011. (EMA/236501/2013). Third ESVAC report. In http://www.ema.Europa.eu/dosc/en GB/document library/Report/2013/10/WC500152311.pdf.99., 2013.
7. Gonzalez-Zorn B., Teshager T., Casas M., Porrero M.C., Moreno M.A., Courvalin P., Dominguez L.: *armA* and aminoglycoside resistance in *Escherichia coli. Emer Infect Dis*, 11, 964-956, 2005.
8. Guerra B., Junker E., Schroeter A., Malorny B., Lehmann S., Helmuth R.: Phenotypic and genotypic characterization on antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J Antimicrob Chemother*, 52, 489-492, 2003.
9. Heinzel P., Werbitzky O., Distler J., Peipersberg W.: A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin -3$^{//}$-phosphotransferase. *Arch Microbiol*, 150, 184-192, 1988.
10. Hollingshead S., Vapnek D.: Nucleotide sequence analysis of the gene encoding a streptomycin/spectinomycin adenylyltransferase. *Plasmid*, 13, 17-30, 1985.
11. Hopkins K.L., Escudero J.A., Hidalgo L., Gonzalez-Zorn B.: 16S rRNA methyltransferase RmtC in Salmonella enterica serovar Virchow. *Emer Infect Dis*, 16, 712-715, 2010.
12. Jakobsen L., Sandvang D., Jensen V.F., Seyfarth A.M., Frimodt-Müller N., Hammerum A.M.: Gentamicin susceptibility in *Escherichia coli* related to the genetic background: problems with breakpoints. *Clinical Microbiol Infect*, 13, (8), 816-842, 2007.

13. Johnson A.P., Burns L., Woodford N., Threlfall E.J., Naidoo J., Cooke E.M., George R.C.: Gentamicin resistance in clinical isolates of *Escherichia coli* encoded by genes of veterinary origin. *J Med Microbiol*, 40, 221-226, 1994.
14. Johnson A.P., Malde M., Woodford N., Cunney R.J., Smyth E.G.: Urinary isolates of apramycin-resistant *Escherichia coli* and *Klebsiella pneumoniae* from Dublin. *Epidemiol Infect*, 114, 105-112, 1995.
15. Kozak G.K., Boerlin P., Janecko N., Reid-Smith R.J., Jardine C.: Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario. *Appl Environ Microbiol*, 76, (3), 559-566, 2009.
16. Liu J.H., Deng Y.T., Zeng Z.L., Gao J.H., Chen L., Arakawa Y., Chen Z.L.: Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(6′)-Ib-cr among 16S rRNA methylase RmtB-producing *Escherichia coli* isolates from pigs. *Antimicrob Agents Chemother*, 52, 2992-2993, 2008.
17. Mazurec J. Pusz P., Bok E., Stosik M., Baldy- Chudzik K.: The phenotypic and genotypic characteristics of antibiotic resistance in *Escherichia coli* populations isolated from farm animals with different exposure to antimicrobial agents. *Polish J Microbiol*, 62, (2), 173-179, 2013.
18. Sãenz Y., Brinas L., Domínguez E., Ruiz J., Zarazaga M., Vila J., Torres C.: Mechanisms of resistance in multiple-antibiotic resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrob Agents Chemother*, 48, (10), 3996-4001, 2004.
19. Sandvang D., Aarestrup F.M. : Characterization of aminoglycoside resistance genes and class 1 integrons in porcine and bovine gentamicin-resistant *Escherichia coli. Microb Drug Resist,* 6, (1), 19-27, 2000.
20. Sholz P., Haring V., Wittmann-Liebold B., Ashman K., Bagdasarian M., Scherzinger E.: Complete nucleotide sequence and gene organization of the broad-host range plasmid RSF1010. *Gene*, 75, 271-288, 1989.
21. Stannarius C., Bŭrgi E., Regula G., Zychowska M.A., Zweifel C., Stephan R.:. Antimicrobial resistance in *Escherichia coli* strains isolated from Swiss weaned pigs and sows. *Schweiz Arch Tierheilk*, 151, 119-125, 2009.
22. Sundin G.W., Bender C.L.: Dissemination of the *strA-strB* streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Molecul Ecology*, 5, 133-143, 1996.
23. Szmolka A., Anjum M.F., La Regione R.M., Kaszanyitzky E.J., Nagy B.: Microarray based comparative genotyping of gentamicin resistant *Escherichia coli* strains from food animals and humans. *Vet Microbiol*, 156, 110-118, 2012.

24. Szmolka A., Nagy B.: Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. *Frontiers Microbiol*, doi:10.3389/ fmicb. 2013.00258, 2013.
25. Vakulenko S., Mobashery S.: Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev*, 16, 430-450, 2003.
26. Wasyl D., Hoszowski A.: *Safepork*, Session 6: Antimicrobial resistance, 506- 509, Verona, Italy, 2007.

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RESISTANCE TO TETRACYCLINE IN *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*: BRIEF OVERVIEW ON MECHANISMS OF RESISTANCE AND EPIDEMIOLOGY

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Abstract

In this work we briefly present the mechanisms of resistance to tetracyclines. Tetracycline's were introduced in clinical practice in 1948, and are used for the therapy in human and veterinary medicine or as growth promoters in livestock industry. There are three major mechanisms of resistance to tetracyclines. Gram negative bacteria utilize efflux pump system of proteinaceous transporters in eliminating the drug from the cell. This mechanism of resistance is encoded by *tet* genes that belong to the group 1. Gram positive bacteria promote resistance to tetracyclines by producing soluble cytoplasmatic ribosomal protection proteins and the most frequent once are TetM and TetO proteins. Enzymatic inactivation is not widespread mechanism and the responsible gene is termed *tetX*. Epidemiological importance of tetracyclines is well documented in number of research papers. We described few works showing that tetracycline's are provoking resistance to other classes of antibiotics or vice versa. This phenomenon is probably due to the fact that resistance determinants are often situated on mobile genetic elements. Withdrawal of the therapy does not exclude resistance in short time frame due to the various environmental factors and animal feeding habits. Most often resistance to tetracycline is reported in *Escherichia coli* isolates from pigs, chickens and turkeys. The TetM and TetK proteins are most often found in methicillin resistant *Staphylococcus aureus*.

Key words: resistance, tetracycline, *Escherichia coli*, *Staphylococcus aureus*, mechanisms of resistance

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REZISTENCIJA NA TETRACIKLINE KOD *ESCHERICHIA COLI* I *STAPHYLOCOCCUS AUREUS*: KRATAK PRIKAZ MEHANIZAMA REZISTENCIJE I EPIDEMIOLOGIJE

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Kratak sadržaj

U radu su prikazani osnovni mehanizmi rezistencije na antibiotike iz grupe tetraciklina. Njihova upotreba u kliničkoj praksi, u terapiji ljudi i životinja otpočela je 1948. godine, a široko su primenjivani i kao promotori rasta u stočarstvu. Bakterije su razvile tri glavna mehanizma rezistencije na tetracikline. Gram negativne bakterije uglavnom koriste mehanizam efluks pumpe preko proteinskih transportera, u cilju eliminacije leka iz ćelije. Mehanizam rezistencije efluks pumpe kodiran je *tet* genima grupe 1. Gram pozitivne bakterije produkuju rastvorljive proteine u citoplazmi koji štite ribozom od tetraciklina, a najčešći su TetM i TetO proteini. Enzimska inaktivacija tetraciklina je treći mehanizam rezistencije povezan sa prisustvom *tetX* gena, i u odnosu na prva dva, ovaj mehanizam nije često ustanovljavana pojava. U literaturi postoji veliki broj podataka o epidemiološkom značaju tetraciklina. Na ovom mestu ukazujemo na značaj tetraciklina u razvoju rezistencije na druge klase antibiotika i obrnuto. Nastanak takvog fenomena objašnjava se činjenicom da su determinante za rezistenciju locirane na mobilnim genetičkim elementima. Ukidanje terapije tetraciklinima ne isključuje pojavu rezistencije u kratkom vremenskom intervalu, zbog uticaja raznih faktora sredine i načina ishrane životinja (antibiotici kao dodaci hrani). Rezistencija na tetracikline najčešće je ustanovljavana kod izolata *Esherichia coli* poreklom od svinja, ćuraka i pilića, a proteini TetM i TetK kod meticilin rezistentnih sojeva *Staphylococcus aureus*-a.

Ključne reči: mehanizmi rezistencije, tetraciklini, *Escherichia coli*, *Staphylococcus aureus*

INTRODUCTION

The tetracyclines are one of the oldest antibiotics that are used in livestock industry. They have been discovered in 1948, and have been used under generic name chlortetracycline and oxytetracyline. Over the years tetracycline antibiotics have been improved and marketed under various trade names. The

range of activities for these antibiotics is very broad encompassing therapy of infections caused by Gram-negative and Gram positive bacteria but also chlamydiae, mycoplasmas, rickettsiae and protozoan parasites. Importantly, they are also used as growth promoters of farm animals worldwide. It is not surprising that resistance to these antibiotics has spread in various bacterial communities. Their mode of action requires inhibition of bacterial protein synthesis by preventing association of the aminoacyl t-RNA to the ribosome A site (Chopra and Roberts, 2001).

Resistance to tetracycline has been developed utilizing various mechanisms but the most prominent is the efflux pump driven by the proton motive force. Such efflux proteins belong to the major facilitator superfamily (MSF) of transporters. Bacteria can produce ribosomal protection proteins and over two decades ago, enzymatic inactivation of tetracycline has been discovered as well (Speer et al., 1991). Detection of the respective genes encoding resistance to tetracycline raises a possibility to analyze epidemiological aspects of the resistance and to discover mobile genetic elements that may have contributed in dissemination of the tetracycline resistance genes (*tet*) in nature. Indeed the *tet* resistant genes are found on mobile genetic elements the transposones. This is the case with the *tet*(A) gene that was found on transposone Tn*1721* while the *tet*M gene was detected on Tn*916* (Frech and Schwarz, 1999; Chopra and Roberts, 2001). Even though Tn*1721* is non-conjugative transposone it is inserted in conjugative plasmids and this is facilitating dissemination of the *tet* genes in the environment. On the other hand the Tn*916* transposone is conjugative so it could be self transferable.

Mechanisms of resistance

Efflux pump

The efflux proteins belong to a large group of transmembrane proteins encoded by *tet* genes. Based on sequence amino acid identity *tet* genes are placed in six groups (Chopra and Roberts, 2001). The *tet* genes belonging to the group 1, are most abundant and represented by *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), tet(H), tet(I), tet(J), tet(K), tet(L), tet(V), tet(Y), tet(Z), *tet*(30), *tet*(31), *tcr*3, *otr*(B), *tet*P(A). The amino acid identity between these proteins is 41 to 78% and for the repressor protein it is 37 to 88%. The TetZ protein is found also in Gram positive bacteria, but all other proteins from the group 1 belong to Gram-negative bacteria (Chopra, 2002). The structure of Tet proteins has been studied and it is evident that they consist of 12 predicted transmembrane

α-helices with nonconserved central loops connecting α-helicis 6 and 7. The efflux proteins are inserted in the lipid bilayer and their hydrophilic amino acid loops are protruding into the periplasm and cytoplasm. Efflux proteins utilize the energy by exchanging a proton for a tetracycline cation complex against a concentration gradient (Chopra and Roberts, 2001). Namely, upon entry to the bacteria cell by diffusion process tetracycline are releasing proton (H+) and become increasingly receptive for divalent cations (the Me+). The monovalent cationic reactive complex strives to binds to repressor - operator complex due to its own instability. This process disables the repressor binding to DNA by triggering conformational changes in the repressor protein. Even very small amount of tetracycline's are sufficient for separation of the TetR from the DNA. This mechanism leads to the constitutive expression of the *tet* genes encoding efflux proteins. In the absence of antibiotic, the repressor protein TetR negatively regulates *tet* gene expression by binding to the operator (TetO). It has been noted that tetracycline-metal complex present one of the most sensitive effector-inducible systems of transcriptional regulators. (Hillen and Barrens, 1994). In Gram negative bacteria two or more efflux genes may be commonly responsible for resistance to tetracycline while in Gram positive bacteria, the efflux mechanism is often coupled with the ribosomal protection mechanism which gives opportunity for development of new generation of drugs aiming at both resistance targets (Chopra, 2002).

Ribosomal protection proteins

Ribosomal protection proteins (RPP) are soluble cytoplasmic proteins and they are protecting ribosome from the tetracycline including doxycycline and monocycline. The best studied mechanisms of the RPP are encoded by *tet*(M) and *tet*(O) genes. TetM proteins induce release of tetracycline from the ribosome by using the energy from GTP hydrolysis. The analysis of the upstream and downstream sequence of the structural gene and GC content (<40%) of the tetM gene supports the postulate that *tet*(M) gene may have originated from Gram positive bacteria and indicate that these genes are been transferred to Gram negative microorganisms (Chopra and Roberts, 2001). TetO proteins are changing the architecture of the ribosome inducing alterations on tetracycline binding site. These alterations are transient but mechanisms involved to prevent rebinding of the tetracycline to the ribosome are presumptive. Two interesting postulates, yet to be experimentally proven, relay on a possibility that Tet proteins promote aa-tRNA binding to the A site or that Tet(O) acts successively before aa-tRNA binding. Even though the conformational

changes at the primary binding site of the tetracycline have been recognized, it is to be answered how ribosome's returns and sustains elongation cycles in the presence of tetracycline antibiotics (Connell et al., 2003).

The *tetM* gene was most frequently found in methicillin resistant *Staphylococcus aureus* (MRSA) from 24 clinical isolates, the *tetK* gene was represented by 21 isolate and the *tetKM* genotype was found in 21 isolates (Trzcinski et al., 2000). In their research, for the first time, a single *tetK* gene in heterogeneous MRSA isolates from Poland was discovered. It was established that all isolates possessing *tetM* gene are resistant to all tetracycline's while *tetK* gene does not induce resistance to monocycline. A high level of resistance to tetracycline's (MIC > 128 mg/L) was obtained when *tetKM* genes were present in *Staphylococcus aureus* isolates comparing to isolates with single determinants. It was elucidated that preincubation with subinhibitory concentration of tetracyclines or monocyclines (except for *tetK* positive isolates where preincubation with monocycline only slightly increased MIC) induced higher MICs in MRSA isolates hence involving improved screening method (the preincubation with antibiotics before MIC analysis).

The abundance of *tet*(M) genes in the collection of clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) was documented in the research of Schmitz et al. (2001). The *tetM* gene was detected in 76% of MRSA isolates while *tetK* gene was present in 73% of the isolates. Both genes the *tetM* and *tetK* were present in half of the MRSA isolates. They have revealed that in methicilin susceptible/tetracycline resistant *S. aureus* (MSSA) the *tet*(K) gene was the most frequently found (in 96% of isolates), while tetM gene was detected in 10% of isolates. In MSSA isolates combination of *tet*(M) and *tet*(K) genes were represented at 6%.

Enzymatic inactivation and other mechanisms of resistance to tetracyclines

TetX protein is responsible for the enzymatic inactivation of tetracyclines. The tet(X) gene encoding the relevant protein was found on transposon of the strict anaerobes of the genus *Bacterodies*. Less research was directed to reveal the actual presence of *tet*(X) genes in nature and because of that the *Bacteroides* group is the only, yet to be known, species having *tetX* genes. In their natural host, the *tetX* gene is probably not functional since it requires oxygen and NADPH to chemically modify tetracycline. There are some unknown mechanisms conferring low level of resistance to tetracycline's encoded by *tet*(U) gene. The sequence of the *tet*(U) gene is however not similar to

other sequences obtained for T*et* determinants and it is difficult to reveal their mechanisms of resistance. The *otr*(C) gene was found in *Streptomycetes*, but the whole sequence was not obtained and the resistance mechanisms are not yet explained (Chopra and Roberts, 2001).

Presence of tet resistance genes in humans and animals

Resistance to tetracycline is widely distributed and occurs because of the application of the antibiotics in human and veterinary medicine. This type of resistance is often found in multi drug resistant isolates and often is the most prevalent resistance in commensal and clinical isolates (Miles et al., 2006). It is not surprising that single resistance to TET is rare among family of *Enterobacteriaceae* (Dolejska et al., 2009). The clinical impact on resistance to tetracycline was estimated during the tigecycline phase 3 clinical trials. Patients all over the world were enrolled in the clinical trial. The incidence of resistance to tetracycline in *E. coli* was 39%. Multiple efflux pump determinants were represented by 33% of isolates. It was concluded that resistance rate to tetracycline depend on its presence in the environment, excessive use of biocides and genetic transfer of resistance elements among bacterial species (Tuckman et al., 2007). Cross selection of resistance to tetracycline occurs in patients treated with other antibiotics, but it is important to note that resistance to tetracycline in *E. coli* from stool specimens of infants that have not received any antibiotic was similar to the *E. coli* from children that were treated with β-lactam antibiotics. Colonization capacity of the TET^r versus susceptible strains of *E. coli* in infants was similar showing that resistance traits was not related to treatment with antibiotics. Rather it was influenced by the presence of virulence determinant, presumably the P fimbriae and aerobactin. Hence the colonizing capacity and invasiveness of the bacteria really on virulence factors in infantile intestinal microbiota (Karami et al., 2006). A longitudinal study on antimicrobial drug resistance in *E.coli* isolates from humans and food animals in the USA has revealed that the most prevalent was resistance to tetracycline. Also this type of resistance was commonly found with the resistance to streptomycin, sulfonamide, ampicillin and chloramphenicol (Tadesse et al., 2012). The withdrawal of antibiotics especially those that have been used as growth promoters, has led to a moderate decrease in resistance rate. So was the case when application of antimicrobial agents was terminated for subtherapeutic use in specific pathogen free Yorkshire herd of pigs, held at the Kentucky Agricultural Experiment Station Research farm in the USA. Decrease of resistance to tetracycline in lactose positive enteric bacteria was 82 to 24%, but this decrease did not show steady trends after 126 months of withdrawal, since

resistance to tetracycline remains at the level of 40% (Langlois et al., 1983). Resistance to tetracycline's was the most commonly found by Miles et al. (2006) in *E. coli* isolates from humans (in 43.8% of isolates) and poultry (in 82.4% of isolates) in Jamaica. The cross resistance of tetracycline with kanamycin and nalidixic acid was limited to avian isolates, while isolates from humans were frequently cross resistant to the aminoglycosides, ampicillin and quinolones. The *tetB* and *tetD* genes, encoding the active efflux proteins, were detected on transferable plasmids in *E. coli*. Authors have discussed that *E. coli* from humans and poultry in Jamaica may not have a common source because they have different resistance patterns. The levels of resistance to tetracycline's was tested on 1263 isolates of *E. coli* from humans, domestic and wild animals in a research work of Bryan et al. (2004). The highest resistance was found in chickens, turkeys and pigs while other species like goats, horses, ducks, geese and deer have shown low level of resistance. The multiplex PCR assay aimed to detect *tet* resistant genes was performed in isolates having MIC to TET ≥ 93 mg/L. The obtained MIC represented high resistance to tetracyclines and presumably offer a possibility of resistance gene detection. In total 325 strains were analyzed by PCR and 97% have been found to contain one or more *tet* resistance genes. The distribution of genes was within the following order: *tetB* gene was detected most abundantly i.e. it was found in 63% of isolates, *tetA* gene was detected in 35% of isolates followed with the various frequencies of *tetC*, *tetD* and *tetM* gene detection. The *tetM* gene was found in *E. coli* from pigs and chickens only. Tetracycline genes in pair were found in 30% of isolates originated from turkey, pigs and horses while 4.5% of isolates from pigs have carried three *tet* resistant genes. The presence of several genes comparing to one *tet* resistant determinant was not correlated to the MIC values.

Even though it is a well established opinion that treatment of humans and animals with antibiotics select for antimicrobial resistance in pathogenic and commensal bacteria, there is a plausible lines of evidence that even in circumstances when antibiotics are not provided on farms or in cases when long withdrawal period of antibiotic use was established, resistance to tetracycline's and/or ampicillin is still documented. This is explained by the interchange of mobile genetic elements among bacteria that present the normal flora of the gastrointestinal tract of animals, age of animals and the diet provided. Another important fact is that resistance to some antibiotics coselects resistance to other and this is usually related to transferable nature of resistance mechanisms common in some bacterial species such as *E. coli* (Mirzaagha et al., 2011).

CONCLUDING REMARKS

The application of antibiotics for the therapy and for growth promotion in livestock industry is the main reason for resistance development in commensal and pathogenic bacteria. In human medicine problems occur if antibiotic treatment is required in life threatening diseases caused by multiresistant bacteria that are difficult to treat with standard antibiotics. The prominent examples now days are resistance of *Staphylococcus aureus* to vancomycin, but also resistance to metallo-β-lactamases of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* as well as *Enterobacter* spp. and *Klebsiella* spp. from the hospital environment. In developed countries patients infected with such microorganism are isolated in special units and this practice has shown to be adequate to restrict spreading of multiple resistant bacteria in the hospital settings (Levy and Marshall, 2004). Because of the broad antimicrobial activity of tetracycline's it is important to continue developing new generation of antibiotics, such as glycylcyclines, competent to efflux and ribosomal protection mechanisms, for treatment of infections caused by Gram positive and Gram negative bacteria (Chopra, 2002).

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LITERATURE

1. Bryan A., Shapir N., Sadowsky M.J.: Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. *Applied and Environmental Microbiology*, 70, 2503-2507, 2004.
2. Chopra I., Roberts M.: Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*, 65, 232-260, 2001.
3. Chopra I.: New developments in tetracycline antibiotics: glycylcyclines and tetracycline efflux pump inhibitors. *Drug Resistance Updates*, 5, 119-125, 2002.
4. Connell S.R., Tracz D.M., Nierhaus K.H., Taylor D.E.: Ribosomal protec-

tion and their mechanism of tetracycline resistance. *Antimicrobial Agents and Chemotherapy*, 47, 3675-3681, 2003.
5. Dolejska M., Bierošová B., Kohoutová L., Literák I., Čižek A.: Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *Journal of Applied Microbology*, 106, 1941-1950, 2009.
6. Frech G., Schwarz S.: Plasmid-encoded tetracycline resistance in *Salmonella enterica* subsp. *enterica* serovar *choleraesuis* and *typhimurium*: identification of complete and truncated Tn*1721* elements. *FEMS Microbiology Letters*, 176, 97-103, 1999.
7. Hillen W., Berens C.: Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. *Annual Review of Microbiology,* 48, 345-369, 1994.
8. Karami N., Nowrouzian F., Adlerberth I., Wold A.E.: Tetracycline resistance in *Escherichia coli* and persistence in the infantile colonic microbiota. *Antimicrobial Agents and Chemotherapy,* 50, 156-161, 2006.
9. Langlois B.E., Cromwell G.L., Stahly T.S., Dawson K.A., Hays V.W.: Antibiotic resistance of fecal coliforms after long-term withdrawal of therapeutic and subtherapeutic antibiotic use in swine herd. *Applied and Envronmental Microbiology*, 46, 1433-1434, 1983.
10. Levy S.B., Marshall B.: Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, 10, S122-S129, 2004.
11. Miles T.D., McLaughlin W., Brown P.D.: Antimicrobial resistance of *Escherichia coli* isolates from broiler chickens and humans. *BMC Veterinary Research*, 2:7, 2006.
12. Mirzaagha P., Louie M., Sharma R., Yanke L.J., Topp E., McAllister T.A.: Distribution and characterization of ampicillin and tetracycline-resistant *Escherichia coli* from feedlot cattle fed subtherapeutic antimicrobials. *BMC Microbiology,* 11:78, 2011.
13. Schmitz F.J., Krey A., Sadurski R., Verhoef J., Milatović D., Fluit A.C.: Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *Journal of Antimicrobial Chemotherapy*, 47, 239-240, 2001.
14. Speer B.S., Shoemaker N.B., Salyers A.A.: Bacterial resistance to tetracycline: mechanisms, transfer and clinical significance. *Clinical Microbiology Reviews*, 5, 387-399, 1992.
15. Tadesse D., Zhao S., Tong E., Ayers S., Singh A., Bartholomew M.J., McDermott P.F.: Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. *Emerging Infectious Diseases*, 18, 741-749, 2012.

16. Trzcinski K., Cooper B.S., Hryniewicz W., Dowson C.G.: Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 45, 763-770, 2000.
17. Tuckman M., Petersen P.J., Howe A.Y.M., Orlowski M., Mullen S., Chan K., Bradford P.A., Jones C.H.: Occurrence of tetracycline resistance genes among *Escherichia coli* isolates from the phase 3 clinical trials for tigecycline. *Antimicrobial Agents and Chemotherapy*, 51, 3205-3211, 2007.

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METHOD VALIDATION FOR DETERMINATION OF ORGANOCHLORINE PESTICIDE RESIDUES IN FOOD AND FEED

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Abstract

Validation of analytical methods ensures the reliability and accuracy of analytical results. To get a reliable result we performed a validation of the method taking into account all factors that may affect of the result. In this paper we optimized QuEChERS method for food and feed sample preparation as well as instrumental method using gas chromatography with mass spectrometry (GC-MS), and we obtained a method for successful determination of pesticides with calibration through the matrix. In this way, applying calibration through matrix, we satisfied the requirements for precision and reproducibility of the method being less than 20%, the accuracy rate in the range of 70-130% and method linearity throughout the range of interest.

Keywords: validation, matrix, GC-MS, QuEChERS

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VALIDACIJA METODE ZA UTVRĐIVANJE REZIDUA ORGANOHLORNIH PESTICIDA U NAMIRNICAMA I HRANI ZA ŽIVOTINJE

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Kratak sadržaj

Validacijom analitičkih metoda se obezbeđuje pouzdanost i tačnost analitičkih rezultata. Da bismo dobili pouzdane rezultate prilikom validacije metode smo uzeli u obzir sve faktore koji mogu uticati na rezultate ispitivanja. U ovom radu pokazana je optimizacija metode za pripremu uzoraka hrane i hrane za životinje QuECHERs metodom pripreme kao i optimizacija instrumentalne metode gasne hromatografije (GCMS) za određivanje pesticida u različitim matriksima. Na ovaj način. kalibracijom kroz matrix, dobili smo rezultate preciznosti i ponovljivosti koji su manji od 20%, tačnost se kretala u opsegu od 70-130% a metoda je bila linearna u celom opsegu od interesa.

Ključne reči: validacija, matrix, GCMS, QuECHERS

INTRODUCTION

Pesticides are chemical compounds, which are used for the removal, suppression and destruction of plant and other pests. Unlike the majority of pollutants that are introduced into the environment without specific targets, pesticides are introduced with the intention to help the man, to increase nutrition and to protect the environment in the "fight" against harmful microorganisms and numerous pests (Stajkovac. 2009). Analysis of organochlorine pesticides (OCP) in food and feed samples comprises application of the number of methods in order to prepare samples for analysis and determination, depending on the type of the samples. Anastassiades et.al (2003) were the first who developed Quick, Easy, Cheap, Effective, Rugged and Safe approach (QuEChERS) method for sample preparation, which gave fast, efficient and reliable result of preparation in optimal time. To eliminate the influence of matrix, calibration through matrix that does not contain pesticide was performed as well. Identification of pesticide residues is complex procedure, and moni-

toring of low concentrations requires the use of highly sensitive instrumental analytical techniques,gas chromatography with mass spectrometry(GC-MS) or high performance liquid chromatography (HPLC).GC-MS predominates in the testing's due to its greater selectivity and sensitivity as compared to other analytical methods. The aim of this study was to validate the method for determination of organochlorine pesticides set down by regulations in Serbia ("Official Gazette"RS. 29/2014) in food and feed by GC-MS technique after sample preparation applying QuEChERS method.

MATERIALS AND METHOD

Solvents and chemicals that were used in the validation process were HPLC gradient grade (Merck. Germany). Calibrant solutions were prepared using the pesticides mix of 20 pesticides (organochlorine pesticides mixture, manufacturer Ultra Scientific. lot CL-1069).Spiked samples were used to the purpose of internal control of the following parameters: recovery, precision, limit of quantification (LOQ) and limit of detection (LOD). Pesticides mix that contains 19 pesticides (Chlorinated Pesticides-herbicides, AccuStandard. Inc. lot: 213091108) was used for spike preparation. In order to eliminate the influence of the matrix, calibration through matrix blank sample was performed as well (SANCO, 2014).

Matrix	Representative sample matrix	Number of samples	Sample amount (g)	Expected concentration in matrix (mg/kg)
Meat and meat products	Meat	10	3	0.005
Milk and milk products	Whey powder	10	3	0.005
Eggs and egg products	Melange	10	3	0.005
Feedstuff	Fishmeal	10	3	0.005

Table 1 The matrix and the amount of working solution for LOQ and LOD determination.

Table 2 Representative matrix and the amount of working solution for spike with the aim of determining the precision and reproducibility

Matrix	Representative sample matrix	Number of samples	Sample amount (g)	Expected concentration in matrix (mg/kg)
Meat and meat products	Meat	20	3	0.05
Milk and milk products	Whey powder	20	3	0.05
Eggs and egg products	Melange	20	3	0.05
Feedstuff	Fishmeal	20	3	0.05

This method of sample preparation is based on the extraction with acetonitrile in the presence of anhydrous magnesium sulfate (MgSO4) and anhydrous sodium acetate (CH3COONa). Sample (3 g) is measured and transferred into centrifuge tube, 3 mL of water is added and 3 mL of acetonitrile. After intensive stirring on a vortex, 3 g of anhydrous magnesium sulphate and 1 g of anhydrous sodium acetate were added. Exothermic reaction occurred within 1 min after the intense stirring on vortex. The sample was then centrifuged until 5 min at 3000 rpm. 1 mL of upper acetonitrile extract is transferred into the 5 ml tube, which contained 150 mg of anhydrous magnesium sulphate, 100 mg of Primary and Secondary Amine (PSA) and 50 mg of C18 (Anastassiades et al. 2003). The tube content was centrifuged for 5 min at 3000 rpm. After centrifuging, purified and clear extract is obtained. Then, 0.5 mL of the extract is evaporated in nitrogen and reconstituted with hexane. A sample prepared in this way is ready for the analysis on GC-MS (Agilent 7890B/5977A).

Organochlorine pesticides are separated on DB-5MS column (30 m· 0.25 μm· 0.25 mm). Sample volume of 4 μL (splitless mode) was injected at the constant pressure of 11.36 psi and and flowed through the column of the carrier gas at the flow rate 1.2 mL/min. The list of analyzed OCP as well as retention time, molecule weight, ions important for the analysis of HCH isomer are shown in Table 3. The target and qualifier abundances were determined by injecting the mixture of pesticide standards under the same chromatographic conditions using full scan with the mass/charge ratio ranging from 60 to 500 m/z. Standards were prepared in blank matrix extracts to counteract the matrix effect

(SANCO. 2014). With the aim of obtaining more reliable results, further pesticide quantification was performed in SIM mode. Pesticide quantification was performed according to mass spectra and characteristic ions defined in SIM mode (Table 4), as well as the retention time of exit components, pesticides (Selvi et al. 2012). The processing of the obtained data was performed applying Mass Hunter Software. The analysis of the method performance is performed in a calibration range from 0.005 to 0.1 mg/kg.

Table 3 Retention time (RT), molecule mass (MW), primary (target) ion (T) and secondary and tertiary ion (Qualifier Ions, Q1, Q2).

Pesticide	RT(min)	MW	T	Q1	Q2
α HCH	11.28	290.8	181	181	219
β HCH	12.47	290.8	183	181	183
γ HCH (lindane)	12.57	290.8	181	183	109
δ HCH	13.74	290.8	109	219	183
heptachlor	15.74	370	272	235	237
aldrine	17.40	362	263	220	291
heptachlorepoxide	19.55	386	353	81	355
trans-chlordane	20.88	406	373	375	-
alpha endosulfan	21.46	404	195	159	133
cis chlordane	21.71	406	373	375	-
pp'DDE	22.84	378	79	277	239
dieldrine	23.09	316	246	176	211
endrin	23.80	378	263	191	226
endosulfan	24.26	404	195	157	159
pp'DDD	24.90	318	235	165	237
endrin aldehyde	25.065	378	67	345	-
endosulfansulfate	25.97	420	272	274	387
pp'DDT	26.26	352	235	165	200
methoxychlor	26.88	344	227	165	184
endrin ketone	27.46	240	317	67	-

Table 4 SIM program was used for the analysis and confirmation (m/z. total dwell time)

Group	Time (min)	Pesticide	m/z	Total dwell time
1	10.78	α HCH, β HCH, γ HCH, δ HCH	181, 219, 109	150
2	14.98	heptachlor	100, 237, 272	150
3	16.66	aldrine	66, 263, 293	150
4	18.84	heptachlor epoxide	81, 353, 237, 263	200
5	20.37	Cis, trans-chlordane, endosulfan I	373, 237, 272, 195, 237, 170	300
6	22.38	Dieldrine. pp´DDE	79, 263, 246, 176, 318	250
7	23.45	Endrin, Endosulfan II	81, 67, 263, 245, 195, 237, 243	350
8	24.62	pp´DDD. endrin aldehyde	235, 165, 67,173, 250	250
9	25.29	pp´DDT. endosulfansulfate	165, 235, 237, 275, 387, 422	300
10	26.56	methoxychlor	227, 152	100
11	27.14	endrin ketone	67, 317, 345	150

RESULTS AND DISCUSSION

Based on tests conducted on five representative matrices and implemented to the internal controls, we obtained the results shown in Tables 5 and 6. Validation plan included determination of linearity, precision, reproducibility, accuracy and LOQ and LOD.

Linearity that was determined by setting a calibration curve was tested by regression analysis to establish the mathematical relationship between concentration and results in a set range of resultant values.

The precision of the method represents an agreement between values obtained in a series of repeated measurements of the same homogenous sample under the same determination conditions by at least 5 repeated measurements of the representative spiked matrix.

The reproducibility of the method represents matching results obtained by successive measurements of the same samples, but under many different conditions, determination is accomplished by analyzing at least 5 spiked samples (representative matrix).

The accuracy represents the mean value of the obtained results and actual or accepted value of the results. It is expressed as the yield (recovery), calculated on the spiked sample in relation to the expected theoretical value of the results.

LOQ is the lowest concentration that can be determined with reasonable accuracy. It is calculated as the sum of the mean values of 10 repetitions and 3 standard deviations.

LOD , granica detekcije , je najniza koncentracija analita koja može biti dokazana ali ne i određena. Izračunava se kao zbir srednje vrednosti od deset ponavljanja na matrix spajku , koji odgovara prvoj tački kalibracije, i 10 standardnih devijacija.

Table 5 The average values of accuracy, reproducibility, accuracy, linearity, LOQ and LOD for all matrices

Pesticides	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (R^2)	LOQ (mg/kg)	LOD (log/kg)
α HCH	4.35	5.22	96.07	0.9990	0.0047	0.0014
β HCH	17.91	8.88	99.14	0.9918	0.0019	0.0006
γ HCH (lindane)	8.98	8.32	99.64	0.9980	0.001	0.0003
δ HCH	0.8	18.2	100.32	0.9993	0.0042	0.0003
heptachlor	3.39	14.64	88.08	0.9979	0.001	0.0003
aldrine	3.57	3.44	98.3	0.9904	0.0046	0.0014
heptachlorepoxide	3.52	3.36	94.37	0.9973	0.0016	0.0005
trans-chlordane	4.37	8.22	90.16	0.9981	0.0012	0.0004
alpha endosulfan	9.32	8.7	87.27	0.9977	0.0028	0.0009
cis chlordane	4.3	8.22	91.55	0.9993	0.0039	0.0012

Pesticides	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (R^2)	LOQ (mg/kg)	LOD (log/kg)
pp'DDE	3.14	3.87	96.87	0.9965	0.0048	0.0014
dieldrine	3.52	3.36	94.37	0.9924	0.005	0.0015
endrin	8.51	16.21	83.41	0.9942	0.0031	0.0009
endosulfan	7.77	10.25	91.24	0.9986	0.0049	0.0015
pp'DDD	5.69	14.29	81.57	0.9991	0.0039	0.0012
endrin aldehyde	5.57	10.46	85.62	0.9979	0.0044	0.0013
endosulfansulfate	13.7	15.3	116.3	0.9990	0.0046	0.0014
pp'DDT	3.52	3.36	94.37	0.9918	0.0048	0.0014
methoxychlor	7.67	1.84	106.09	0.9980	0.0021	0.0006
min	**0.8**	**1.84**	**81.57**	**0.9904**	**0.001**	**0.0003**
max	**17.91**	**18.2**	**116.3**	**0.9993**	**0.005**	**0.0015**

Table 6 Data obtained from internal quality control for the different matrices (mean values Xsr, STD, RSD, Bias Recovery, N number of measurements for each pesticide)

Matrix	N	Xsr (mg/kg)	STD	RSD (%)	Bias (%)	Recovery (%)
Fishmeal	20	0.053	0.010	19.438	7.095	106.330
Whey powder	20	0.055	0.005	9.818	9.697	109.183
Meat	20	0.048	0.010	18.807	11.176	96.607
Melange	20	0.047	0.008	16.052	7.814	93.916
Honey	20	0.052	0.005	10.523	3.960	103.960
Xsr	**20**	**0.051**	**0.0076**	**14.927**	**7.948**	**101.999**

According to study of Maštovská et al. (2005), as compared to matrix-matched standardization, the analyte protectant approach offers a more convenient solution to the problems associated with calibration in routine GC/MS analysis of pesticide residues and possibly other susceptible analyte types in diverse samples. In a study on Alternative calibration techniques for coun-

teracting the matrix effects in GC–MS-SPE pesticide residue analysis, Rimayi (2015) shows descriptive and inferential statistics proving that the matrix-matched internal standard calibration was the best approach for samples of varying matrix composition since it produced the most precise average mean recovery of 87% across all matrices tested. We demonstrated the same in our testing. Kartalovic et al. (2015) suggested application of gas chromatography with mass detector for the determination of pesticide traces, as it provides us with a confirmation of result reliability by comparing the obtained spectrum with that from the library. GCMS analysis offers good precision and recovery rate for determination of pesticides in hake fillets when applying matrix calibration (Kartalovic et al., 2015b).

CONCLUSION

Based on the conducted research and appropriate preparation, calibration and verification of the representative matrix we can conclude that the method for determination of pesticide residues in food and feed meets the eligibility criteria required by SANCO (2014). The method is linear in the range of 0.005 to 0.1 mg/kg. The linearity factor (R^2) is higher than 0.99. The precision and reproducibility rate for pesticide determination is a greater than 20%. The accuracy of the method is in the range 70-130%. The method can be successfully used for the determination of pesticide residues in food and feed.

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LITERATURE

1. Anastassiades M. Lehotay S.: Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase extraction" for the Determination of Pesticide Residues in Produce. *Journal of AOAC International*. 86(2). 2003.
2. Cornelius Rimayi .DavidOdusanya·Fanyana. Mtunzi.Shepherd Tsoka· Alternative calibration techniques for counteracting the matrix effects in GC–MS-SPE pesticide residue analysis – A statistical approach. *Chemosphere* Volume 118. Pages 35–43. 2015.

3. Jugoslav Stajkovac. Branka Amidžić. Jelena Biočanin: Pesticidi i izvori zagađenja u životnoj sredini i značaj remedijacije u sanaciji kontaminacije. 1st International Conference 'Ecological safety in post-modern environment'. Banja Luka.RS. BiH. 26-27. Juny 2009.
4. Kateřina Maštovská . Steven J. Lehotay . Michelangelo Anastassiades: Combination of Analyte Protectants To Overcome Matrix Effects in Routine GC Analysis of Pesticide Residues in Food Matrixes. *Anal. Chem. 77* (24). pp 8129–8137. 2005.
5. Kartalović B. Babić J. Prica N. Živkov-Baloš M. Jakšić S. Mihaljev Ž. Ćirković M.: The presence of Lindane in different types of honey in the Pannonian region. *Bulgarian Journal of Agricultural Science*. ISSN 1310-0351. 208-212. 2015
6. Kartalović B.. Živkov-Baloš M.. Prica N.. Babić J.. Jovanić S.. Petrović J.. Ćirković M.: Presence of organochlorine pesticides in hake fillets. VII International Conference 'Water & Fish'. Belgrade-Zemun. Serbia June. 10-12. 2015. editors in chief Vesna Poleksić and Zoran Marković. Conference Proceedings. 293-297. Belgrade. University. Faculty of Agriculture. 2015b
7. Pravilnik o maksimalno dozvoljenim količinama ostataka sredstava za zaštitu bilja u hrani i hrani za životinje i o hrani i hrani za životinje za koju se utvrđuju maksimalno dozvoljene količine ostataka sredstava za zaštitu bilja. Beograd. JP „Službeni glasnik"Republike Srbije. 29/2014.
8. SANCO. Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed. Supersedes SANCO/12495/2011 Implemented by 01/01/2014.
9. Selvi C. Paramasivam M. Rajathi D. S. Chandrasekaran S.: Multiresidue analysis of organochlorine pesticides in milk. egg and meat by GC-ECD and confirmation by GC-MS. *Bull Environ Contam Toxicol*. 89: 1051–1056. 2012.

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FORENSIC EXAMINATION OF A BOAR DIED DURING TRANSPORT AIMED AT REIMBURSEMENT OF INSURED ANIMAL

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Abstract

The long lasting transport of high-value animals during the import represents the significant stress factor for animals, which can lead to severe health problems and even animal death. Thus, insurance of animals against the event of death is highly recommended. In case of the damage of the insured animal (death) it is necessary to implement all statutory and contractual measures for determining the reasons of the damage in order to effectively compensate the loss in the country, in which the animal is insured. In this article, we described the case control study, a death of breeding animal (boar) during the transport (import) from Denmark to Serbia. The animal was insured in exporting country and the forensic expertise was necessary to the purpose of adequate insurance compensation. On the basis of applied methods, which included medical history, records of clinical findings, gross pathological findings and laboratory examination, the cause of death of imported boar was identified (heart failure and suffocation). The Danish insurance company accepted the results of the examination and compensation has been successfully implemented on behalf of the dead boar.

Key words: animal insurance, transport, mortality, pigs

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FORENZIČKO ISPITIVANJE TRANSPORTNOG UGINUĆA NERASTA U SVRHU NAPLATE ŠTETE OSIGURANE ŽIVOTINJE - PRIKAZ SLUČAJA

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Kratak sadržaj

Dugotrajni transport visokovrednih životinja koji se odvija prilikom uvoza predstavlja stres za životinje, koji može prouzrokovati narušavanje zdravlja pa i uginuće životinja. Upravo zbog navedenog, preporuka je da se visokovredne jedinke osiguraju u toku transporta**.** U slučaju nastanka štete (uginuća) kod osiguranih životinjaneophodno je sprovesti i sve propisane i ugovorene mere u cilju utvrđivanje razloga nastanka štete, kako bi se uspešno nadoknadio gubitak i u drugoj državi, u kojoj su životinje osigurane. U radu je opisan slučaj transportnog uginuća visokovrednog priplodog nerasta,prilikom uvoza iz Danske u Srbiju. Jedinka je bila osiguranau državi izvoznici i u cilju naplate osiguranja bio je neophodan argumentovan, forenzički nalaz. Na osnovu primenjenih metoda ispitivanja, koje su obuhvatale anamnestičke podatke, kliničku sliku, patomorfološki pregled i laboratorijski nalaz, utvrđen je uzrok uginuća nerasta (srčanaslabost, ugušenje). Osiguravajuće društvo države izvoznice je prihvatilo nalaz, čime je omogućena realizacija naplate štete za uginulog nerasta.

Ključne reči: osiguranje životinja, transport, uginuće, svinja

INTRODUCTION

Transport of animals represents an important stress-inducing situation that may lead to subclinical changes, clinical manifestations of poor health and even death (*Malena et al., 2007; Stojanac et al., 2014*). During the transport of animals, special care should be taken to ensure the welfare of animals. The transportation needs to provide appropriate conditions and satisfy physiological and other needs specific to particular animal species, such as feeding, accommodation, physical, psychological and thermal comfort and safety. Also, conditions enabling animals to demonstrate the basic behavior

patterns, social contact with animals of the same species, absence of negative experiences (pain, suffering, fear, stress, illness and injuries) need to be provided (*Official Gazette of RS, 2009*). During the transport on the long distances, the stress certainly occurs (*von Borell and Schaffer, 2005*) and the overall ambient conditions for animals are changed. The transportation of an animals is not a narrowly defined stressor; it represents a combination of several factors since the animal is exposed to different and unknown materials, smells, light intensity, sounds and vibrations, manipulation by people, separation from the group and mixing with unfamiliar individuals, changing temperatures and air circulation, injuries, limited space, deprivation of food and water. It is suggested that, inevitably, the fact that all principles of wellbeing cannot be respected during transportation (*EC, 2004*).

The livestock insurance is a special branch of insurance characterized by a number of specificities, which primarily relate to the subject of insurance (animals) that function under specific biological laws. If owners or farmers want to insure their pigs (subject of insurance) against various risks, they have an obligation to pay the appropriate compensation for risk-taking. This entails additional costs and certainly increases the cost for animals insurance (*Marković and Jovanović, 2010*).

Insurance including coverage for risk requires fulfillment of some basic insurability criteria, including the capability to estimate the severity of risk with associated uncertainty. Conventional empirical methods are of limited use to estimate the impact of new risk data, are usually scarce and/or may not be relevant to the new disease. Hence, the feasibility of alternative methods to quantify the impact of risk should be further explored (Coble et al., 2006; Zagmutt et al., 2013). The price of risk (insurance) is a premium, which exists in all types of insurance. Farmers set aside significant funds for the livestock insurance (crop insurance) and they pay the full amount of premium (gross premium). The whole issue of livestock insurance is intended to enable faster development of livestock and to reduce damages in animal husbandry to the minimum. In the selected period, technical premium is not sufficient to cover the damages and it is necessary to try to increase it in the future. This can be achieved through better education of farmers, who could use certain preventive measures to partially mitigate the development of specific damages, and on the other hand with appropriate livestock insurance packages to try to encourage more farmers to insure their animals (Marko, 1989; Marković, 2007).

In the majority of cases, the highly valuable breeding pigs are transported on long distances (over 1000 km). The long distance transportation represents substantial expenses, which increase the cost of sold goods, and therefore the-

re is no financial justification to transport the commercial (slaughter) pigs. Depending on the final agreement, the animal insurance in the international transport may be completed by the exporter or importing country. All rights and obligations should be regulated by the contract, which clearly indicates, which contracting party bears the risk during the transport of animal. In case of any damage, the implementation of all prescribed and agreed measures for determining the cause of damage should be taken in order to compensate the loss in another country, in which the animal was ensured.

As described in this study, all animals (11 hogs) imported to Serbia were clinically examined by an authorized veterinarian before loading, and no clinical signs of disease or visible defects were detected. However, the exporting country, i.e., the insurance company did not agree to accept the damage on the basis of statements of the driver and the customer, and they demanded an official forensic report. In this article, the case, of death of breeding animal (boar) during the transport (import) from Denmark to Serbia was described. The animal was insured in exporting country, and with an aim of obtaining the insurance compensation, the complete forensic expertise was necessary.

MATERIAL AND METHODS

The material for this research included 11 breeding hogs, Duroc breed, imported to Serbia and settled in quarantine facility according to the official decision of the Veterinary Directorate of the Ministry of Agriculture and Environmental Protection of the Republic of Serbia. When unloading the animals, it was discovered that one of the boars died. The applied research methods included epidemiological and medical history, clinical finding records, gross pathological findings and laboratory examination of sera samples sampled from live boars as well as the tissue samples (liver, spleen, kidney, lungs, heart, mandibular, mesenteric and mediastinal lymph nodes, tonsils) derived from dead animal (bacteriological and virology testing). The serological examination for relevant bacterial (antibodies against leptospirosis, Brucella sp.) and viral diseases (antibodies against Classical Swine Fever (CSFV), Morbus Auijeszky (MA), Porcine Respiratory and Reproductive Syndrome virus (PRRSV)) was performed in line with the Quarantine Decision of the Veterinary Directorate of the Ministry of Agriculture and Environmental Protection.

Isolation of bacteria from tissue samples deriving from dead pigs was performed by standard aerobic and microaerophilic cultivation (*Quinn et al., 2011*).

The titer of MA specific antibodies was determined by serum neutralization test (SNT) following standard procedure as described before (*O.I.E. Manual of Epizootic, 2004, section 2.1.2.*).

Determination of the presence of antibodies against the PRRSV and CSFV was performed applying ELISA (*OIE, section 2.8.7*) and ELISA (*OIE, section 2.8.3*), respectively.

RESULTS AND DISCUSSION

Pursuant to relevant medical and other documents supporting the importation license, 11 breeding boars, Duroc breed, were transported from Denmark to Serbia. All accompanying import documentation for animals was checked by the Veterinary Inspection. When unloading boars, the veterinarian discovered that one boar died. Clinical investigation revealed no signs of disorders or disease in remaining 11 boars and animals were placed in a quarantine facility. Other boars did not manifest any clinically apparent disorders. The transport of animals lasted in total 23 hours and transport routes went through several countries: Germany, the Czech Republic, Slovakia and Hungary.

The Veterinary inspector applied the official control of the certificate issued by the Danish Veterinary and Food and established the following: the health status of the boars was checked before loading, and there was no suspicion on the presence a contagious disease that can be transmitted to pigs. The certificate stated that the State of Denmark has conducted testing on all diseases requested by the Veterinary Directorate of the Ministry of Agriculture and Environmental Protection, thus confirming freedom from certain viral (MA, TGE, PRRSV, African swine fever, CSFV, foot and mouth disease, swine vesicular disease) and bacterial swine diseases (atrophic rhinitis, brucellosis, leptospirosis, swine dysentery, tuberculosis, actinobacillosis, enzootic pneumonia). Finally, it was stated that breeding boars were not transported (on the way from the place of loading to the place of unloading) through an area where contagious diseases were detected. The transport vehicles have previously been appropriately cleaned and disinfected, and meet all requirements considering animals' welfare during transport.

Official control of transport vehicles

The breeding boars were shipped by the vehicle (truck) owned by the seller from Denmark. The total floor area was about 6.9m^2 and was divided into two unequal parts (4.5 and 2.4m^2) with 1m high fence. In the larger part, seven boars were placed, and four boars were put in the back part of the vehicle. Additional control of animal space revealed a small quantity of sawdust sprinkled on the rough floor was found. The transport vehicle was equipped with the water supply (separated in two parts) as well as the ventilation system.

Clinical investigation

While unloading the live boars from the vehicle, one carcass of dead boar was found. Clinical examination revealed no clinical signs of the disease in remaining animals. Examination of the respiratory and digestive tract as well as the locomotor and reproductive system revealed no signs of diseases in 11 breeding boars. The animals were settled in quarantine stables.

Gross pathological finding

The pathomorfological examination of dead breeding boar was performed according to standard gross pathology veterinary farm protocol. By external examination, the Duroc breed and good body condition was established. The animal body weight was estimated to some 100 kg. The skin of dead animal was cyanotic, particularly in the area of the scrotum, preputium, chest and neck. After external examination, pathological control of internal cavities was done. In the abdominal cavity, distinct meteorism of the stomach and small intestine was discovered as well as small amount of free bloody content in the abdominal cavity. Gross pathological control of the chest cavity revealed distinct emphysema of the complete lung lobes and the presence of bloody content. After detailed examination and opening of the airways, reddish color of tracheal and mucosa of major bronchi brochs (Picture 1-A) along with the presence of blood content was observed. On lung cross-section, dark red color of the lung tissue and large amount of blood were observed (Picture 1-B).Pathological examination of the heart muscle revealed markedly expanded and significantly enlarged right ventricle (about 1.5 times larger than the left one) (Picture 1-C). In the right atrium and the heart chamber, a large amount of coagulated blood, dark red in color was discovered (Picture 1-D).

Pathomorphological diagnosis was established: *Emphysema pulmonum, Hyperaemia passiva acuta pulmonis, Dilatatio passiva cordis*.

The results of laboratory examination

Bacteriological testing of tissue samples obtained from dead breeding boar revealed the presence of the following bacteria: *Escherichia coli, Clostridium perfringens*. Serological investigation did not confirm antibodies against the examined swine diseases (MA, PRRSV, CSF, Brucela spp., Leptospirosis).

Before transporting high-value animals over long distances it is necessary to precisely define all conditions of the insurance, to specify the responsibi-

lities of each party and what is included in the insurance. This is very important when it comes to international trade because management systems of veterinary service differ from country to country. The responsibility of the veterinary service is particularly important when assessing the damage and performing forensic testing to identify the causes of damage. In this case, the boars have been insured in Denmark and it was necessary, factually and with good arguments, to identify the cause of the death of a boar in order to charge for the loss.

In this paper, the examination of the reason for death of the boar indicated that beside the changes observed on the animal itself, the means of transport as well as the conditions under which the animals were transported played an important role. During the transportation of animals, it is necessary to ensure their well-being (Official Gazette of RS, 2009). One of the most important issues concerning the welfare of animals on long lasting transport is the adequate surface area per animal. Animals in the transport vehicle should be able to lie down and to stand when they need (RSPCA, 2010; EC, 2004). To fulfill this requirement, the minimum required space for pigs should not exceed 235 kg/ m^2, or by transported pig (100 kg) should be provided 0.42 m^2 of floor area. Available floor area (A) can be calculated from the formula A = 0,0192W0.67, where "W" is animal body weight (Voslářováisar., 2010).

Several studies reported that high stocking density reduces animal welfare and is associated with greater mortality during transport and lairage (Ritter et al., 2007; Fitzgerald et al., 2009). The spatial area in the truck, in which they boars from Denmark were transported, was divided into two parts, so the space available per one animal was 0.64 m^2 in the larger part and 0.60 m^2 in the smaller part of the vehicle. The boars were of an average weight of about 90 kg, which suggests that the boars had enough floor space per animal, but one should take into account that the trip took quite a long time (23h) and that all boars did not originate from the same herd. A review of the Common Veterinary Entry Document (CVED) established that the truck entered Serbia 4 hours prior to unloading and that all animals were inspected by the border veterinary inspector, who reviewed the identity and performed physical examination of the boars, marked them as "satisfactory" and accepted the import and transport to the quarantine. This indicates that the death occurred in the last part of the transportation, that is, between 19th and 23rd hour spent in the truck, and before unloading.

The severity of transport stress depends on aspects of loading and unloading as well as length of the journey, stocking density, group social hierarchies, genotype, and climatic condition (Nanni Costa, 2009). Different studies have

been aimed at investigating the effects of season, air temperature, and length of the journey on in-transit market pig losses (Averos et al., 2008; Sutherland et al., 2009). Import of boars was carried out in July, which according to many other authors (Werner et al., 2007; Vitali et al., 2014) is the month with the highest mortality rate during pig transport. Also, the distances that are crossed during import of boars are very long, which affects the increase in mortality during transport (Haley et al., 2008; Sutherland et al., 2009) and obviously resulted in the death of boars also in this study.

CONCLUSION

On the basis of the certificate issued by the Danish Veterinary and Food Administration, Common Veterinary Entry Document (CVED), medical history, clinical presentation of other boars, autopsy findings and laboratory results it is concluded that the cause of death of the boar was heart insufficiency. Death came due to long lasting transport (23h) and large number of boars in a small space, which caused further stress in the boar. The animal obviously fell down, and traces of injuries on the skin on one side of the body indicated that the animal was trampled by other animals. In the present case, it was a very common cause of death in pigs. When animals are transported in the correct way and in compliance with the procedures that are necessary to meet the requirements for compensation, cases of animal death during transport are successfully solved within the framework of international trade obligations. Danish insurance company has accepted this finding and compensation payment for dead boar was successfully implemented.

LITERATURE

1. Averos X., Knowles T.G., Brown S.N., Warriss P.D., Gosálvez L.F.: Factors affecting the mortality of pigs being transported to slaughter. *The Vet Rec*, 163(13), 386-390, 2008.
2. von Borell E., Schaffer D.: Legal requirements and assessment of stress and welfare during transportation and pre-slaughter handling of pigs, review article. *Livest Prod Sci*,97, 81-87,2005.
3. Coble K.H., Hanson T.R., Sempier S.H., Shaik S., Miller J.C.: Investigating the feasibility of livestock disease insurance: A case study in aquaculture. Pp. 252–271 in Koontz SR, Hoag DL, Thilmany DD et al. (eds). The Economics of Livestock Disease Insurance: Concepts, Issues and International Case Studies. Wallingford, UK: CABI Publishing, 2006.

4. EC. Counsil Regulation (EC) No 1/2005 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97, 2004.
5. Fitzgerald R.F., Stalder K.J., Matthews J.O., Schultz Kaster C.M., Johnson A.K.: Factors associated with fatigued, injured, and dead pig frequency during transport and lairage at a commercial abattoir. *J Anim Sci*, 87, 1156-1166, 2009.
6. Haley C., Dewey C.E., Widowski T., Poljak Z., Friendship R.: Association between in-transit loss, internal trailer temperature, and distance travelled by Ontario market hogs. *Can J Vet Res*, 72, 385-389, 2008.
7. Malena M., Voslářová E., Kozák A., Bělobrádek P., Bedáňová I., Steinhauser L., Večerek V.:Comparison of Mortality Rates in Different Categories of Pigs and Cattle during Transport for Slaughter. *Acta Vet Brno*,76: S109-16, 2007.
8. Marko J.: Osiguranje useva (plodova) i naknada štete, Agroekonomika, 18(1), 98,1989.
9. Marković T.: Razvoj osiguranja stoke u Srbiji. *Letopis naučnih radova*, 31(1)158-166, 2007.
10. Marković T., Jovanović M.:Livestock insurance as a factor of economic stability in the agriculture. *Contemporary Agriculture*, 59(3-4), 287-92,2010.
11. Nanni Costa L.: Short-term stress: The case of transport and slaughter. *Ital J Anim Sci*, 8, 241-252, 2009.
12. OIE. Manual of Diagnostic Test and Vaccines for Terrestrial Animals. Fifth Edition, 2004.
13. Quinn J. P., Markey K.B., Leonard C. F., Fitz S.E., Fanning S., Hartigan J.P.: Veterinary microbilology and Microbial disease, *Wiley Blackwell*, 196-287, 2011.
14. Ritter M.J., Ellis M., Bertelsen C.R., Bowman R., Brinkmann J., DeDecker M., Keffaber K.K., Murphy C.M., Peterson B.A., Schlipf J.M., Wolter B.F.: Effects of distance moved losses of market weight pigs on arrival at the packing plant. *J Anim Sci*, 85, 3454-3461, 2007.
15. RSPCA, Welfare standards for pigs. The Royal Society for the Prevention of Cruelty to Animals, UK, 2010.
16. Službeni glasnik RS. Zakon o dobrobiti životinja. *Sl. glasnik RS*, br. 41/2009, 2009.
17. Stojanac N., Stevančević O., Cincović M. R., Stančić I., Potkonjak A., Toholj B., Vračar V.: Forensic investigation of sow deaths in the intesive breeding system. *Archives of Veterinary Medicine*, Vol. 7, No. 1, 29 - 37, 2014.
18. Sutherland M.A., McDonald A., McGlone J.J.:Effects of variations in the

environment, length of journey and type of trailer on the mortality and morbidity of pigs being transported to slaughter. *Vet Rec* 165, 13-18, 2009.
19. Vitali A., Lana E., Amadori M., Bernabucci U., Nardone A., Lacetera N.: Analysis of factors associated with mortality of heavy slaughter pigs during transport and lairage. *Journal of animal science*, 92(11), 5134-5141, 2014.
20. Voslářová E., Chloupek P., Steinhauser L., Havlíček J., Večerek V.: Influence of Housing System and Number of Transported Animals on Transport-induced Mortality in Slaughter Pigs. *Acta Vet Brno*, 79, S79-84, 2010.
21. Werner C., Reiners K., Wicke M.: Short as well as long transport duration can affect the welfare of slaughter pigs. Animal welfare, 16(3), 385-389., 2007.
22. Zagmutt F., Sempier S., Hanson T.: Disease Spread Models to Estimate Highly Uncertain Emerging Diseases Losses for Animal Agriculture Insurance Policies: An Application to the US Farm-Raised Catfish Industry. *Risk Analysis* 33.10, 1924-1937, 2013.

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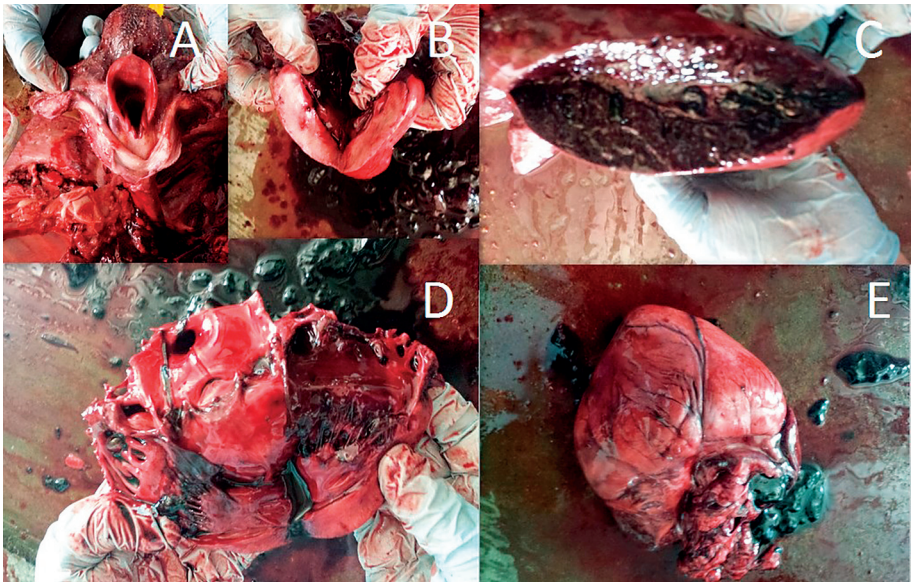
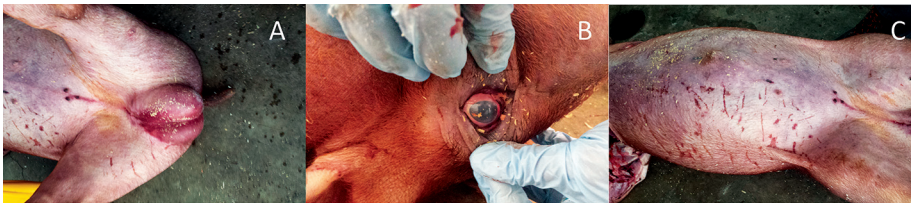


Picture1. Post-mortem examination: A, B-lung, C, D-heart.


A
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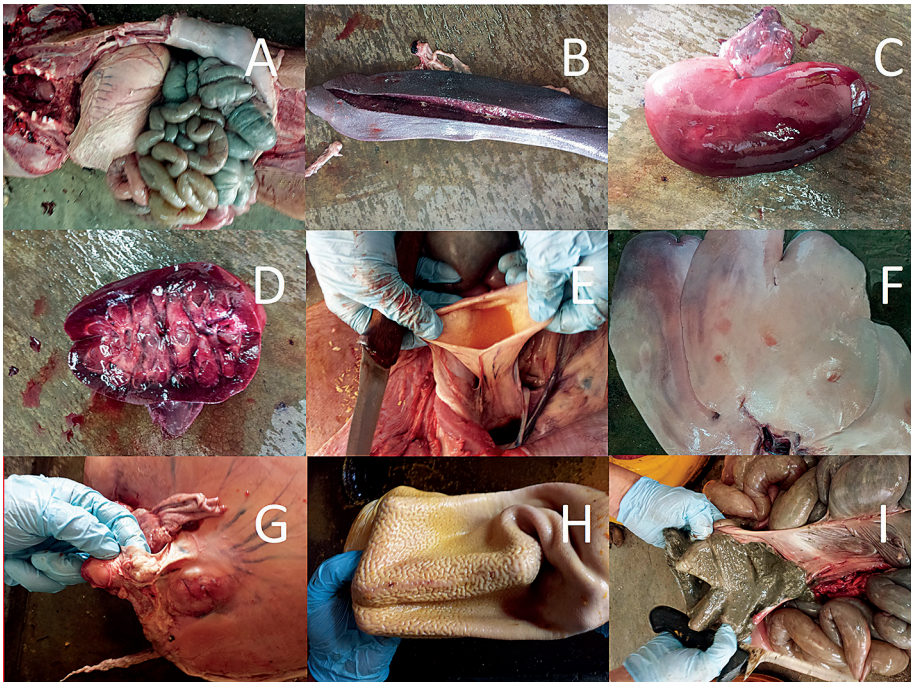
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CORYNEBACTERIUM RENALE CYSTITIS IN COW - CASE REPORT -

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Abstract:

Corynebacterium renale is a common inhabitant of the the vulva, vagina and prepuce of apparently normal cattle, but also an opportunistic pathogen and the cause of cystitis and purulent pyelonephritis in cows. In this paper, we show the isolation of *C. renale* from the urine of cows with clinical cystitis, colonial, microscopic and biochemical characteristics of the isolates, relevant data on virulence factors, clinical manifestations of disease and basic principles of therapy.

Key words: cow, *Corynebacterium renale*, cystitis

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CORYNEBACTERIUM RENALE CYSTITIS KOD KRAVE -PRIKAZ SLUČAJA-

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Kratak sadržaj:

Corynebacterium renale je uobičajeni deo mikrobiota sluzokože vulve, vagine i prepucijuma klinički zdravih goveda, ali i oportunistički patogen i uzročnik *cystitisa* i purulentnog *pyelonephritisa* krava. U ovom radu prikazujemo izolaciju *C. renale* iz urina krave sa kliničkim cistitisom, osnovne kulturelne, mikroskopske i biohemijske karakteristike izolata, relevantne podatke o faktorima virulencije uzročnika, kliničkim manifestacijama bolesti i osnovnim principima terapije.

Ključne reči: krava, *Corynebacterium renale*, *cystitis*

INTRODUCTION

Bovine cystitis is an inflammatory process of the urinary bladder. The causative agents are most commonly bacteria which are an integral part of the microbiota of the genital and gastrointestinal tract, such as: *Escherichia coli*, *Corynebacterium renale* group (*Corynebacterium renale*, *cystidis* and *pilosum*), *Trueperella* (formerly *Arcanobacterium*) *pyogenes*, rarely *Staphylococcus* spp., *Streptococcus* spp., *Proteus* spp., *Klebsiella* spp., (Yeruham et al., 2006; The Merck Manual, 2014). Infections are more common in cows, compared to male cattle (Andrews and Williams, 2004). The reasons are related to the anatomical features (short urethra), hormonal status (high levels of estrogen may affect the functional integrity of the epithelium in the urethra and urinary bladder), risks associated with pregnancy or iatrogenic procedures (Stevens et al., 2007)

Corynebacterium renale belong to the *Corynebacterium renale* group, genus *Corynebacterium*, family *Corynebacteriaceae* (Quin et al., 2013). The family contains a large number of ubiquitously widespread species that are commensals on skin and mucous membranes and opportunistic pathogens to humans and animals (Stevens et al., 2007). The main representative of the

species, is a highly contagious human pathogen *Corynebacterium diphtheriae.* In domestic animals, nondiphtherial *Corynebacteria* cause different infections: *Corynebacterium pseudotuberculosis* causes caseous lymphadenitis in sheep and goats; *C. pseudotuberculosis* causes ulcerative dermatitis in cattle, while *C. ulcerans* and *C. bovis* cause mastitis. According to this new classification, the *Corynebacterium renale* group contains three species (previously three types): *C. renale* (type I), *C. pilosum* (type II) and *C. cystitidis* (type III). The *C. renale* is the cause for cystitis and pyelonephritis in cows, ulcerative (enzootic) balanoposthitis in sheep and goats and osteomyelitis in goats (Markey et al., 2013; Quin et al., 2013). *C. cystitidis* causes haemorrhagic inflammation of the bladder in cows with ulceration of the mucous membrane of the bladder, urethritis and pyelonephritis. *C.pilosum* has lower virulence and the infection typically results in a less severe clinical picture or in an uncomplicated cystitis. In natural infections, pyelonephritis develop less frequently than *C. renale* (Hayashi et al., 1985).

CASE REPORT

Sample: Catheterized urine of cows (Fig. 1).

Laboratory examination: An amount of 100 mL of the urine sample was inoculated on 2 plates of Columbia blood agar base supplemented with 5% sheep blood and the MacConkey agar (CM0007,Oxoid, Basingstoke, UK).

Incubation atmosphere: The plates are incubated aerobically (blood and i MacConkey agar) and in 5-10 per cent CO2 conditions (blood agar).

Incubation temperature: 37°C

Incubation time: 48h.

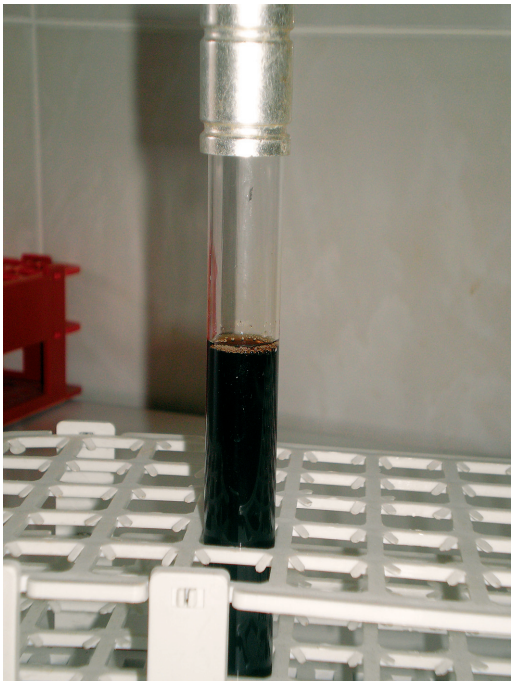

Fig. 1. Blood urine samples

Expected results: The isolation of the folowing causal agents of cow cystitis: *Escherichia coli*, *Trueperella pyogenes, Corynebacterium renale* group (*C renale*, *C cystitidis*, and *C pilosum*), *Staphylococcus* spp., *Streptococcus* spp., *Proteus* spp., *Enterobacter* spp., *Pseudomonas aeruginosa.*

RESULTS

Colonial apperance of isolates: After 24 hours of incubation under aerobic and microaerophylic conditions, a significant increase of very small (up to 1mm), non-transparent (opaque), non-haemolytic colonies were noted on the blood agar. After the incubation for 48 hours, colonies turned a pale yellow color (pigment production). There was no growth on the MacConkey agar.

Microscopic apperance: Gram stained smears reveal small, Gram-positive bacteria, short, slightly curved rods, grouped under different angles (looking like "*Chinese characters*").

Biochemical reactions: catalase test, oxidase test, urease test (Christensen urea agar), growth in broth (pH 5.4), fermentation of glucose and xylose (acid production) and aesculin hydrolysis.

Identification criteria for isolates*: a pale yellow color of colony, absence of haemolysis, absence of growth on the MacConkey agar, coryneform Gram-positive rods, oxidase negative, catalase positive, strong urease production, growth in broth at pH 5.4, glucose fermentation, non acid production from xylose and negative aesculin hydrolysis.*

Identification of the etiological agent: *Corynebacterium renale*.

Relevant data of Corynebacterium renale cystitis

Infection: *C. renale* is a common inhabitant of the vulva, vagina and prepuce of apparently normal cattle, and the occurrence of the infections contribute to certain predisposing factors. In most cases, trauma, damage to the integrity of the epithelium of the genito-urinary tract or stress (parturition, lactation peak) contributes to the infection (Quin et al., 2013; Merck Manual, 2014). These infections are therefore common in cows that have already calved as well as immediately after parturition (parturition). The stasis of urine is also an important factor of dispositions, occurring in permanent or temporary obstruction of the urinary tract due to the presence of calculi or pressure of the gravid uterus (Andrews and Williams, 2004). A diet rich in protein contributes to the development of the infection because it increases urine pH, which enhances the expression of the flagella *C. renale* and provides favorable conditions for the proliferation of bacteria (Stevens et al., 2007). The adhesion of C. renale bacteria to the epithelial cells of the bovine urinary tract is higher at pH of urine above 7.6 (Andrews and Williams, 2004).Non-sterile catheterization may contribute to the spread of infection from cow to cow.

Virulence factors: All species from the *Corynebacterium renale* group (*Corynebacterium renale, cystidis* and *pilosum*) possess fimbriae which enable the attachment to the urogenital mucosa, epithelial cells of the urinary bladder and renal pelvis (Hayashi et al., 1985; Yeruham et al., 2007; Markey et al., 2013; Quin et al., 2013). Only *C. renale* (most isolates) possess an extracellular protein that is referred to as renalin. Renalin reacts with ceramides, the integral part of the sphingomyelin cell wall of the red blood cells of mammals. Therefore, it is referred to as renalin "CAMP like" protein because it produces synergistic haemolysis on blood agar with sphingomyelinase of *Staphylococcus aureus* (beta-haemolysin). It is believed that renalin plays an important role in the lysis of the host cells (Markey et al., 2013). Enzyme urease is an important virulence factor in all three species from the *Corynebacterium renale* gruop.

The urease quickly (within 1 hour) and vigorously hydrolyze the urea, and the resulting products (such as ammonia) stimulate the mucosal inflammation.

***Clinical signs*:** Hematuria is usually the first symptom of infection, and in uncomplicated cystitis, can be the only, permanent or temporary symptom. The ascending spread of the infection results in the development of purulent pyelonephritis (inflammation of the renal parenchyma and the renal pelvis). Clinical signs of pyelonephritis include fever, anorexia, colic, frequent attempts to urinate, poliuria, pyuria, agitation, decreased milk production and anemia. In chronic infections, due to the inflammatory process, the bladder wall becomes thickened, the ureters expands and fills with purulent exudates. *Corynebacterium* spp. are pyogenic bacteria, and purulent inflammation can affect the kidneys and result in the development of multiple abscesses (The Merck Manuals, 2014). In the urinary sediment, a large number of leukocytes and bacteria has been found (Merck Manual., 2013)

Although *C. renale* is widespread and commonly present in the mucosa of the genital tract, the affected individual should be isolated of the herd, in order to prevent an increase in the number of pathogens in the environment and in the prevention of the spread of infection (Merk Manual., 2013). *C. renale* cystitis is established across Europe and North America, but the prevalence is unknown (Andrews and Williams, 2004).

Therapeutic recommendations:

Corynebacterium renale is sensitive to the majority of antibiotics, such as the penicillins, ampicillin, cephalosporins, quinolones, chloramphenicol, tetracyclines, cefuroxime and trimethoprim. The treatment of choice for pyelonephritis due to *Corynebacterium* spp is penicillin, because penicillin is excreted in the urine (e.g. 10 000-15 000 iu/kg daily for at least 10 days) (Andrews and Williams, 2004) or trimethoprim-sulfadoxine (16 mg combined/kg, IM, for ≥3 wk) (Merk Manual, 2014). Treatment should begin as early as possible, before the development of serious tissue damage.

CONCLUSION

This is also the only case of *C. renale* cystitis in cows which was etiologically confirmed in the laboratory for clinical bacteriology of the Scientific Veterinary Institute "Novi Sad" for a period of 10 years. Cow's urine samples are usually very rarely submitted to the laboratory for bacteriological exami-

nation. The assumption is that clinical cystitis in the field is treated empirically. The exact prevalence of *C. bovis* cystitis therefore remains unknown, and research of the etiology of cystitis in cows, to our knowledge, and on our epizootic area has not been implemented.

Clinically, *C. bovis* cystitis is indistinguishable from bladder urinary infections caused by other, mostly Gram-negative bacteria (eg, *Escherichia coli*). For proper treatment, etiological diagnosis is necessary, because in this case penicillin preparations are the drug of choice. The treatment is successful if starts on time, hence preventing the development of purulent pyelonephritis.

ACKNOWLEDGMENT

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LITERATURE

1. Andrews A.H. and Williams B.M.: Contagious bovine pyelonephritis, Chapter 44: Bacterial Conditions. In: Andrews A.H., Blowey R.W., Boyd H., Eddy R.G.: *Bovine Medicine*, Diseases and Husbandry of Cattle, Blackwell Publishing Company, 725-726, 2004.
2. Hayashi A., Yangawa R., Kida H.: Adhesion of *Corynebacterium renale* and *Corynebacterium pilsom* to the epithelial cells of various parts of the bovine urinary tract from renal pelvis to the vulva, Veterinary Microbiology, 10: 287–292, 1985.
3. Markey B., Leonard F., Archambault M., Cullinane A., Maguire D.: *Corynebacterium* spp. and *Rhodococcus equi,* Section 2: Bacteriology. In: *Clinical Veterinary Microbiology*, second ed., Edwards, R. and Hewat, C. (Ed.), Mosby, New York, 135-145, 2013.
4. Merck Manuals, The Merk Veterinary Manual: Infectious Diseases of the Urinary System in Large Animals, Bovine cystitis and Pyelonephritis, Last full review/revision by Peter D. Constable, October 2014. http://www.merckvetmanual.com/mvm/urinary_system/infectious_diseases_of_the_urinary_system_in_large_animals/bovine_cystitis_and_pyelonephritis.html
5. Stevens E.L., Twenhafel N.A., MacLarty A.M., Kreiselmeier N.: Corynebacterial Necrohemorrhagic Cystitis in Two Female Macaques, *Journal of the American Association for Laboratory Animal Science,* 46(6): 65-69, 2007.

6. Yeruham I, Elad D, Avidar Y, Goshen T: A herd level analysis of urinary tract infection in dairy cattle, *The Veterinary Journal,* 171:172-176, 2006
7. Quin P.J., Markey B.K., Leonard F.C., FitzPatrick E.S., Fanning S., Hartigan P.J.: *Corynebacterium* species, Section III: Pathogenic Bacteria, Chapter 17. In: *Veterinary Microbiology and Microbial Disease*, Second Edition, Wiley-Blackwell, 207-212, 2013.

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THE EPIZOOTIOLOGICAL IMPORTANCE OF *SALMONELLA* SPP. ISOLATED IN VARIOUS ASPECTS OF POULTRY PRODUCTION IN THE SOUTHERN BAČKA AND SREM REGION*

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Abstract

Salmonella causes local and systemic infections of poultry, which may lead to substantial direct and indirect economic losses, presenting also significant risk to human health. The aim of this study was to monitor the occurrence of certain serotypes of *Salmonella spp.* isolated on poultry farms in Southern Bačka and Srem regions in the period from 2010 to 2014, as recommended by the Book of rules of early detection, diagnostic, prevention of spreading and eradication of Salmonella spp. We analyzed the results obtained from the laboratory for clinical bacteriology to determine number of salmonella cases. From all samples that have been submitted for bacteriology analysis, salmonellas were isolated from 7.3% samples. *Salmonella infantis* was isolated from 50.3% of all salmonella-positive samples, mostly from materials supplied from broiler farms. *Salmonella enteritidis* was most frequently isolated in broiler chickens at the rate of 48.2%. There is an increasing trend in the occurrence of *Salmonella enteritidis* and *Salmonella infantis* on poultry farms from year to year. Our research revealed the highest incidence of salmonella isolates in broilers that died during transportation or within the first three days upon arrival of chickens. During the five-year research period, 65 samples from parent flocks (63 from broiler breeders and 2 from parent flocks of layers) were salmonella positive, which ma-

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kes 8.03% of all positive isolates. It is most likely that salmonella infection occurs due to infection of parent flocks and young chickens are infected trough both vertical and horizontal transmission.

Keywords: salmonella, broiler chickens, samples

EPIZOOTIOLOŠKI ZNAČAJ *SALMONELLA SPP.* IZOLOVANIH U RAZLIČITIM VIDOVIMA ŽIVINARSKE PROIZVODNJE U JUŽNOBAČKOM I SREMSKOM OKRUGU

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Kratak sadržaj

Salmonele kod živine izazivaju infekcije lokalnog i sistemskog karaktera, dovodeći do značajnih kako direktnih tako i indirektnih ekonomskih gubitaka u industrijskom živinarstvu i predstavljaju opasnost po zdravlje ljudi. Cilj ovog rada bio je monitoring određenih sojeva *Salmonella spp.* propisanih Pravilnikom o utvrđivanju mera za rano otkrivanje, dijagnostiku, sprečavanje širenja, suzbijanje i iskorenjivanje infekcija živine određenim serotipovima salmonella, na teritoriji Sremskog i Južnobačkog okruga u periodu od 2010. do 2014. godine. Analizirali smo rezultate ispitivanja laboratorije za kliničku bakteriologiju. Od ukupno ispitanih bakterijskih infekcija iz kliničkog materijala u ovom petogodišnjem periodu, salmonele su izolovane u 7,3% slučajeva. Ustanovljeno je da je *Salmonella infantis* izolovana u 50,3% svih pozitivnih uzoraka, najčešće iz materijala dostavljenih sa farmi brojlerskih pilića. *Salmonella enteritidis* izolovana je u 48,2%, takođe najčešće kod brojlerskih pilića. Prisutan je trend porasta izolata *Salmonella enteritidis* i *Salmonella infantis* iz godine u godinu. Našim istraživanjem ustanovili smo da je najviše pozitivnih uzoraka bilo kod brojlera, u transportnim uginućima i uginućima iz prva tri dana starosti. Kod roditeljskih jata bilo je pozitivno 65 uzoraka u ovom petogodišnjem period (63 kod

teške linije i 2 kod lake linije), što iznosi 8,03 % od svih pozitivnih izolata. Pretpostavlja se da je do infekcije salmonelama moglo doći usled horizontalne i vertikalne infekcije preko roditeljskih jata.

Ključne reči: salmonela, brojlerski pilići, uzorci

INTRODUCTION

Salmonella does not cause clinical symptoms in poultry, but seldom may lead to increased mortality usually during rearing and induce a decrease in egg production. People become infected with salmonella by consuming contaminated food. These infections may be manifested in humans through lighter or heavier clinical symptoms which may be accompanied with a long convalescing period (Dačić et al., 2004). *Salmonella* are a facultative Gram - negative aerobes, rod-shaped, and classified as the *Enterobacteriacae* family. They do not create spores and they do not encapsulate.

Salmonellas are excreted through feces, contaminating the environment so reinfections are frequent. Certain serotypes such as *Salmonell*a Enteritidis (*S*. Enteritidis) and *Salmonella* Typhimurium (*S*. Typhimurium) persist inside parenchymal organs and ovaries which can cause vertical transmission (Ilić i sar., 2010). *Salmonella* can cause embryo death and early mortality in chickens (in the first seven days of life). If the parent stock has been infected, incubators for hatching chickens may also be contaminated which may lead to a large number of hatched chickens to be infected. The process of animal feed pelleting can decrease the *Salmonella* contamination only to a certain extent therefore it is crucial to maintain cleanliness in animal feed factories. Since the *Salmonella* is so widespread and difficult to eliminate from the environment, it is on the eradication program priority list throughout the world. The goal of this research was to determine the prevalence of certain serotypes of *Salmonella* spp. in Srem and Southern Backa district from year 2010 - 2014, specified by the Book of Rules for poultry salmonellosis RS number 7/10, and to briefly highlight control, prevention measures, and goals which should be achieved in order to eradicate these persistent infections.

MATERIAL AND METHODS

During the timeline from 2010 - 2014, samples for *Salmonella* examination from the Srem and Southern Bačka district were delivered to the Veterinary

Institute in Novi Sad. The following categories of poultry flocks were included in this research:

- rearing broiler breeders and rearing layer breeder flocks
- broiler breeders and layer breeders in production
- hatcheries
- Broilers
- Turkey pullets
- Layer chickens
- Pheasants raised on farms

In this research 11.044 samples from poultry were analyzed. These samples were: parenchymatous organs, embryonated chicken eggs, unhatched eggs, mortality during transport, and paper pads. *Salmonella* spp. was isolated in a laboratory for clinical bacteriology, by using liquid media for enrichment, selective media, differential media, and for serological typing the slide aglutination test with specific serums (with poly-somatic and, flagellar antigens) was done.

The results were processed by descriptive statistics and presented in percentages.

RESULTS

During the 2010 - 2014 timeline, 11.044 samples were examined, 809 (7.32 %) tested positive for *Salmonella*. Most frequently detected were *S.* Enteritidis and *Salmonella* Infantis (*S.* Infantis). In a smaller number of samples, *S.* Typhimurium was isolated as well (Table 1).

Table 1: The number of isolates from *Salmonella* Enteritidis, Typhimurium and Infantis in poultry flocks from Southern Bačka and Srem region from 2010-2014.

Year	*Salmonella* Enteritidis	*Salmonella* Typhimurium	*Salmonella* Infantis
2010	**85**	**5**	**46**
2011	**32**	**/**	**39**
2012	**59**	**1**	**92**
2013	**89**	**2**	**124**
2014	**125**	**4**	**106**
Total	390	12	407

*values in the table represent the numbers of positive samples

The results from this research show that the number of positive *S.* Enteritidis isolates had increased in the 2011-2014 timeframe. A total of 390 samples were isolated, which is approximately 48.2% of all the positive samples. Most of the positive samples originated from broilers (244) and layers (96). The prevalence of positive samples from years 2010 to 2014 is presented in Fig 1 and 2.

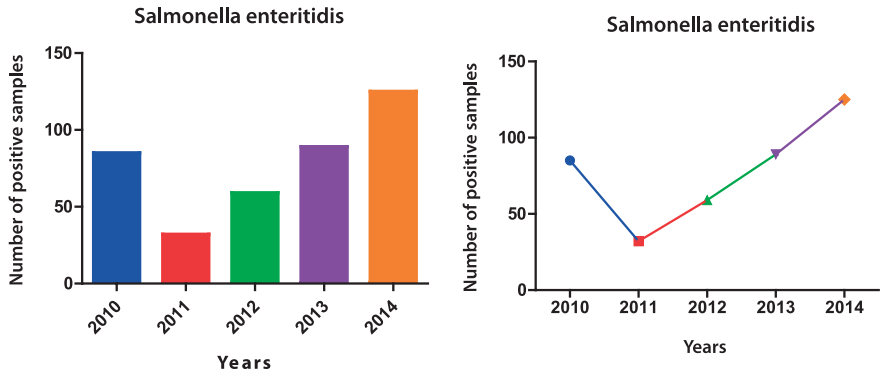

Fig 1 and 2: The prevalence of positive samples
for *S.* Enteritidis from years 2010-2014.

S. Typhimurium was isolated in 12 samples at the rate of 1.48%. During the year 2011 there were no positive cases. The prevalence of positive samples throughout the years 2010-2014, is shown in Fig 3 and 4.

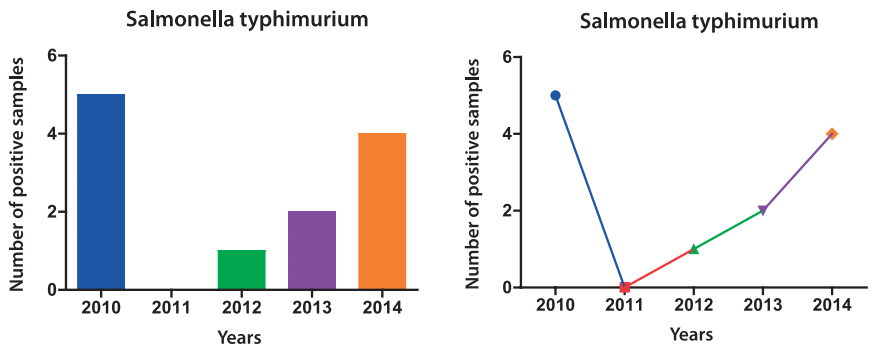

Fig 3 and 4: Prevalence of positive samples
for *S.* Typhimurium from years 2010-2014.

The number of positive isolates for *S.* Infantis was the highest in 2013 and it was isolated from 50.3 % of all the positive samples. Most of the positive samples came from broiler farms (271) and layers (81), which is similar to the occurrence of *S.* Enteritidis. The prevalence for positive samples per year is shown in Fig 5 and 6.

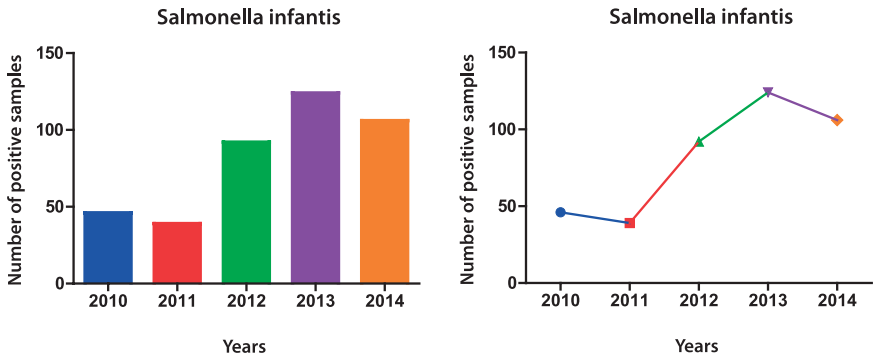

Fig 5 and 6. The prevalence of positive samples for *Salmonella* Infantis from 2010-2014.

DISCUSSION

The prevalence of *Salmonella* spp. in diagnostic materials is similar to reports from previous years (Stojanov et al., 2006). Most of the positive samples were from broilers, mortality during transportation, and mortality at three days old. From all of the positive samples, 521 were from broiler farms, which is 64.40% of the whole number of samples. Broiler breeders had 65 positive samples, which is 8.03% of all the positive samples. Sixty three positive samples were from rearing layer breeders and only two from flock of layer breeders. Samples that came from layer chickens were positive in 178 cases, which is 22.12% from the total number of *Salmonella* isolation. *Salmonella* was found in pheasants in seven samples, during this five year run, and turkey pullets had 25 positive samples.

Salmonella is isolated in high percentages in poultry flock throughout the Srem and Southern Backa district, as well as in other regions of Serbia. Stojanov et al., (2011) were examining the presence of *S.* Enteritidis and *S.* Infantis in poultry farms during 2009 and 2010. They had established that *S.* Enteritidis

was present in poultry specimens by 61.42 % in 2009 and 56.63% in 2010, in relation to the total number of positive results. During the same period, *S.* Infantis was isolated at the rate of 3% (2009) and 38.05% (2010). Our findings regarding the incidence of *Salmonella* in farms during the period from 2010-1014 are similar but the number of *S.* Infantis is increasing with regards to the period of 2010.

During the research that was done by Ilić et al., in 2010, internal organs in poultry were examined, as well as embryionated eggs and table eggs. A total of 1289 samples were examined from which 9 tested positive for *S.* Enteritidis and 9 for S. Typhimurium, showing the incidence of 0.7% (Ilic et al., 2010). Matović and his associates examined 48182 specimens from poultry during the years 2000-2005, in the Raška and Zlatibor epizootiological area. *Salmonella* spp, was diagnosed in 476 samples (0.98%). Similar to our finding *S.* Enteritidis was most often isolated (453 samples) and in 23 samples *Salmonella* of other serotypes was found. In their work *Salmonella* was isolated from fertile and table eggs, paper pads, mortality during the first three days, pheasant chicks, mortality during transport, carcasses and broiler chickens as well as layers and adult pheasants (Matovic et al., 2006).

Al-Nakhli and colleagues (1999) examined the presence of *Salmonella* spp. in poultry farms in Saudi Arabia, from 1988-1997. In their research 25.759 samples were examined and 1.052 i.e. (4%) were positive for *Salmonella*. These samples were taken from broiler farms, layer farms, rearing broiler breeders and rearing layer breeders. *S.* Enteritidis, *Salmonella* Virchow (*S.* Virchow) and *S.* Infantis were the most frequently found. Poppe et al., (1991), had isolated several types of *Salmonella* from a layer farm in Canada in 1991. They examined 295 samples, and isolated *Salmonella* Heidelberg 59/295 (20%), *S.* Infantis 18/295 (6.1 %), *Salmonella* Hadar 17/295 (5.8 %) and *Salmonella* Schvarzengrund 21/295 (7,2%). From 2007-2011 in a research performed by Rahmani et al., (2013), 36 serotypes of *Salmonella* were isolated from broiler farms which are located in three different provinces in north Iran. *S.* Infantis and *S.* Enteritidis were most frequently isolated. Lassing et al., (2012) performed a research on 363 broiler flocks with at least 5000 broilers in Austria. The sampling was done during the whole year. The *Salmonella* spp infections were registered in 28 flocks (7.7%). In six farms *S.* Enteritidis (1.7%) was isolated while in 2 flocks *S.* Typhimurium (0.6%) was found. In the remaining 20 flocks: *Salmonella* Montevideo (4,1%), S. Infantis (0,6%), *Salmonella* Senftenberg, *Salmonella* Tennessee and *S.* Virchow (0.3%) were isolated. Data shows that the risk of horizontal transmission of *Salmonella* spp. to broiler chickens is very high. The study that was conducted by Rusul and associates (1996) in

Malesia, in order to determine the prevalence of *Salmonella* in 230 samples from broiler chickens, has shown that the most prevalent are *S.* Enteritidis, *Salmonella* Muenchen, *Salmonella* Kentucky and *Salmonella* Blockley.

Salmonella are one of the most important bacteria which cause zoonoses. Worldwide, they are considered to be very significant in epidemiology, and the program for eradication is mandatory in all the EU states. In order to eliminate salmonella from the food chain it is essential to minimize the incidence of *Salmonella* in breeder flocks and in other poultry farms as well (Velhner et al., 2011). The directive, EC number 1003/2005 has required that the incidence of *S.* Enteritidis and *S.* Typhimurium as well as other significant serotypes of Salmonellas in breeder farms should not exceed an amount of ≤ 1% (EC, 1003/2005). For these reasons, *Salmonella* monitoring needs to be more comprehensive while better management practice on farms has to become one of the primary goals in the livestock industry in Serbia.

CONCLUSION

- From 2010 – 2014, we have established an increase in *S.* Infantis in poultry samples. The highest number of samples was from broiler chickens, death during transport, and mortality in the first three days of life.
- An increase in positive samples for *S.* Enteritidis was also proven for broiler farms.
- *S.* Typhimurium was isolated in a smaller percentage, 1.48% of all the positive samples.
- The highest amount of positive samples has come from broiler chickens (64.40%).
- In layers, positive samples were found in 22.12 % of samples.
- *Salmonella* Hadar and *S.* Virchow which are category 2 according to the Book of rules number 7/10, were not isolated at this time.

In order to prevent salmonella infections in chickens, it is essential to apply the following measures: to purchase chickens only from farms that are free of salmonella; avoid mixing chickens from different flocks; thermal processing of feed ingredients; to provide water not contaminated with salmonella; prevent wild birds and rodents from accessing farms; thoroughly clean and disinfect objects between poultry production cycles; disinfect by using fumigation for hatching eggs; disinfect incubators after every hatching. Farm workers need to wear protective clothing and regularly conduct personal hygiene. Treatment

with antibiotics is not recommended in poultry, unless diarrhea and fever occur, when they can be used to reduce mortality. In laying hens antibiotics are contraindicated.

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LITERATURE

1. Al-Nakhli H. M., Al-Ogaily Z. H., Nassar T. J.: Representative Salmonella serovars isolated from poultry and poultry environments in Saudi Arabia. *Revue scientifique et technique*, 18, 3, 700-9, 1999.
2. Commission regulation (EC) No 1003/2005 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in breeding flocks of Gallus gallus and amending regulation (EC) No 2160/2003. *Official Journal of the European Union 2005*, L 170/12, 01. 07. 2005.
3. Dačić M., Petković J., Simonović Lj., Račić Z.: Salmonella enteritidis in laying hens with emphasis on presence / absence of the agent in eggs. VI Episootiology Days with International Patricipation, Book of Abstracts, March 31 – April 2, 2004, Vlasinsko lake, Serbia, organized by Section of Zoonoses SVA and Depart. of Infec. Animal Disease and Bee Disease FVM, Belgrade, 2004, 159
4. Ilić Ž., Jovičić D., Žugić G., Pavlović I.: Salmonella spp. as part of the ecosystem. XII Episootiology Days with International Patricipation, Book of Abstracts, April 7-10, 2010, Oplenac, Serbia, organized by Section of Zoonoses SVA et al, 2010, 222
5. Lassnig H., Much P., Schliessnig H., Osterreicher E., Kostenzer K., Kornschober C., Köfer J.: Prevalence of Salmonella spp. in Austrian broiler flocks in the context of the EU-wide baseline survey 2005-2006. *Berl Munch Tierarztl Wochenschr*, 125, 3/4, 129-37, 2012.
6. Matović K., Žarkovic A., Tornić A., Šekler M., Debeljak Z., Vasković N., Kolarević M.: Prevalence of Salmonella in Raška and Zlatibor Epizootiologic region from 2000 to 2005. VIII Episootiology Days with International Patricipation, Book of Abstracts, April 5-8, 2006, Beograd, Serbia, organized by Section of Zoonoses SVA and Depart. of Infec. Animal Disease and

Bee Disease FVM, Belgrade and Scientific Institut of Veterinary Medicine, Novi Sad, 2006, 127.
7. Poppe C., Irwin R. J., Forsberg C. M., Clarke R. C., Oggel J.: The prevalence of Salmonella enteritidis and other Salmonella spp. among Canadian registered commercial layer flocks. *Epidemiology & Infection* , 106, 2, 259–270, 1991.
8. Pravilnik o utvrđivanju mera za rano otkrivanje, dijagnostiku, sprečavanje širenja, suzbijanje i iskorenjivanje infekcija živine određenim serotipovima salmonella. „Službeni glasnik Republike Srbije", broj 7/10, od 19. februara 2010.
9. Rahmani M., Peighambari S. M., Svendsen C. A., Cavaco L.M., Agersø Y., Hendriksen R. S.: Molecular clonality and antimicrobial resistance in Salmonella enterica serovars Enteritidis and Infantis from broilers in three Northern regions of Iran. *BMC Veterinary Research*, 9:66, 2013.
10. Rusul G., Khair J., Radu S., Cheah C. T., Yassin R. M.: Prevalence of Salmonella in broilers at retail outlets, processing plants and farms in Malaysia. *International Journal of Food Microbiology*, 33, 2/3, 183-94, 1996.
11. Stojanov I., Orlić D., Došen R., Prodanov J., Velhner M.: Finding of Salmonella in materials originating from Poultry and Pigs during 2002-2005. VIII Episootiology Days with International Patricipation, Book of Abstracts, April 5-8, 2006, Beograd, Serbia, organized by Section of Zoonoses SVA and Depart. of Infec. Animal Disease and Bee Disease FVM, Belgrade and Scientific Institute of Veterinary Medicine, Novi Sad, 2006, 141-142.
12. Stojanov I., Potkonjak D., Kapetanov M., Ratajac R., Maljković M., Pušić I., Jovičin M.: Changes in prevalence of some Salmonella serotypes in materials originating from poultry. First International Epizootiology Days, Book of Abstracts, April 6-9, 2011, Sijarinska spa, Serbia, organized by Section of Zoonoses SVA et al, 2011, 86.
13. Velhner M., Potkonjak D., Stojanov I., Petrović J., Kozoderović G.: Salmonella control in poultry production and resistance monitoring in Serbia. *Arhiv Veterinarske Medicine*, 4, 2, 11-22, 2011.

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