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SEASONAL DYNAMICS OF THE PRESENCE OF CULICOIDES SPP. IN SERBIA IN THE PERIOD 2015-2016

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Abstract

Genus *Culicoides spp*. includes small insects 0.5-2 mm in length, usually grey or black and at first glance very similar to mosquitoes. They are strictly hematophagous, feeding by attacking hosts outdoors and indoors (although they are less susceptible to the stationing like *Aedes* mosquitoes do). The sting is very painful at the injection site and often followed by hypersensibilisation with the consequent formation of allergic dermatitis. In addition, the insects carry and transmit a multitude of diseases, often of a zoonotic character, and therefore are of great epidemiological importance. In our country, continuous monitoring of *Culicolides spp*. has been carried out and seasonal dynamics of their appearance in the period 2015-2016 is presented in this article. During October 2015, the presence of *Culicolides* spp. was confirmed in 10.00% of samples; in November, their presence was not established, whereas in December, 2.35% of samples proved positive for the presence of *Culicolides* spp. During 2016, from January to March, no *Culicolides spp*. were found in any of the examined samples. During April, their prevalence was 9.63%, in May - 6.74%, in June - 3.70%, in July - 15.78%, in August - 18.07%, in September - 27.27%, and in October - 45.65%. In Serbia, the dominant *Culicoides spp*. species are Obsoletus complex and Pulicaris complex established in 57.21% and 33.37% of samples, respectively. Other species are present in lesser extent. In Obsoletus complex, the dominant species was *Culicoides obsoletus/scoticus*. The percentage of *Culicoides obsoletus /scoticus* males in samples was 25.52%. Non-pigmented (young)

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females were present in 66.06% of samples; females who took blood in 7.55% and 0.87% were gravid females. In Pulicaris complex, the dominant species was *Culicoides pulicaris*. Males of *Culicoides pulicaris* were found in 19.23% of samples, non-pigmented (young) females in 70.96%, females who took blood in 9.08% while 0.73% were gravid females.

Key words: *Culicoides spp*., Seasonal dynamics, Serbia

SEZONSKA DINAMIKA PRISUSTVA CULICOIDES SPP. U SRBIJI U PERIODU 2015-2016. GODINE

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Kratak sadržaj

Rod *Culicoides* spp. su mali insekti veličine 0,5-2 mm, većinom sivkaste ili crne boje i na prvi pogled veoma slični komarcima. Oni su striktne hematofage. Ubod je veoma bolan, a mesta uboda su često hipersenzibilisana uz posledično nastajanje alergijskih dermatitisa. Uz to oni prenose mnoštvo oboljenja često zoonotskog karaktera tako da su od izuzetnog epidemiološkog značaja. U našoj zemlji se vrši stalni monitoring Culicolides-a i ovde dajemo prikaz sezonske dinamike njihovog pojavljivanja u periodu 2015-2016.godine. Tokom 2015. godine u oktobru je njihovo prisustvo ustanovljeno u 10.,00% uzoraka, u novembru ih nije bilo, a tokom decembra su bili prisutni u 2,35%. Tokom 2016. godine tokom januara, februara i marta nisu nađeni ni u jednom prispelom uzorku. Tokom aprila meseca njihova prevalencija je iznosila 9,63%, u maju je bila 6,74%, u junu 3,70%, u julu 15,78%, u avgustu 18,07%, u septembru 27,27% i u oktobru 45,65%. U Srbiji su dominantne vrste *Culicoides* spp. iz Obsoletus kompleksa i ustanovljeni su u 57,21%. *Culicoides* spp. iz Pulicaris kompleksa ustanovljeni su 33,37% dok su ostale vrste manje zastupljene. Kod Culicoides spp. iz Obsoletus kompleksa dominantna vrsta je bila *Culicoides*

obsoletus/scoticus. Mužjaci su nađeni u 25,52%, nepigmentisane (mlade) ženke u 66,06%, ženke koje su uzele krv u 7,55%, a 0,87% su bile gravidne ženke. Kod *Culicoides* spp. iz Pulicaris kompleksa dominantna vrsta je bila *Culicoides pulicaris*. Mužjaci su nađeni u 19,23%, nepigmentisane (mlade) ženke u 70,96%, ženke koje su uzele krv u 9,08%, a 0,73% su bile gravidne ženke.

Ključne reči: *Culicoides spp*., sezonska dinamika, Srbija

INTRODUCTION

Culicoides spp. are small insects whose females sting and suck blood (Blackwell, 2009; Pavlović et al., 2009). The first report of these insects dates back from 1731, when the priest, naturalist and philosopher William Derham (1657-1735) described their biological cycle and gave details of their stings (Pavlović, 2016a). *Culicoides spp.* currently contains 1343 existant and 44 extinct species, representing the largest genus of the *Ceratopogonidae* and comprising 21.5% of all Ceratopogonid species (Borkent, 2014).

Epidemiological importance of *Culicoides spp.* was described in 1944 by Rene du Toit from ARC - Onderstepoort Veterinary Institute, who believed that these insects can play an important role in the transmission and spread of viruses that cause animal diseases such as bluetongue and acute allergic dermatitis in horses (Meiswinkel et al. 2008). Later, the genus received considerable attention through the role of several species as biological vectors of pathogens of medical and veterinary importance. In addition to several nematode and protozoan species, over 50 arboviruses have been isolated from species of *Culicoide*s and their role in the transmission of veterinary and human pathogens has been reviewed (Blackwell, 2001; Pavlović et al., 2002; Borkent, 2004).

Genus *Culicoides* has not been investigated in Serbia, thus, there were conflicting opinions about its presence in our region. It was only with the emergence of Bluetongue disease in 2006, when research of these insects begin to gain importance, leading to the first survey aimed at determining the presence and extent of these insects. The studies carried out in Serbia during 2006-2007 confirmed the presence of these insects in our region. Later studies conducted from 2011 to 2012 enabled the determination of Culicoides types in Serbia (Rajković et al., 2009; Pavlović et al, 2009, 2014; Pavlović, 2016b). Finally, the research performed in 2014, after the re-emergence of Bluetongue disease, revealed that this type of insects became widespread and covers the entire area

of Serbia as was expected after the results of previous investigation. Since then, continuous epidemiological monitoring of these insects in the entire territory of Serbia has been performed with an aim of establishing their biodiversity, spread, abundance and seasonal dynamics.

In our paper, we presented results of examination of biodiversity and seasonal dynamics of *Culicoides spp.* in Serbia in the period 2015-2016.

MATERIAL AND METHODS

Based on the instructions of Veterinary Directorate on performing entomological and virological tests for the monitoring and surveillance of Bluetongue disease (BTD) in the Republic of Serbia No. 323-02-7461/2015-05 dated 14/09/2015 in the period from 01/10/2015 to 30/09/2016 entomological tests were carried out in order to control Bluetongue disease.

In the period from 01/10/2015 to 31/10/2016, a total of 775 entomological check-ups were made. *Culicodes spp.* samples were collected from the epizootiological areas such as Belgrade – 36 samples, Šabac - 61, Zaječar and Jagodina - 66, Požarevac - 96, Kraljevo - 112, and Niš - 227 samples. In Vojvodina Province, samples of Culicodes spp were collected from several epizootical areas, i.e., Novi Sad -29 samples, Zrenjanin - 8, Sombor - 24, Pančevo - 45 and Subotica - 71.

Determination of *Culicodes spp* insects was made by morphometric method recommended by the Italian National Reference Centre for Exotic Diseases (National Reference Centre for the study of Exotic Animal Diseases (CESME) Reference Laboratory for Bluetongue OIE, Istituto Sperimentale Zooprofilattico dell'Abruzzo e del Molise "G. Caporale" (IZSAM) from Teramo, Italy. Species definition of *Culicoides spp.* has traditionally been based on the morphology of adult insects. Adult individuals of *Culicoides spp.* are notable for their characteristic wing pigmentation pattern and distribution of wing microtrichia, which in certain species can be used as the principle diagnostic feature. In practice, however, the requirement is that specimens should be slide mounted, image-captured, measured and analysed which is time consuming and therefore the use of morphometrics for identification purposes in high-throughput systems such as surveillance programs is recommended (Weeks et al., 1999).

RESULTS

Of the total number of insect samples, the presence of *Culicoides* spp. was established in 11.22% (87/775). In the epizootiological area of Belgrade, the

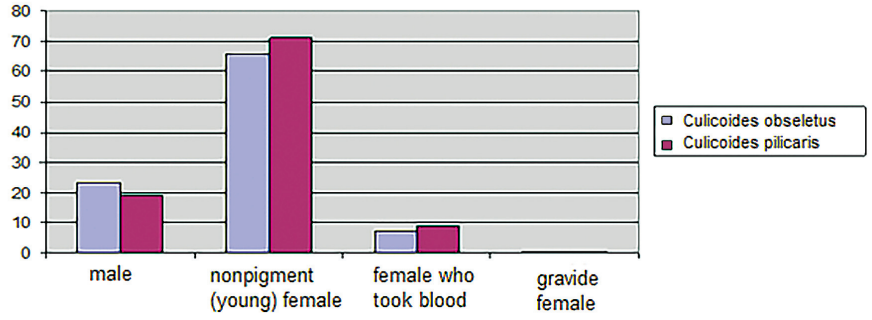
presence of *Culicoides spp.* was established in 8.33% (3/36) samples, Požarevac 1.04% (1/96), Kraljevo 2.67% (3/112), Jagodina 6.06% (4/66), Niš 11.89% (27/227), Zaječar 15.15 % (10/66) and Šabac 31.14% (19/61).

In the area of Vojvodina, in the epizootical area of Novi Sad, the presence *Culicoides spp.* was established in 13.79% (04/29) of samples, Pancevo 13.3% (6/45), Subotica 11.26% (8/71) and Sombor 8.33% (2/24). In the epizootiological area of Zrenjanin, the presence of *Culicoides spp.* was not established (0/8).

During this research, *Culicoides spp.* from Obsoletus complex were detected in 57.21% of the total catch and the dominant species was *Culicoides obseletus/scoticus*. Of the total population, male individuals made 25.52%, non-pigmented (young) females 66.06%; females who took the blood 7.55% and 0.87% were gravid females (Figure 1).

Pulicaris complex was established in 33.37% of total catch and the dominant species was *Culicoides pulicaris*. Males made 19.23%, non-pigmented (young) females 70.96%, females who took the blood 9.8% of the population, whereas 0.73% were gravid females (Figure 1).

Figure 1. The relationship between gender and stages of female *C. obsoletus/scoticus* and *C. Pulicaris*

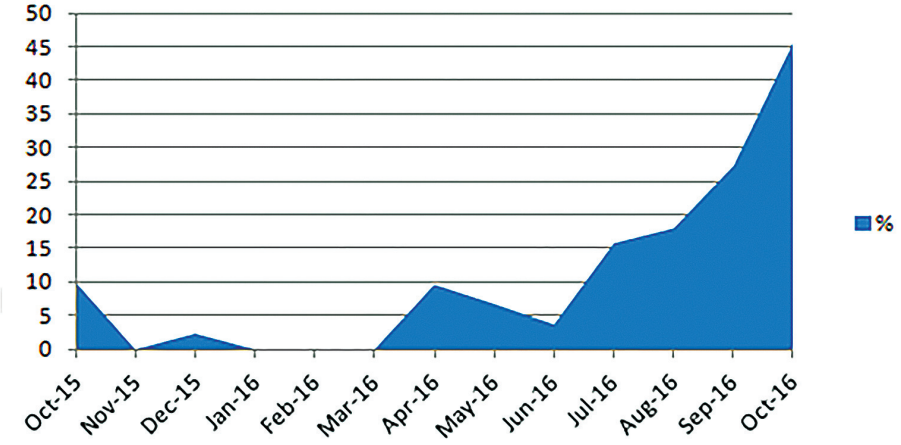


The ratio of gender and stages of female *Culicoides spp*. from Obsoletus complex (*Culicoides obsoletus/scoticus*) and Pulicaris complex (*Culicoides pulicaris*) are shown in Figure 1. Other types of Culicoides are set up in less than 10% of the examined samples (9.42%).

In this paper, seasonal dynamics of the presence of *Culicoides spp*. is monitored during the one-year period. During 2015, in October, their presence is established in 10.00% (4/40) samples, in November there were not any positive findings, and in December the insects were present in 2.35% (2/85) of samples.

During 2016, in January, February and March, *Culicoides spp*. were detected in neither of samples. During April, insects were present in 9.63% (8/83), in May in 6.74% (6/89), in June in 3.70% (2/54), in July in 15.78% (9/57), in August in 18.07% (15/83), in September in 27.27% (18/66), and in October in 45.65% (21/46) of samples. Results seasonal dynamics of occurrence *Culicoides spp.* are shown in Figure 2.

Figure 2. Seasonal dynamic of *Culicoides spp*. in the period October 2015-October 2016



DISCUSSION

Genus *Culicoides spp.* belongs to the class *Insecta*, order *Diptera*, family *Ceratopogonidae*, subfamily *Ceratopogoninae* and genus Culicoides. These are small insects the size of 0.5-2 mm, usually grey or black and at first glance very similar to mosquitoes. Nevertheless, they are morphologically different from mosquitoes and *Simulidae* by long antennae with 13-14 segments and Palpae, which have 3-4 segments. The dorsal side of the insect has a protrusion similar to *Simulidae*, width of the body and mild elongation which are clear morphological characteristics but which can also cause confusion in determination (Rawlings, 1996). The most obvious difference from the genera *Aedes, Culex* and *Phlebotomus* is in the wings with characteristically spotted pattern (Pavlović, 2009).

Systematics and taxonomy of this genus is still confusing and there are many subgenera and species which are not in the most accurate taxonomic sites (Blackwell, 2009). The current sub-generic classification of *Culicoides* consists of 31 subgenera containing 63% of existent species, 38 unplaced groups of species containing 24% of existent species and a further 13% of existent species that are not placed into any of these groupings by now (Blackwell, 2009; Bosnić, 2011; Pavlović, 2016a).

The entire genus *Culicoides* are strictly hematophagous and attack their hosts outdoors and indoors (although they are less susceptible to the stationing like Aedes mosquitoes are). They use an attractant to locate the host. One of the most important characteristics of hosts is carbon dioxide emission. As vertebrates breathe, the air of carbon dioxide is released and stimulates female *Culicoides* to fly upwind to the source of carbon dioxide. They are most active at sunset and in case of strong infestation and favourable weather conditions they attack even during the day. Female *Culicoides* feed on a wide range of hosts including reptiles, mammals, birds, humans, and even blood from fed mosquitoes. Southern *Culicoides spp.* prefer to feed on the blood of some animals species, mostly in Europe, they are known for their habit biting humans (Blackwell, 2001). They pose a serious threat to humans in certain parts of the world due to their ability to transmit deadly human diseases and some researchers tend to consider them cause of two of the ten biblical plagues of ancient Egypt.

Studies of the ecology of adult *Culicoides* is primarily focused on two areas: seasonal occurrence and feeding pattern. Many species reach their peak population in spring and summer months in moderate temperature regions and some species occur constantly during the year. Some species have two population peaks so that the first peak population is in the spring followed by a second peak in the autumn (Conte et al, 2007; De Liberato et al., 2010). Primary ecological factors affecting the *Culicoides* include rain, temperature and relative humidity, insolation, vegetation composition and pedological soil composition. This is why in many parts of the world insects of this genus occur seasonally. The air temperature in part affects the seasonal fluctuation of the population of some species of *Culicoides* and rain or other sources of water are crucial for the development of immature stages (Ducheyne et al., 2006, Hendrickx et al., 2006).

It is believed that rain is the most influential factor for the occurrence *Culicoides*, which are vector of BTD virus. For example, in Australia, it has been observed that the vector of BTD virus is prevalent usually in border areas where the levels of precipitation during rainy seasons are over 700 to 800 mm

(Meiswinkel et al., 2008; Pavlović et al., 2016). The same is observed in Europe where similar climatic conditions exist - in some parts of Italy and the Mediterranean (Conte et al, 2007; De Liberato et al., 2010).

The influence of climatic conditions is also observed in the Western Balkans (Croatia, Serbia, and Bulgaria) during the outbreak of the BTD in recent years (Bosnić, 2011; Maksimović-Zorić et al., 2016; Pavlović et al., 2016b). Unlike Mediterranean conditions, where *C. immicola* is most present and abundant species, continental climate contributed to the prevalence of species of Culicoides obsoletus Complex followed by Pulicaris Complex (Ducheyne et al., 2006; Hendrickx et al, 2006).

CONCLUSION

The *Culicoides spp.* are present in Serbia and they occur regularly throughout entire territory with high prevalence from June to October. The most abundant species is *C. obsoletus/scoticus*. The global environmental factors play a key role in expanding and changing biodiversity of insects of the genus *Culicoides* and therefore, given the exceptional vector potential of these small but dangerous insects, the epidemiological situation in the world has to be monitored.

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INCIDENCE OF HYDROMETRA IN GOATS AND THERAPEUTIC EFFECTS

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Abstract

The presence of hydrometra was analyzed on five dairy goat farms (one Saanen and four Alpine) in a period of one to three years, from 2013 to 2016. Total of 3,355 goats were scanned by ultrasound for pregnancy diagnosis after breeding season or out-of-season upon hormonal synchronization. Overall incidence of hydrometra was 1.37%. One farm of Saanen goats demonstrated statistically higher incidence of hydro/pyometra as compared to other four Alpine farms (3.25% vs. 0.56%; $p < 0.001$). Seasonal synchronization of anestric goats and occasional out-of-season synchronization in nulliparous Saanen goats probably contributed to higher incidence of this pathological condition (7/67; 10.45%), thus leaving unclear if this results can be attributed to breed affinity and/or to intensive reproductive management. Treatment with double prostaglandin injections and antibiotic treatment of hydrometra/pyometra resulted in relatively good conception rate of 64% (16/25). In conclusion, regular ultrasonography 40-70 days after mating or insemination is of crucial importance for intensive farm reproductive management on large dairy goat farms. After the treatment, significant percentage of goats with hydrometra can be successfully rebred.

Key words: goat, reproduction, hydrometra.

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ZASTUPLJENOST HIDROMETRE KOD KOZA I EFEKTI TRETMANA

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Kratak sadržaj

Prisustvo hidrometra je analizirano na 5 farmi mlečnih koza u periodu od jedne do tri godine. Ukupno 3.355 koza je skenirano ultrazvukom radi detekcije graviditeta nakon sezone parenja ili van sezone, nakon hormonske sinhronizacije. Ukupna učestalost hidrometra iznosila je 1,37%. Jedna farma sanskih koza je pokazala statistički veću učestalost hidro/piometre u odnosu na ostale 4 Alpina farme (3,25% : 0,56%; $p < 0.001$). Sezonska sinhronizacija anestričnih koza i povremena vansezonska sinhronizacija nuliparih jarica verovatno je doprinela većoj učestalosti ovog patološkog stanja (7/67; 10,45%), ostavljajući neodređenim da li se ovi rezultati mogu pripisati rasnoj sklonosti ili/i intenzivnom sistemu upravljanja reprodukcijom.Tretman sa dvostrukim injekcijama prostaglandina i antibiotika kod hidrometri/piometri doveo je do relativno dobre stope koncepcije od 64,00% (16/25). Kao zaključak, redovni ultrazvučni pregled sa 40-70 dana nakon parenja ili oplodnje je od presudnog značaja za intenzivni reproduktivni menadžment u velikim farmama muznih koza. Nakon tretmana, značajan deo koza sa nalazom hidrometre može se uspešno upariti.

Ključne reči: koza, reprodukcija, hidrometra

INTRODUCTION

Hydrometra is an important pathological condition in goats and represents one of the main causes of temporary infertility. It occurs mainly in dairy goats and is characterized by excessive accumulation of sterile fluid in the uterus in the absence of fetuses and placentomes associated with persistent corpus luteum (Hesselink, 1993; Wittek et al., 1998). By ultrasonography, it can be seen as non-echogenic fluid compartments separated by thin tissue wallstrabecules (Hesselink and Taverne, 1994). Although Smith (1980) proposed a difference between pseudopregnancy and hydrometra, most authors consider them synonyms (Pieterse and Taverne, 1986; Martel, 2001).

In the pathogenesis of this condition, the presence of fluid is the result, but not the cause of a prolonged progesterone secretion (Taverne et al., 1988), probably due to a failure in the luteolytic mechanism.

According to Brice et al. (2003), two important mechanisms are needed to establish hydrometra: 1) spontaneous persistence of the corpus luteum (CL) after an ovulation without fertilization, and 2) the persistence of the CL after an early embryonic mortality.

According to Chemineau et al. (1999), at least 50% of the cases of hydrometra in goats occur as a consequence of embryo mortality that apparently takes place at a gestational age of about 40 days (Wittek et al., 1998). Also, it could be linked to indiscriminate use of hormones or mating outside the breeding season (Pugh, 2002). However, this can also be observed in goats with spontaneous or synchronized ovulation, even if they have not been mated (Pieterse and Taverne, 1986; Wittek et al., 1998). Nonetheless, Hesselink and Elvin (1996) identified a genetic influence on its occurrence.

MATERIALS AND METHODS

In this study, a collection of ultrasound pregnancy control results obtained from five commercial goat milk farms from different locations in the Republic of Serbia in the period 2013-2016 were analyzed in order to determine the incidence of hydrometra. One farm was located in central part of the country, at a latitude 800 meters above sea level. Other farms were located in the flatland, i.e., northern part of Serbia. The goats were kept in an intensive system, in stalls, and fed and milked twice daily. Average production on these five farms ranged from 500 to 850 liters of milk in 280 days of lactation. The animals were between 1 and 8 years of age, mostly with good body condition score (BCS) of 3.5 out of 5. They were fed various available forages: corn silage, Lucerne

haylage, hay, soy and wheat straw, concentrates with different balanced formulations fed according to milk production. Drinking water was available *ad libitum*.

Annual buck fertility control was done before breeding season on two of these farms including clinical tests and semen control after electro-ejaculation. Semen was evaluated by CASA method, flow cytometry (viability test and sperm chromatin structure assay) and cyto-morphology. The number of goats per buck was determined according to breeding value, and then adjusted to scrotal circumference, body condition and semen quality.

Picture1. Portable ultrasounds facilitate and speed up scanning practice. About 100 animals can be scanned in an hour.

Picture 2. Goats presented to ultrasound check: left pregnant goat with swollen vulva after injury; goat in the middle characterized by dirty tail (vaginal/uterine exudate) are often recognized indicating barren goats with reproductive problems.

All examinations were performed applying a B-mode transrectal ultrasonographic scanner with 5MHz transducer (WED 3000 Vet Palm hand held veterinary animal ultrasound scanner, WELD, China), 1.5 months after the end of the breeding period. Goats were scanned to determine pregnancy, number of fetuses and its approximate gestation length if no farm-data records were available or to confirm correctness of hand mating dates. At ultrasound control, the does were kept in a standing position. Additional check was done by transrectal scan if no fetuses were diagnosed after transabdominal scanning. Fecal pellets were removed manually (with a gloved, gel-lubricated fingers). The tip of the probe was protruded into the rectum supported with index finger, then pushed through the entire its length and left-right rotation was done to widescan intrapelvic area. Using the rectal control method, pregnancy can be detected as early as 20 days. Up to 5% of early pregnancies not diagnosed

at transabdominal check can be detected after transrectal ultrascanning, thus increasing precision of pregnancy diagnosis.

The majority of ultrasound checks for pregnancy were performed in the period November-January (after natural seasonal breeding) or during the May-June period (for out-of-season breeding).

After control, unpregnant animals were segregated according to diagnosed problems and management decision:

- Old goats (≥ 6 lactations) with lower production results or with doubtful prognosis, requiring long recovery period were culled. This group also included goats with udder problems (limps on udders because of non-differential *Corynebacterium* infection, inactive mammary glands), bad teeth, lameness, etc.
- Anestric goats were stated as dominant problem (according to strict evidence of hand mating system). The percentage of goats diagnosed at ultrasound check as non-pregnant without obvious reason was 5-7% of all animals tested;
- Hydrometra was the most prominent reproductive diagnosis among all reproductive disorders visible at ultrascan;
- Cystic ovarian disease or dead fetus (no heart contraction, undulating membranes with flakes) were rare conditions

The protocol for hydrometra treatment included injection of Cloprostenol at the dose of 250 μg each, intramuscularly (IM), twice at 11-12 days intervals. Supporting therapy consisted of three injections, two days apart, of Baytril® Max (100 mg/ml Enrofloxacin, KVP Pharma, Germany) for hydro-myxo-pyometra and one 5 ml shot of AD_3E vitamin for all other treated-unpregnant animals.

Anestric goats have been vitaminized 10 days before synchronized with 30 mg flugestone acetate polyurethane vaginal sponges (Synchro-part, Ceva, France) for 11 days. At day 9 after sponging, 400 IU PMSG (Sugonal, VZ Subotica, Serbia) together with 5 mg dinoprost (Enzaprost, Ceva, France) were applied i/m, 48 hours before sponge removal. Bucks were joined to goats 42 hours after sponge removal and left in group for the next two days.

After the treatment and rebreed, one more ultrasound check was done to confirm the effects of proposed therapy and repeated mating.

Chi-square test was performed to determine statistical significance of the difference between hydrometra incidence in Alpine and Saanen goats.

RESULTS

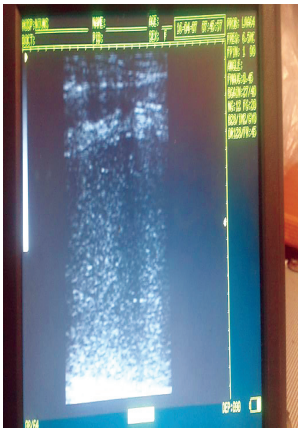
After routine pregnancy diagnosis of 3,355 scanned goats, 46 animals were diagnosed with hydrometra (incidence of 1.37%). One farm of Saanen goats had statistically higher incidence of hydro/pyometra complex as compared to other four Alpine farms (3.25% vs. 0.56%; *p <0.001)* (Table 1).

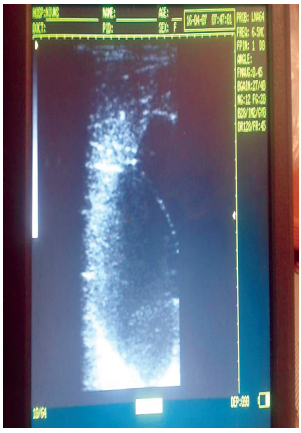
Table 1. Description of farm breeds, origin, number of scanned and hydrometra "positive" animals

Farm No.	Farm name	Breed	Origin of goats	No. of scanned goats	No. of goats with hydrometra	(%)
1.	Farm "A" 2016	Alpine	Domestic	320	2	0.63%
2.	Farm "B" 2016	Alpine	Direct import (France)	750	3	0.40%
	Farm "B" 2015			708	4	0.56%
3.	Farm "C" 2015	Alpine	Domestic	210	2	0.95%
4.	Farm "D" 2015	Alpine	Direct import (France)	140	1	0.71%
- Total for Alpine goats:				***2,128***	***12***	***0.56%***
5.	Farm "E" 2016	Saanen	Direct import (Austria)	365	6	1.64%
	Farm "E" 2015			279	6	2.15%
	Farm "E" 2014 multiparous			237	4	1.69%
	Farm "E" 2014 nulliparous			86	8	9.30%
	Farm "E" 2013			260	10	3.85%
- Total for Saanen goats:				***862***	***28***	***3.25%***
TOTAL for ALL GOATS:				**3,355**	**46**	**1.37%**

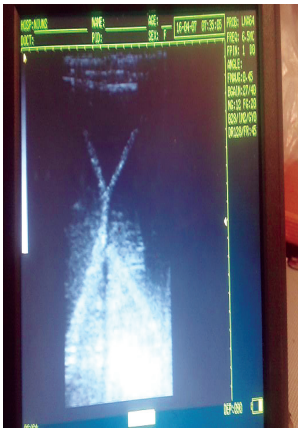
As presented in Table 1, the incidence of hydrometra ranged from 0.40% to 0.95% in Alpine goats and 1.64% to 9.30% in Saanen goats. The highest percentage of hydrometra incidence in Alpine goats was observed in 2015, while on Saanen goat farm nulliparous does had highest hydrometra percentage in 2014.

Characteristic of hydrometra at scanning was evident by absence of cotyledons, hypo-echogenic fluid accumulation, and straight nets-like hyper-echogenic membranes, undulating in this fluid, with clear or turbid flakes that are slowly circling like clouds (*Pictures 3-5.*).





Picture 3 and 4. Pseudopregnancy (*hydrometra*); the uterine wall is thin, tensed with clear or slightly turbid fluid, absence of caruncles on fetal membranes.



Picture 5. Pseudopregnancy (*pyometra*); distinctly turbid fluid in uterus.

Treatment with double prostaglandin injections and antibiotic treatment of hydrometra/pyometra resulted in the conception rate of 64% (16/25) (*Table 2*).

Table 2. Results of prostaglandin + antibiotic treatment in animals with hydrometra

Treatment	No. of hydrometra cases	No. of treated animals	Conception rate, %
Prostaglandin +antibiotic	40	25	64% (16/25)

At the end of breeding season, overall annual conception rate is achieved in 88-90% of goats (ranging from 79.32% to 95.66%).

DISCUSSION

The diagnosis of hydrometra and other genital conditions in unpregnant animals (cystic ovarian disease, enlarged uterus indicating on endometritis)

can be routinely determined by ultra sound scanning by experienced practitioner. Animals manifested with fluid in the uterine lumen in the absence of fetuses and placentomes were diagnosed with hydrometra. This condition was recognized by hypo-echoic fluid accumulation. Often, no cases of fetus skeleton were diagnosed by ultra scanning. More frequently, we noticed hyperechogenic mass on the bottom of uterus, if any other structure that indicate the presence of a dead fetus.

The prevalence of pseudopregnancy widely ranges by different authors. Lyngset (1968) reported three cases after examination of 1,020 reproductive tracts at a slaughter house (0.3%). Holdsworth and Davis (1978) found four animals after 98 checks (4%). A large investigation in France that included over 10 000 she-goats revealed the incidence of the disorder ranging between 2 and 3% of the females and spread over >55% of the flocks. However, the incidence was >5% in 11% of flocks. Significantly higher number of pseudopregnancy cases was observed in out-of-season goats after kidding in autumn and in goats subjected to hormonal treatments for synchronization of estrus (Duquesnel et al., 1992).

Hesselink (1993) found up to 9% of pseudo-pregnancies on 3 farms in Netherlands, and in some A.I. groups, pseudo-pregnancy may reach even 20.8%. Lopes Junior et al. (2004) reported an incidence of up to 30% in Saanen dairy goats raised in Northeast Brazil. Similar to our investigation, Wittek et al. (1997) scanned a larger group of animals (2,434 goats) using transrectal ultrasonography throughout the period of three years. The mean incidence of hydrometra was determined to be 5.78%

In our study, an overall incidence of hydrometra was 1.37%, which is lower as compared with the majority of authors particularly when speaking of Alpino milking goats, where the number of scanned animals is more relevant as it included data from 4 different farms with 2,128 checked animals and only 12 cases of hydrometra (0.56%).

Higher incidence in Saanen breed can be expected to be related to genetic influence (breed) compared to Alpino herds (3.25% compared to 0.56%, respectively), as this is diagnosed almost 6 times more often. Breed affinity is discussed to some extent by Duquesnel et al. (1992) in a study on a large animal population after 2 consecutive years, yet without any specific conclusion. This can also be attributed to intensive farm management within breeding season or out-of-season hormonal synchronization and keeping older animals in stalls for genetic reason.

As described in the article of Milovanovic et al. (2016), a group of 47 nulliparous Saanen goats that did not show signs of heat were subjected to hor-

monal synchronization and 37 were mated, resulting with 27 pregnant goats (pregnancy rate of 57.45%); however, the presence of high percentage of hydrometra and pyometra complex in nulliparous goats in this study was obvious (7 (10.45%) goats were diagnosed with hydro/pyo-metra at pregnancy check). Ultrasound check of goats was not performed before sponging or breeding season, so, initial prevalence of pseudopregnancy remained unknown.

Hormonal treatment protocol with double prostaglandin injections 11 days apart and antibiotic treatment of hydrometra/pyometra complex was satisfactory therapy and resulted in conception rate of 64.00% (16/25). Salles and Araújo (2008) treated dairy goats with a single dose of PGF and demonstrated that it was sufficient to induce uterus drainage in all animals. Moraes et al. (2007) observed that all female goats (n = 11) diagnosed with hydrometra and treated with PGF showed estrus within 120 h, were mated and were positive for pregnancy after 30 days. Conversely, goats treated with PGF and then subjected to artificial insemination had an average fertility rate of 48%, that is, lower than 73% obtained in goats without hydrometra in the same herds (Leboeuf et al., 1998). Hesselink (1993) cited that reproductive performance improves when a second treatment is applied. After the first PGF, only 3 out of 20 does conceived but, after the second dose administered 12 days later, 14 out of 29 became pregnant. It is believed that if the disorder is maintained for a long time it could irrevocably damage the endometrium, possibly altering uterus capacity for hormone secretion and leading to subfertility in affected goats. The possibility of using consecutive services with higher interval from the end of the treatment could improve pregnancy rate (Wittek et al., 1997). In the article of Reddy et al. (2014), a total of 20 hydrometra cases were diagnosed, while 18/20 goats exhibited estrus within 2-5 days after second injection of Cloprostenol, and 14 out of 18 estrus goats become pregnant after breeding.

Spontaneous recovery is possible (after hormonal treatment, high producing goats that were not pregnant in this year got pregnant in the next breeding season), if the farmers are willing to keep them without milking for the next breeding cycle (aimed at obtaining progeny from mothers with good milking genetics). Four goats spontaneously recovered in the next breeding season and got pregnant.

CONCLUSION

Regular ultrasonography is of crucial importance for intensive farm reproductive management in large dairy goat farming.

Average incidence on of hydrometra on five farms in a period of one to

three years was 1.37% after scanning 3,355 goats for pregnancy diagnosis after breeding season or out-of-season hormonal synchronization.

Six-time higher incidence was observed on a farm of Saanen goats as compared with the other four Alpine goat farms (3.25% vs. 0.56%), but this can be attributed also to intensive farm management with synchronization and keeping older animals in stalls for genetic reasons.

Out-of-season synchronization in primiparous goats probably leads to higher incidence of this pathologic condition.

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USUTU VIRUS: AN EMERGING FLAVIVIRUS IN EUROPE

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Abstract

Among various arthropod-borne viruses (arboviruses), the flaviviruses stand out with regard to their number, geographic distribution and importance in both human and veterinary medicine. West Nile virus is flavivirus, present endemic in many European countries as well as in Serbia where it circulates in horses, birds, humans and mosquitoes. Usutu virus (USUV) is flavivirus morphologically, antigenically, genetically and ecologically very similar to WNV, which circulates in neighbouring countries (Hungary, Croatia, Austria). The USUV is maintained in transmissible cycle between birds and mosquitoes mainly from the genus *Culex*. Mammals (humans, horses, rodents) can also be infected. Humans and other mammals are "dead end" hosts. Virus is isolated from numerous bird species. The USUV infections are asymptomatic in wild African birds, while for European birds, the virus is very virulent causing necrotizing focal encephalitis, degenerative myocarditis and fatal encephalitis. It is assumed that the virus was introduced into Europe by the migratory birds that have been infected by living or passing through endemic areas in Africa. First human cases were recorded in Italy in 2009. The genome of USUV was detected in cerebrospinal fluid of woman suffering from B-cell lymphoma with meningoencephalitis and in plasma of a female, who was subjected to a liver transplantation and subsequently developed fever, headache, and fulminant hepatitis which progressed to coma. In Austria, USUV infections were confirmed

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in people with a skin rash of unknown aetiology using plaque reduction neutralization test. The circulation of USUV has been proven in humans in many European countries by serological studies (Germany, Italy, and Croatia). Serological study performed in 2015 revealed that USUV is present in inhabitants of South Bačka District of Vojvodina, Serbia. Serum samples were tested using commercial ELISA IgG test for USUV and IgG antibodies against USUV were detected in 5% (4/88) of patients. The molecular investigation included 216 pools of mosquitoes collected in the period from June to September, in the South Bačka District. The USUV genome was detected in two mosquito pools (2/216). In human samples tested by RT PCR, USUV genome was not found.

Key words: USUTU virus, morphology, biology, diagnosis

USUTU VIRUS: NOVI FLAVIVIRUS U EVROPI

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Kratki sadržaj

Među Arbovirusima po brojnosti, rasprostranjenosti i značaju za humanu i veterinarsku medicinu izdvajaju se flavivirusi, RNK virusi koje prenose komarci i krpelji. Virus Zapadnog Nila (VZN) je flavivirus, endemski prisutan u mnogim evropskim zemljama, pa i u Srbiji gde cirkuliše u konjima, pticama, ljudima i komarcima. Usutu virus (USUV) je flavivirus morfološki, antigenski, genetski i biološki vrlo blizak VZN i cirkuliše u zemljama u okruženju (Austrija, Mađarska, Hrvatska). Virus se održava u transmisivnom ciklusu između ptica i komaraca uglavnom iz roda *Culex*. Sisari (čovek, konji, glodari) takođe mogu biti inficirani i slučajni su domaćini. Virus je izolovan iz različitih vrsta ptica. Kod afričkih ptica

infekcija je asimptomatska. Za evropske ptice virus je veoma virulentan i izaziva nekrotizirajući fokalni encephalitis, degenerativni miokarditis i fatalni encefalitis. Pretpostavlja se da su virus u Evropu donele migratorne ptice koje su se inficirale tokom boravka ili prolaska kroz endemska područja u Africi. U Italiji 2009. godine registrovan je prvi slučaj infekcije USUV kod ljudi. Genom USUV dokazan je u likvoru žene sa limfomom B ćelija, kao i iz plazme žene kojoj je transplantirana jetra i u koje su se razvili groznica, glavobolja, fulminantni hepatitis i koma. U Austriji kod osoba sa osipom na koži nerazjašnjene etiologije utvrđena je infekcija ovi virusom, neutralizacionim testom redukcije plakova. Serološke studije sprovedene na stanovništvu nekoliko evropskih zemalja ukazuju na prisustvo ovoga virusa kod ljudi (Nemačka, Italija, Hrvatska). Primenom metode ELISA uz korišćenje komercijalnog ELISA IgG testa na USUV, utvrđeno je prisustvo specifičnih IgG antitela protiv navedenog virusa kod 5% ispitanih uzoraka krvnog seruma ljudi (4/88). U periodu od juna do septembra 2015.godine ispitano je ukupno 216 zbirnih uzoraka komaraca prikupljenih na teritoriji Južnobačkog okruga. Sekvenca RNK USUV je dokazana u dva pula (2/216) komaraca. Testiranjem humanih uzoraka na USUV nisu dobijeni pozitivni rezultati.

Ključne reči: usutu virus, morfologija, biologija, dijagnostika

INTRODUCTION

Among various arthropod-borne viruses (arboviruses), the flaviviruses stand out with regard to their number, geographic distribution and importance in both human and veterinary medicine. They belong to the family *Flaviviridae*, genus *Flavivirus*. Flaviviruses are enveloped viruses with a positive sense single-stranded RNA genome, transmitted by mosquitoes and ticks. According to their antigenic characteristics, flaviviruses were classified into 8 serogroups: tick - borne encephalitis, Rio Bravo, Japanese encephalitis, Tyuleniy, Ntaya, Uganda S, dengue and Modoc (Calisher et al., 1989).

The Japanese encephalitis serogroup includes West Nile virus (WNV) which has now spread globally throughout Africa, Asia, Europe and America, Japanese encephalitis virus that is endemic in Southeast and East Asia, and Oceania, Marray Valley encephalitis virus prevalent in Australia, Saint Louis encephalitis virus spread over American continent, and Usutu virus (USUV). In recent years, a great expansion of WNV was confirmed. In just few years,

the virus has become endemic in the United States, involving thousands of human cases and hundreds of neuroinvasive disease cases reported annually. Endemic circulation of WNV has also been reported in many European countries. The results of serological and molecular studies revealed that WNV is present in Vojvodina and other regions of Serbia and that the virus circulates in horses, birds, humans and mosquitoes (Lupulović et al., 2011; Petrić et al., 2012; Petrović et al., 2013). No confirmed human cases of WNV infection were recorded in our country until 2012, when the first outbreak of WNV occurred in Serbia. Between August and October 2012, 58 people with WNV infection were hospitalized at the Institute of Tropical Medicine in Belgrade. Most of them (52) had neuroinvasive disease and 9 patients died (Popović et al., 2013). During the 2012 and 2013, 32 patients with WNV infection were treated at the Clinic for Infectious Diseases of the Clinical Centre of Vojvodina, and 17 of those had developed a neuroinvasive form of disease (Sević et al., 2015). Previously, WNV has been regarded as exotic virus from distant tropical countries with minor health importance. However, over the last several years, WNV has shown an increasing ability to spread beyond its established geographic ranges and has become an important public health concern in our country.

Increase in knowledge and understanding of WNV as an emerging human pathogen in our region extended our investigations to USUV, a virus taxonomically, morphologically, antigenically, genetically and ecologically very similar to WNV. USUV was first identified in 1959 in South Africa, when Mcintosh isolated the virus from *Culex neaevii* mosquitoes by intracerebral inoculation of new-born mice. The virus was named after the river Usutu in Swaziland. For decades after the discovery, the USUV was restricted to the African region, and it was considered to be unimportant in terms of pathogenicity. Increased attention was paid to USUV in 2001, when the first recognized outbreak of USUV outside of Africa occurred among blackbirds *Turdus merula* in Vienna, Austria (Weissenböck et al., 2002).

MORPHOLOGY OF USUV

Like other members of the family *Flaviviridae*, USUV is small, spherical virus with icosahedral symmetry. USUV virions are composed of a lipid envelope surrounding a nucleocapsid which harbours a positive single-stranded RNA genome with a length between 10,488 and 10,976 nucleotides. The untranslated, non-coding regions at 5' (5'UTR) and 3' (3'UTR) terminal ends of the genome flank the coding sequence. A single open reading frame encodes a polyprotein precursor that is co- and post-translationally processed into three

structural proteins named C (capsid protein), prM (precursor of the membrane protein M) and E (envelope protein) and 7 non-structural proteins with regulatory and enzymatic functions (NS1, NS2A, NS2B, NS3, NS4A, NS5B and NS5). Like in other flaviviruses, the adsorption of the virus to the cell is performed with E protein expressed on the surface of viral particles. During viral replication, the precursor polyprotein is cleaved by cellular and viral protease to the individual proteins (ICTV, 2016).

ISOLATION OF USUV

In the laboratory, the USUV multiplies in newborn mice up to 7 days of age, in which it causes fatal neurological disease. After inoculation, geese and chickens may have asymptomatic infection and occasionally excrete virus. The virus can be propagated in VERO E6, PK15, EGF, Hela cell cultures (Bakonyi et al., 2005). In infected cells, inclusions can be observed.

BIOLOGY OF USUV

The USUV is maintained in transmissible cycle between birds and mosquitoes mainly from the genus *Culex*. Birds are the reservoir (amplification host) and mosquitoes serve as vectors. The virus has been isolated from various species of mosquitoes: *Culex neavei*, *Culex perfuscus*, *Mansonia africana*, *Mansonia aurites* and *Aedes minutus* in Africa and *Aedes albopictus* and *Culex pipiens* in Europe. The infected mosquito transmits the infection to humans, but other mammals (horses, rodents) can also be infected. Humans and mammals are "dead end" hosts for USUV without significance for maintaining the virus in nature, as the low level of viraemia is usually not sufficient to allow the transmission of the virus to mosquitoes.

The USUV has been isolated from different African birds including *Bycanisters harpei*, *Andropadus virens*, *Turdus libomyanus*, and *Andropadus virens*. It has also been detected in number of European bird species as well. For example, in Italy, USUV was isolated in *Turdus merula, Sturnus vulgaris, Garrulus glandarius, Pica pica* (Tamba et al., 2011) In Austria, it was detected in *Turdus merula*, *Turdus philomelos*, *Strix nebulosa*, *Parus caeruleus*, *Passer domestic*us, *Parus major*, *Sitta europea*, *Erithacus rubeculain (*Weissenböck et al., 2003). In Germany, the presence of USUV was confirmed in *Turdus merula, Sturnus vulgaris, Serinus canaria domestica, Passer domesticus, Strix nebulosa, Alcedo atthis* (Jöst et al., 2011). In Switzerland, the virus was found in *Passer domesticus, Turdus merula, Passer caeruleus, Carduelis chloris, Erithacus rubecula,*

Aegolius funereus, Strix nebulosa lapponica, *Surnia ulula, Glaucidium passerinum (*Steinmetzd et al., 2011). In Hungary, it was detected in *Turdus merula (*Bakonyi et al., 2007).

The USUV has been present in Africa for a substantial period of time. Long-term co-evolution of USUV and its avian hosts resulted in natural selection of birds able to survive and become resistant to infection. As a consequence, the USUV infections are asymptomatic in wild African birds, while for European birds, the virus is very virulent causing necrotizing focal encephalitis, degenerative myocarditis and fatal encephalitis (Bakonyi et al., 2007). It is assumed that the virus was introduced into Europe by migratory birds that have been infected by living or passing through endemic areas in Africa. The virus also infects rodents. It was isolated for the first time from rodent *Praomys sp.* in Central African Republic.

HUMAN CASES OF USUV INFECTION

The first human case of infection with USUV has been registered in the Central African Republic, where the virus was isolated from serum sample of patient with fever and skin rash. Another case has been registered in Burkina Faso in the 10-year-old patient with fever and jaundice.

The first human case of USUV infection in Europe was recorded in 2009 in Italy. The virus was detected in cerebrospinal fluid (CSF) of a 60-year-old woman suffering from B-cell lymphoma, with meningoencephalitis, using RT-PCR assay with primers specific for NS5 and prM genes. Sequencing of amplification products revealed 98% homology with strains Vienna-2001 and Budapest-2005 (Pekorari at al., 2009). In the same year, the second case of human USUV infection was registered in Italy. It was a female, age 40, who was subjected to a liver transplantation and subsequently developed fever, headache, and fulminant hepatitis which progressed to coma. USUV was isolated from plasma sample on VeroE6 cell culture and identified by real-time RT PCR test. The virus genome was completely sequenced and the virus was named Bologna (Cavrini et al., 2009). It is interesting that both patients were from the Northeast region of Italy called Emilia-Romagna, where the first transmission of Chikungunya virus in Europe was established. Another three human cases of USUV infections were confirmed also in Italy, when the genome of the USUV was found in CSF samples of 3 out of 44 patients with meningoencephalitis (Cavrini et al., 2011). In Austria, USUV infections were confirmed by plaque reduction neutralization test in 25% of 203 people with an increased risk of USUV infection and a skin rash of unknown aetiology (Weissenböck et al., 2007).

SEROLOGICAL INVESTIGATIONS OF USUV IN HUMANS

Serological studies performed in several European countries indicated the circulation of USUV among humans. In a study conducted in southwest Germany, the antibodies against USUV were found in one out of 4,200 blood donors using enzyme linked immunosorbent assay (ELISA), immunofluorescent test (IFT) and neutralization test (Allering et al.,2012*).* In Italy, USUV IgG antibodies were found in 4 out of 359 blood donors via ELISA test (Gaibani et al., 2012). In Croatia, antibodies against USUV were detected in 3 out of 95 patients with fever and neuroinvasive symptoms, using virus neutralization assay (Vilibic - Cavlek et al., 2014). Seroepidemiological investigations detected the virus in mosquitoes and birds in Austria, Germany, Italy, Spain, Switzerland, Hungary, Poland, Belgium, Greece, the Czech Republic and England. During the study of WNV in horses, antibodies against USUV were discovered by neutralization test in serum samples of horses also positive to anti-WNV antibodies (Lupulović et al., 2011*).*

SEROLOGICAL INVESTIGATIONS OF USUV IN VOJVODINA

Serological investigation performed in 2015 revealed that the USUV is present in inhabitants of South Bačka District of Vojvodina, Serbia. The study included 88 persons with risk factors for infection with arboviruses transmitted by mosquitoes. Serum samples were tested using commercial ELISA IgG test for USUV ("Euroimmun", Germany), strictly according to the manufacturer's recommendations. IgG antibodies against USUV were detected in 5% (4/88) of patients (Hrnjaković Cvjetković et al., 2014).

MOLECULAR INVESTIGATION OF USUV IN VOJVODINA

Molecular investigations for the presence of USUV specific RNA in pooled samples of mosquitoes and human samples were conducted during the 2015. The investigation included 216 pooled samples of mosquitoes collected in the period from June to September in the South Bačka District. Pooled samples of mosquitoes were homogenized and viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany) kits. For the detection of USUV genome, real-time RT PCR was applied. Amplification was performed with oligonucleotide primers and TaqMan probe ("Invitrogen", USA) specific for the NS5 gene, using *SuperScript* III *One-Step RT-PCR* ("Invitrogen", USA) on Applied Biosystems 7500 thermocycler ("Applied Biosystems", USA). The USUV

genome was detected in two mosquito pools (2/216). Samples of patients with neurological symptoms and symptoms of fever (39 serum samples and 20 samples of CSF) were also examined by real-time RT PCR test. None of the samples tested were positive for USUV RNA (Hrnjaković Cvjetković et al., 2015).

DIAGNOSIS OF USUV INFECTION

Serological diagnosis of USUV infection is often difficult due to extensive cross-reactivity between different flaviviruses, particularly in geographic regions where circulation with other flaviviruses, such as WNV and tick-borne encephalitis virus (TBEV) occur. The cross-reactivity is higher for IgG than for IgM detection (Makino et al., 1994). According to available information, there are only commercially available USUV-specific IgG ELISA tests, at the moment. Consequently, development of commercially available USUV-specific IgM ELISA test is needed most urgently. The neutralization test can be used as specific and sensitive tool to overcome the flavivirus cross-reactivity. However, the test is time-consuming and labour-intensive. It is performed using cell cultures and live viruses and requires the biosafety level III facilities, which are available in limited number of laboratories. An additional problem in serological diagnosis of arboviral infections is a long-term persistence of IgM antibodies in serum, sometimes months after the infection (Solomon, 2004).

Molecular tests are now accepted as standard tests for diagnosis of USUV infections in acute phase of the disease. At present, the real-time RT PCR tests are used most frequently. Molecular tests allow the most specific, sensitive and rapid detection of USUV genome in serum and CSF samples within the first days after the onset of infection.

CONCLUSION

The results of many studies indicated that USUV circulates in mosquitoes, birds and humans in Europe. USUV is active in Vojvodina. Genome of USUV was detected in two pools of mosquitoes collected in South Bačka District. Serological investigation showed that USUV is active among humans. The assessment of actual risks associated with USUV for humans and animals in Vojvodina strongly requires further investigations. Large-scale veterinary, human and entomology based surveillance programs should be established to prevent the emergence of USUV in Vojvodina.

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AFLATOXIN M1 IN MILK AND ASSESSING THE POSSIBILITY OF ITS OCCURRENCE IN MILK PRODUCTS

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Abstract

Aflatoxin M1 (AFM1) is a hepatocarcinogenic derivative of aflatoxin B1 excreted into the milk after ingestion of contaminated feed. The presence of AFM1 in milk and milk products is of huge concern for human health. In this paper, the results on long term assessment of AFM1 in milk produced in Serbia are presented. In the period 2013 to 2016, 427 milk samples were examined for AFM1. In 34.4 % of samples, the content of AFM1 was higher than 0.05 μg/kg. The article also offers a review of the fate of aflatoxin in milk products during the different operations in milk processing. The evaluation of the influence of processing on AFM1 stability can propose economic strategy for resolving cases of accidents due to AFM-1contamination of milk.

Key words: aflatoxin M1, milk, milk product

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AFLATOKSIN M1 U MLEKU I PROCENA MOGUĆNOSTI NJEGOVE POJAVE U PROIZVODIMA OD MLEKA

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Kratak sadržaj

Aflatoksin M1 (AFM1) je hepatokarcinogeni derivat aflatoksina B1 koji se izlučuje mlekom nakon uzimanja kontaminirane hrane. Prisustvo AFM1 u mleku i mlečnim proizvodima je od ogromnog značaja za ljudsko zdravlje. U ovom radu su prikazani rezultati dugoročnog ispitivanja AFM1 u mleku u Srbiji. U periodu od 2013. do 2016. godine, u 427 uzoraka mleka je ispitan sadržaj AFM1. U 34,4% uzoraka sadržaj AFM1 je bio veći od 0,05 μg/kg. U radu je takođe dat pregled istraživanja o sudbini aflatoksina u mlečnim proizvodima tokom različitih procesa tokom prerade kontaminiranog mleka. Utvrđivanje uticaja prerade mleka na stabilnost AFM1 može da obezbedi ekonomsku strategiju za rešavanje problema u slučaju kontaminacije mleka sa AFM1.

Ključne reči: aflatoksin M1, mleko, proizvodi od mleka

INTRODUCTION

Aflatoxins (AF) are the most important mycotoxins, and besides cereals, they can contaminate milk and milk products. International Agency for Research of Cancer evaluated aflatoxin M1 (AFM1) as proved carcinogen to humans, and considered it as belonging to Group 1 (IARC, 2002). Contamination of milk and milk products can occur by indirect contamination via contaminated feed or rarely by direct contamination, when molds grow on milk. There are different data about carry-over rate of aflatoxin B1 (AFB1) from feed to AFM1 in milk. According to Creppy (2002), approximately 0.3 – 6.2% of the total ingested AFB1 from feed is transformed into milk. Some investigations revealed different carry-over rate in different animals. For example, in cows, it ranges from 0.35% to 3%, while in sheep this rate is 0.08 to 0.33% (Bakirci, 2001).

AFM1 binds to the protein fraction of milk (casein (Brackett, 1982) and may be present in milk products produced from the contaminated milk in higher concentrations than in milk itself. Only combined action of heat and low pH is able to denature whey proteins to a point where they lost their AFM1-binding capacity (Barbiroli et al., 2007). Due to its semi polar character, AFM1 predominate in the nonfat fraction (Van Egmond and Paulish, 1986; Galvano et al., 1996; Prandini et al., 2009). AFM1 was found in pasteurized milk, UHT milk, powdered milk, infant milk formulas, yoghurt, feta cheese, white cheese, traditional cheese from Turkey, Iran and Brazil, ice-cream, butter (Campagnollo et al., 2016). Different factors can influence the amounts of free AF in milk and milk products: aflatoxin concentration, pH, heat processes, ionic strength, fermentation temperature, storage temperature, storage time, protein content, titratable acidity, strain utilized (Arab et al., 2012). Pasteurization processes, even those using UHT techniques, do not drastically affect AFM1 concentration because of its heat stability (Bakirci 2001; Galvano et al., 1996; Rama et al., 2015). The data from published studies showed variable findings regarding AFM1 reduction during different unit operations used in milk products processing (Camagnollo et al., 2016). There are opinions that these contraindications in aflatoxin stability studies are due to the differences in initial levels of contamination, the range of temperature and analytical methods for AF determination (Campagnollo et al., 2016).

The legislation for AFM1 in EU (EC 1881/2006) is very strict. Maximum level is 0.05 μg/kg. In the USA (FDA, 2011), maximal limit for AFM1 in milk is 0.5 μg/kg as well as in Asia (China) and South America (Brazil). In Serbia, legislation for AFM1 in milk was in accordance with the EU in 2011, when the new Regulation about maximum permissible residues in food and feed was adopted („Službeni glasnik RS" 28/2011). However, since then, maximum level of AFM1 has been changed several times (Table 1). Currently, there is valid modification of the Regulation under which the maximum acceptable amount of AFM1 in milk is 0.25 μg/kg. Except for milk, regulatory limits were established in some countries also for the presence of AFM1 in milk products (Table 2, Campagnollo, 2016).

Table 1. Maximum permissible limits of AFM1 in milk in Serbia

Maximum permissible concentration (μg/kg)	Reference	Period of validity
0.05	„Službeni glasnik RS" 28/2011	04.05.2011. – 01.03.2013.
0.5	„Službeni glasnik RS" 20/2013	01.03.2013. – 20.03.2014.
0.5	„Službeni glasnik RS" 29/2014	20.03.2014. – 01.07.2014.
0.05	„Službeni glasnik RS" 39/2014	01.07.2014. – 14.07.2014.
0.25	„Službeni glasnik RS" 72/2014	15.07.2014. – 31.12.2014.
0.05	„Službeni glasnik RS" 29/2014	01.01.2015. – 06.10.2015.
0.25	„Službeni glasnik RS" 84/2015	07.10.2015. – 05.04.2016.
0.25	„Službeni glasnik RS" 35/2016	06.04.2016. – 05.10.2016.
0.25	„Službeni glasnik RS" 81/2016	06.10.2016. – 05.03.2017.
0.25	„Službeni glasnik RS" 21/2017	05.03.2017. – 06.09.2017.

Table 2. Maximum permissible limits for AFM1 in milk and milk products in different countries (Campagnollo, 2016; Škrbić et al., 2015)

Country	Milk	Milk product
USA	0.50	
EU	0.050	
Iran	0.050	0.50 (milk powder)
		0.020 (butter and butter milk)
		0.250 (cheese)
Turkey	0.050	0.250 (cheese)
Brazil	0.50	5 (milk powder)
		2.5 (cheese)
Italy	0.050	0.250 (soft cheese)
		0.450 (hard cheese)
China	0.5	0.5 (milk powder)
Pakistan	0.05	0.050
Switzerland	0.050	0.250 (cheese)
Austria	0.050	0.250 (cheese)
France	0.050	0.250 (cheese)
The Netherlands	0.050	0.020 (butter and cheese)

The presence of AF is typical for the warm tropical areas, and therefore the occurrence of these toxins has not been characteristic for the climate in Serbia (Živkov-Baloš et al., 2008). Due to climatic changes, as well as the development of novel analytical methods for its determination, the detection of aflatoxins in Serbian corn became more frequent (Jakšić et al., 2015). Consequently, the problems about the presence of AFM1 in milk are more pronounced. After the incident in 2013, when high contamination of corn and milk was recorded, special attention was given to the monitoring of food safety in terms of the presence of aflatoxins. As obvious from the introduction paragraph, the legislation in Serbia regulates the presence of AFM1 in milk, but not in milk products. Only few papers have been published on the topic of monitoring AFM1 in milk products in Serbia, but there are no specific studies on the transfer of AFM1 from milk into traditional and most widely consumed products in Serbia. This paper provides a long-term assessment of AFM1 in milk produced in Serbia and implications for possible fate of AFM1 in milk during its processing.

MATERIAL AND METHODS

In a period from 2013 to 2016, in the laboratory of Scientific Veterinary Institute „Novi Sad", 427 milk samples were analyzed for the content of AFM1. Milk samples were collected from milk collecting points or dairy plants, directly on the production line. Samples were collected directly from the production in dairies or sampled by an official of control.

The presence of AFM_1 was analyzed by enzyme-linked immunosorbent assay method, using Ridascreen® Aflatoxin M_1 (Art. No. R1121) test kit (R-Biopharm, Germany). The color intensity is measured photometrically at 450 nm (Multiskan FC, Thermo Scientific, China) and is inversely proportional to the mycotoxin concentration in the sample. Special software Rida® Soft Win (Art. No. Z9999, R-Biopharm, Germany) was used for the evaluation of enzyme immunoassays. According to the manufacturer´s description, the detection limit (DL) for AFM_1 was 0.005 μg/kg. Because of high toxin concentration and maximum permitted level of 0.5 μg/kg, in a part of examinations the samples were diluted, thus in that case DL was 0.05 μg/kg, while determination range encompassed concentrations from 0.05 to 0.80 μg/kg.

The analytical quality of the ELISA method was assured by determination of spiked samples as well as by participation in proficiency testing scheme (milk powder sample FAPAS 04224). Recovery for AFM_1 was 105%.

RESULTS

The results of AFM1 content in milk samples (2013–2016) are presented in Table 3.

Table 3. Contents of AFM_1 in milk samples in Serbia in 2013–2016

Year	Positive/ total no. of samples	Positive samples* (%)	No. of samples				
			< 0.05 (μg/ kg)	0.05-0.25 (μg/kg)	0.26–0.50 (μg/kg)	0.51–0.80 (μg/kg)	> 0.80 (μg/ kg)
2013	55/75	73.3	20	28	9	8	10
2014	26/66	39.4	40	22	3	1	/
2015	51/178	28.6	127	38	8	2	3
2016	15/108	13.9	93	12	1	1	1
Total	147/427	34.4	280	100	21	12	14

* above 0.05 μg/kg

Characteristic climatic conditions in Serbia during 2012 affected particularly corn production (Jakšić et al., 2015). Corn contamination with high levels of AF has led to consequent milk contamination with M_1. In 73.3% of samples, maximum EU level for AFM1 in milk (0.05 μg/kg) was exceeded. This result is in accordance with published results of Torovic (2015) where 75% of samples exceeded concentration of AFM1 of 0.05 μg/kg. Somewhat lower frequency of AFM1 contamination was recorded by Tomaševic et al. 2015, while some higher levels were reported by Kos et al., 2015. During the period 2013-2014, Tomašević et al. (2015) analyzed a total of 1,438 milk samples. AFM1 levels exceeded the EU maximum residue limit in 56.3% of raw milk and 32.6% of heat-treated milk samples. In the study conducted by Kos et al. (2015) during the first half of 2013, 176 samples of different types of milk were examined in Serbia, and 86.0% contained AFM1 greater than 0.05 μg/kg. After that, in the period 2014 to 2016, gradual decline in the percentage of contaminated samples was observed, ranging from 39.4% to 13.9%. Although corn gender 2014 and after was not significantly contaminated with AF (Nešić et al., 2015), occasional occurrence of samples with high concentrations of AFM1 was still evident, reaching levels of even over 0.8 μg/kg.

DISCUSSION

During the crisis with AF in 2013 and even later years, the producers and milk processors faced the problem what to do with contaminated milk. Although regulations on maximum levels of AFM1 do not include milk products, they still refer to the milk intended for processing. Besides that, questions were also asked about the possibility of processing such milk into the safe products. Results of AFM1 examination of milk products indicate that there is no available process which can completely destroy AFM1 (Table 4.). Contrasting data have been reported on the influence of milk products preparation. Numerous investigation showed that the increase of AFM1 levels in cheese is a function of cheese type, the type of unit operations and the amount of eliminated water during processing (Nilchian and Rahimi, 2012; Bakirci, 2001; Deveci 2007). According to Manetta et al. (2009), there is direct correlation between the AFM1 in milk and its level in the final product. Experimental data showed that compared to milk, AFM1 concentration increases in yogurt (sour milk) 2 times, in cheese with a long ripening period 4.5 times, while the concentration of AFM1 in whey decreases by 40% (Manetta et al., 2009). The study of Deveci and Szegin (2006) revealed that the total AFM1 contents were reduced by about 59-68% when original skimmed milk was spray-dried. Other authors have different conclusions, i.e., transformation of fluid milk into powder will result in great increase in AFM1 concentration (Campagnollo, 2016). During the production of cheese, AFM1 crosses into the cheese (because it is bound to casein) and whey (as it is soluble in water) (Tokar and Vengust, 2008). There was less AFM1 in cream and butter than in milk. In the soft cheese, the content of AFM1 was 2.5 to 3.3 times higher and in hard cheese 3.9–5.8 times higher than in milk from which the cheese is made (Yousef and Marth, 1989). According to Mohammadi et al. (2009), rennet temperature, press time, and saturated brine pH affected the amount of AFM1 in cheese production. The combination of pasteurization, the conversion of milk into feta cheese and at least 50 days of preservation in brine leads to a 50% reduction in initial concentration of AFM1 in milk (Motawee and McMahon, 2009). Studies about the stability of AFM1 in yoghurt during fermentation are controversial, similar as in case of cheese production. In some investigations, AFM1 proved resistant to thermal treatment and slightly acidic conditions in the production of cheese and yoghurt (Colak 2007 Oruc et al., 2006) and an increase of its concentration in yoghurt was observed (Bakirci 2001), while other studies recorded a decreased levels as compared to milk (Govaris et al., 2002). Possible reasons include different pH of yoghurts and fermentation conditions (Govaris et al., 2002).

Table 4. Selected studies about stability and fate of AFM1 during dairy processing

Result	Reference
The AFM1 content in ice-cream and in sherbet remained stable through 8 months of frozen storage.	Wiseman and Marth, 1983
No significant trends for short- and long-term stability of AFM1 in milk powders for 6 years at -20°C.	Josephs et al., 2005
Pasteurization at 63°C for 30 min caused <10% destruction of AFM1.	Motawee and McMahon, 2009
64.4% of AFM1 concentration from milk was found in cream. Mean AFM1 level of skim milk was 3% higher than those of milk.	Bakirci, 2001
Pasteurization and concentration on 30-33% dry mater reduced 35-40% of AFM1.	Deveci and Sezgin, 2006
Fermentation with different starters to pH 4.0 and 4.5 has impact on reducing AFM1 concentration by 25%.	Jasutiene et al., 2006
AFM1 remained at 42.87% and 34.73% in Turkish White and Kashar cheese samples, respectively. The change of AFM1 concentration during the white cheese ripening of 0-90 days was averagely 9.8%.	Colak, 2007
Loss of the initial amount of AFM1 in milk was estimated at about 13% and 22% by the end of the fermentation, and 16 and 34% by the end of storage for 4 weeks at 4°C, for yoghurts with pHs 4.6 and 4.0, respectively.	Govaris et al., 2002
During cheese making, the remaining AFM1 in milk was partitioned: 2/3 retained in the curd and 1/3 going into whey. 22-27% AFM1 reduced during storing feta cheese in brining solutions (8-12% w/w salt) during 10 days, 25-29 % after 60 days.	Motawee and McMahon, 2009

There are no studies on the effects of processing of contaminated milk on the concentration of AFM1 during manufacturing of traditional Serbian products, but there are few studies on the presence of AFM1 in milk products in Serbia. Fifty four samples of white and hard cheese were analyzed in Serbia in May-Jun, 2013. Seven samples (23%) exceed maximum acceptable level of

0.25 μg/kg (Škrbić et al., 2015). In addition to milk, Tomašević et al. (2015) analyzed milk products. Milk powders had the highest mean concentration of AFM1 (0.847±1.948 μg/kg) and were followed by hard (0.379±0.509 μg/kg) and white cheeses 0.146 ±0.170 μg/kg. However, based on these studies it is difficult to conclude about the degree of AF transfer into milk products. As can be seen, there is high standard deviation of AFM1 results in milk products, and there is also large difference between the concentration in the milk and milk products. Obviously, the lowest concentration of AFM1 was in yogurt as compared with other products which is in accordance with the presented studies from other countries.

Good nutrition in appropriate animal production system is essential to economically produce a healthy, high-quality product (Mirilović et al., 2015). It is necessary to act preventively in order to avoid milk contamination. Undoubtedly, it is very important to control the quality of feed and storage conditions in view of the presence of aflatoxin. Grains contaminated with AFB1 should not be fed to lactating animals to avoid contamination of milk.

CONCLUSSION

There is a factual risk of contamination of corn, milk and milk products with AFs in Serbia. In general, one can conclude that neither storage nor processing can fully eliminate AFM1 from milk. Further studies addressing the occurrence and stability of AFM1 in milk products should be carried out in order to evaluate the fate of AFM1 in traditional Serbian milk products. Avoiding economic losses due to processing of contaminated milk is possible if taking into consideration the following conclusions:

1. During milk processing, AFM1 passes from raw milk into cheese, yoghurt and whey.
2. The concentration of AFM1 in cheese depends on the production process and the process of cheese ripening and brining. Recent studies show that AFM1 concentrates in cheese in a large percentage - in contrast to older studies. This is perhaps influenced by the development of new and more accurate methods for determination of AFM1 in cheese. Variations in different studies about AFM1 content in cheese are partially due to the differences in analytical method used to quantify AFM1.
3. The content of AFM1 in yoghurt depends on the pH.
4. The temperature has little effect on reducing the concentration of AFM1, but studies on the presence of AFM1 in pasteurized and UHT milk have always revealed smaller number of contaminated samples of UHT milk.

5. AFM1 is concentrated in milk powder and remains stable in it for a long time.
6. Sour cream and butter have less AFM1, but there is little information on the percentage.
7. According to several studies, the storage of frozen milk and yoghurt results in the reduction of AFM1concentration of.
8. Milk pasteurization and cheese manufacturing process do not eliminate AFM1, so it is prudent to check the AFM1 incidence in cheese.

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CYPRINID HERPESVIRUS DISEASES

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Abstract

Cyprinidae, the largest known fish family including carp and minnows, has worldwide distribution with many species that are economically important in aquaculture. As would be expected, many viral pathogens can affect this group. The most pathogenic of these are the rhabdoviruses, a reovirus and three herpesviruses. Cyprinid herpesviruses can cause significant economic losses in aquaculture, and some of these viruses are oncogenic. The three herpesviruses are closely related but cause distinctly different diseases. Fish pox, caused by cyprinid herpesvirus 1 (CyHV-1), is one of the oldest known fish diseases, being recorded as early as 1563. It takes the form of a hyperplastic, epidermal papilloma on common carp. Cyprinid herpesvirus 2 (CyHV-2) is causative agent of herpesviral hematopoietic necrosis (HVHN). The herpesvirus was first isolated from cultured goldfish in Japan. It causes a severe epizootic but no external clinical signs were apparent on affected fish. One of the most economically important and researched viral diseases of carp is koi herpesviral disease caused by cyprinid herpesvirus 3 (CyHV-3). The aim of this paper is to present the current knowledge on herpesvirus diseases of the cyprinids.

Key words: cyprinids, CyHV-1, CyHV-2, CyHV-3

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HERPESVIRUSNE BOLESTI CIPRINIDA

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Kratak sadržaj

Ciprinide, najveća poznata familija riba, koja obuhvata šaranske vrste riba, je rasprostranjena širom sveta, sa mnogim vrstama koje imaju veliki ekonomski značaj u akvakulturi. Kao što bi se moglo očekivati, mnogi virusi mogu delovati na ovu grupu, među kojima su od najvećeg značaja pripadnici familija rabdovirusa, reovirusa i herpesvirusa. Herpesvirusi ciprinida mogu izazvati značajne ekonomske gubitke u akvakulturi, a neki od njih virusa su onkogeni. Iako su veoma srodni herpesvirusi ciprinida izazivaju različite bolesti sa veoma različitim karakteristikama. Boginje riba, koje izaziva herpesvirus 1 (CyHV-1), je jedno od najstarijih poznatih bolesti riba, utvrđeno još 1563. godine. Ovo oboljenje se po pravilu ispoljava u vidu hiperplastičnih papilomatoznih promena epiderma šarana. Herpesvirus 2 (CyHV-2) je uzročnik herpesvirusne hematopoezne nekroze (HVHN). Ovaj virus je prvi put izolovan iz zlatnih ribica u Japanu. Kao posledica delovanja uzročnika dolazi do pojave značajnih epizootija bez izraženih kliničkih znakova kod obolelih riba. Jedno od ekonomski najznačajnijih virusnih oboljenja šarana je koi herpesviroza izazvana ciprinim herpesvirusom 3 (CyHV-3). Cilj ovog rada je da se predstave aktuelna saznanja o oboljenjima ciprinida izazvanih herpesvirusima.

Ključne reči: ciprinidi, CyHV-1, CyHV-2, CyHV-3

INTRODUCTION

Herpesviruses constitute a large group of large DNA virus with the same structure and biological properties of the virion (McGeoch et al., 2008). They are host-specific pathogens. The family *Herpesviridae* contains mammalian, avian and reptilian viruses; the family *Alloherpesviridae* contains herpesviruses of fish and amphibians, and the family *Malacoherpesviridae* comprises single

invertebrate herpesvirus (*Ostreid herpesvirus*). The family of *Alloherpesviridae* is highly diverse, with a genome size ranging from 134 kbp of channel catfish virus - the smallest sequenced genome, to 295kbp of cyprinid herpesvirus-3, which is the largest known genome among the order *Herpesvirales* (McGeoch et al., 2006; Davison, 2010). The members of the fam. *Alloherpesviridae* are widespread among the fish and frogs and are grouped into four families: *Batrachovirus*, *Cyprinivirus*, *Ictalurivirus* and *Salmonivirus* (Davison et al., 2009). Genus *Cyprinivirus* includes four viruses: eel herpesvirus 1 (AngHV-1) isolated from the European eel (*Anguilla anguilla*), and three cyprinid herpesvirus (CyHV-1, CyHV CyHV-2 and-3) (Hanson et al., 2011). These viruses are capable of causing serious diseases in cyprinids. Size of the genome of these viruses is 291.144 bp (CyHV-1), 290 304 bp (CyHV-2) and 295,146 bp (CyHV-3), and their genomes have a 80% homology (Aoki et al., 2007; Davison et al., 2013). Herpesvirus virion consists of four morphologically distinct portions: the core, which consists of a linear double-stranded DNA; icosahedral capsid; protein coating; and lipid envelope with viral proteins (Davison, 2010).

CYPRINID HERPESVIRUS-1 (CyHV-1)

Cyprinid herpesvirus 1, also known as a carp pox virus, causes benign papillomatous lesions in the epithelium of common carp, *Cyprinus carpio*, and its ornamental form koi (Plumb and Hanson, 2011). This virus was detected in fish in most European countries, the USA, Israel, Russia, and parts of Asia (China, Japan, Korea, and Malaysia). In Serbia, this disease is present and represents one of the most common diseases of cultivated carp in the area of Vojvodina (Jeremić et al., 2005). The changes are initially flat, solid, smooth and transparent, but soon become thicker and resemble drops of paraffin on the skin. Further proliferation leads to the formation of papillomatous formation of an irregular shape, with thickness of 4 to 6 millimeters. They become milky white to gray in color, sometimes with a pink hue that comes from capillary vasodilation. The lesions may be present in different parts of the body but usually begin in the fins. Proliferated cells are not invasive and do not metastasize. Infected adults show no change in behavior or clinical signs; however, in younger fish, CyHV-1 infection can lead to clinically manifested disease with high mortality. In infected carp fry, loss of appetite, dilatation of abdomen, exophthalmos, bleeding on operculum and abdomen and dark skin pigmentation may occur (Plumb and Hanson, 2011). Growth retardation and emaciation are continuously present signs of the disease in advanced cases. The tail of such carp can be easily bent to touch its head. Such flabby fish have a reduced

muscular tone and osteomalatia with very low levels of ash, calcium and phosphorus in the vertebrae. After recovering, deformations of the spine are often visible. The disease is seasonal and lesions usually occur when the water temperature drops below 15°C, and disappear with the rise in temperature. Sano et al. (1993) showed that the CyHV-1 genome is present in the nervous system and the subcutaneous tissue after the termination of the disease, suggesting that the virus becomes latent, which may explain the recurrence of lesions in the convalescent fish. Virus has not been isolated from diseased carp. However, herpesvirus was isolated from koi carp on FHM cell line at 15°C in Japan. It has been demonstrated that this virus, like other herpes viruses, is lethal to juvenile carp and the survivors, after several months, develop papilomatous changes characteristic of carp pox. Japanese CyHV-1 isolate did not cause disease in grass or crucian carp (Sano et al., 1991).

CYPRINID HERPESVIRUS-2 (CyHV-2)

Cyprinid herpesvirus-2 (CyHV-2) is the causative agent of the disease called herpes hematopoietic necrosis (HVHN). Although it does not cause massive epizootic, it is clear that CyHV-2 is present globally and very prevalent in populations of goldfish.

The disease occurs when the fish are exposed to stress and the water temperature allows the replication of the virus. The disease was first identified in Japan in 1992 and 1993 in goldfish (*Carassius auratus*) (Jung and Miyazaki, 1995). Later, the occurrence of the disease in goldfish has been reported worldwide, in the USA (Groff et al., 1998; Goodwin et al., 2006), Taiwan (Chang et al., 1999), Australia (Stephens et al., 2004), the UK (Jeffery et al., 2007), China (Li et al., 2013), Switzerland, Germany (Haenen et al., 2016), France (Boitard et al., 2016). Recently, the disease in crucian carp (*C. carassius*) and Prussian carp (*C. gibelio*) caused by CyHV-2 has been found in several European and Asian countries (Danek et al., 2012; Luo et al., 2013; Fichi et al., 2013; Ito and Maeno, 2014). The presence of the CyHV-2 virus was not detected in susceptible fish populations in the fishfarms of the Republic of Serbia. Although it is generally accepted that CyHV-2 has been circulating in populations of goldfish around the world for a long time (Goodwin et al., 2006; Waltzek et al., 2009; He et al., 2013) the exact mechanism of its occurrence in the new host is not completely clear. In an outbreak of the disease in the goldfish, diseased fish do not show typical signs of the disease. The only noticeable changes are apathy and pallor of gills, with hypertrophy and hyperplasia of the gill epithelium. Internally, spleen and kidney necrosis are present (Jung and Miyazaki, 1995;

Groff et al., 1998). The disease incidence is dependent on the temperature of the water and occurs between 15-25°C, with a mortality rate of 50-100% (Groff et al., 1998). In the outbreak of the disease in Prussian carp, diseased fish are lethargic, with dark skin pigmentation and stay on the bottom of the pond. The disease occurs at 20 to 30°C and disappears when the water temperature rises above 30°C (Luo et al., 2013). In addition, the virus caused neither death nor symptoms of the disease in silver or bighead carp. Bearing in mind the increasing number of cases that indicate the spread of the virus as well as the fact that CyHV-2 can infect other species of the genus *Carassius*, knowledge of its characteristics and application of preventive measures is of vital importance.

CYPRINID HERPESVIRUS-3 (CyHV-3)

The disease caused by Cyprinid herpesvirus-3 (CyHV-3), named koi herpesvirus disease (KHVD), was first described in Germany in 1997 (Bretzinger et al., 1999), and shortly thereafter in Israel and the United States (Hedrick et al., 2000). There is evidence that CyHV-3 has been present in carp in the UK since 1996 (Aoki et al., 2007).

The disease is present in our neighboring countries, and the latest cases were recorded in Croatia in 2016, in Czech Republic in 2016, in Romania in 2017 (OIE, 2017). The assumption that the virus is present in carp populations in Serbia is established in 2004, when a high mortality of one-year and two-year-old carp on three ponds on the territory of the Republic of Serbia was recorded. In two fish farms, mortality of one-year and two-year-old carp was observed in the spring, with clinical symptoms and pathomorphological alterations characteristic to KHV (Jeremic and Radosavljevic, 2007).

Since CyHV-3 causes a disease in all age groups (Hedrick et al., 2000) with huge mortality - up to 100%, it is considered to be the major viral pathogen in carp. The disease seriously challenges the trade of ornamental koi and carp production worldwide (Rakus et al., 2013) bearing in mind that the common carp is the world's fourth most-farmed fish (Ronen et al., 2003).

Based on sequence analysis of CyHV-3 isolated worldwide, Kurita et al. (2009) have determined the existence of the Asian (two variants) and the European genotype (seven varieties).

The outcome of the infection is highly dependent on water temperature, and deaths occur within water temperature range from 18-25°C (Gilad et al., 2003) to 17-26°C (Haenen et al., 2004) or 17-28°C (Ilouze et al., 2006). The maximum water temperature tolerated by the virus is 28°C (Gilad et al., 2003). Undoubtedly, the disease occurs most often in spring, with a water tempera-

ture increase (Hedrick et al., 2000), and is primarily due to the inability of the immune response, suppressed by low temperatures in winter, to adequately react to the replication of the virus.

In studies of the virus ability to survive in water, Perelberg et al. (2003) found that CyHV-3 causes the death of fish within 4 hours after the addition of the virus into the water, while the virus loses its activity after 21 hours. It was found that the virus persists in water for 3 to 7 days after inoculation (Shimizu et al., 2006), as well as to survive longer in fish feces and mud (Dishon et al., 2005).

The study of Minamoto et al. (2011) revealed that in 90.3% of river water samples viral DNA was present 4-5 years after the onset of the disease, i.e., that 5 years after the occurrence of the disease all rivers in Japan were contaminated with CyHV-3. The same authors found that this virus is present in the plankton in outbreak waters.

In addition, CyHV-3 DNA can be present in approximately 100-fold higher concentration in sediment than in water, suggesting that the sediment can be a reservoir of the virus (Honjo et al., 2012). The skin of carp acts as the portal of entry of CyHV-3 and the site of early replication (Costes et al., 2009). The early replication of the virus at the portal of entry could contribute not only to the spread of the virus within infected fish but also to the spread of the virus throughout the fish population. On the third day after infection, the fish cease feed intake and become lethargic. After that, nervous signs of infection appear (shaking, twitching, uncoordinated movements, erratic swimming in shallow water). Also, infected fish rub against other fish or against objects. Such behavior could contribute to a skin-to-skin mode of transmission. To date, no evidence of vertical transmission of CyHV-3 has been found. Death occurs within three to four days after the onset of these signs, about 7 days after infection (Hedrick et al., 2000).

Among clinical signs endophtalmus, areas of pale patches on the skin, increased mucus secretion, multifocal loss of the epidermis giving the skin a "sandpaper-like texture" and changes to the gills in the form of irregular discoloration of the gills, swelling and necrosis of gills are present (Pikarsky et al., 2004; Gilad et al., 2004).

In clinically healthy fish, CyHV-3 can remain latent and the infection can be reactivated by temperature stress (Gilad et al., 2004; Eide et al., 2011).

CONCLUSIONS

Most of herpes viruses in fish cause a mild infection in natural environment, but in the intensive aquaculture these viruses can cause serious diseases

with high mortality. Since the number of identified herpesviruses in fish increases, development of effective prophylactic measures and control of these diseases became much more important.

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AUJESZKY'S DISEASE IN A DOG - CASE REPORT

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Abstract

This article reports on the occurrence and diagnosis of Aujeszky's disease in a dog. The procedure for isolation and identification of Aujeszky's disease virus was described. A dog of unknown breed aged about two years died of Aujeszky's disease after consuming animal offal (internal organs: lungs, spleen, kidneys) fed by the owner after slaughtering piglets and preparing meat for cooking. As early as 24 hours after consuming the offal, the dog manifested characteristic symptoms of Aujeszky's disease, which were immediately recognized by the veterinarian. The death occurred within less than 24 hours upon first clinical signs of disease. Aujeszky's disease virus was isolated and identified from brain and internal organ (lung and spleen) samples of the dog at the Department of Virology of the Scientific Veterinary Institute „Novi Sad". Isolation and identification of the virus was performed on PK-15 porcine kidney cell line and using nested PCR technique.

Key words: Aujeszky's disease, dog, virus isolation and identification

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AUJESKIJEVA BOLEST KOD PSA - PRIKAZ SLUČAJA

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Kratak sadržaj

U radu je dat opis nastanka, razvoja i dijagnostikovanja Aujeskijeve bolesti kod psa. Opisan je postupak izolovanja i dokazivanja virusa Aujeskijeve bolesti. Pas nepoznate rase, uzrasta oko 2 godine uginuo je od Aujeskijeve bolesti nakon konzumiranja iznutrica (unutrašnji organi: pluća, slezina, bubrezi), koje je psu dao vlasnik nakon obrade prasadi i pripreme mesa za pečenje. Pas, je već 24 sata posle konzumiranja iznutrica ispoljio karakteristične simptome Aujeskijeve bolesti, što je veterinar, koji je pregledao psa odmah uočio. Uginuće psa je usledilo za nepunih 24 sata nakon ispoljavanja simptoma. Na Odeljenju za virusologiju, Naučnog instituta za veterinarstvo „Novi Sad“ iz uzoraka mozga i unutrašnjih organa (pluća i slezina) obdukovanog psa izolovan je i dokazan virus Aujeskijeve bolesti. Izolovanje i dokazivanje virusa Aujeskijeve bolesti vršeno je na kulturi ćelija PK-15 i upotrebom nested PCR tehnike.

Ključne reči: Aujeskijeva bolest, pas, izolovanje i dokazivanje virusa

INTRODUCTION

Aujeszky's disease (AD), also known as *Pseudorabies,* is an infectious disease caused by a Suid herpesvirus 1 (SHV-1) from the family *Herpesviridae*, subfamily *Alphaherpesvirinae*. The virus infects the central nervous system and other organs, such as respiratory tract of dogs, cats, cattle, sheep, rabbits, foxes, etc. Clinical signs do not occur in humans i.e. the disease is not zoonosis. Seroconversion has been reported, but there is no evidence that the virus replicates significantly or is shed from people (CFSPH, 2017). Pigs are considered as a natural host and the main reservoir of the virus but the characteristic clinical picture is manifested only in the suckling and sometimes in the weaned piglets. *Aujeszky's* disease in dogs was first described in Hungary in 1902. Pets can get infected by consuming contaminated raw pork meat. So far, there are

no reports on direct dog-to-dog transmission. In dogs, after consuming meat contaminated with AD virus, the virus enters nerve endings in the mucosa and spreads to the brain along nerve axons (Kotnik et al., 2006). The incubation period is usually 2-6 days in the category of suckling pigs and less than 9 days in cattle and sheep. Reported incubation periods in dogs and cats range from 2 to 10 days, but most cases probably become apparent in 2-4 days (CFSPH, 2017). The infection of non-adapted species (such as dogs), results with death within few hours after showing of the first symptoms (Kotnik et al., 2006).

The diagnosis of *Aujeszky's* disease can be confirmed considering the observed clinical picture, epidemiological data and in the laboratory by virus isolation from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from tissue samples (brain, tonsils, lung, mandibular and mediastinal lymph nodes) from dead pigs. On susceptible cell cultures, *Aujeszky's* disease virus induces cytopathogenic effect (CPE) within 24-72 hours. Polymerase chain reaction (PCR) is applicable for identification of the isolated virus. The PCR is based on the selective amplification of a specific part of the genome using two primers located at each end of the selected sequence. A real-time PCR has been developed for virus identification and differentiation between vaccinal and wild-type viruses based on specific detection of gB and gE genes. Virus neutralisation (VN) has been recognised as the reference method for serology, but for monitoring and surveillance diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) (Moennig et al., 1982; OIE, 2012).

There is no specific treatment for AD, except supportive care and treatment for secondary infections (CFSPH, 2017). Specific control strategies for eradication of AD are conducted in pigs and are based on application of marker vaccines. The EU countries have successfully eradicated AD or have been conducting relevant programs for its eradication thus being considered AD-free (COMMISSION DECISION 2008/185/EC). Accordingly, AD does not occur in most of the EU countries and is observed only in the population of wild boars, which are nowadays considered major virus reservoir in Europe (Meier et al., 2015). The disease has been identified in hunting dogs in Croatia after consumption of wild boar meat (Keros et al., 2015). *Aujeszky's* disease has also been reported in dogs in several European countries such as Hungary, Austria, Spain, Italy, Belgium and Croatia (Quiroga et al., 1998; Cay and Letellier 2009; Thaller et al., 2006; Sozzi i sar., 2012; Pizzurro et al., 2016).

The Aujeszky's disease is enzootic in swine population in Vojvodina region. Pušić and co-workers (2011) pointed out that AD intermittently occurs in the population of domestic pig in Vojvodina; however, it has been identified in

another six animal species – cattle, sheep, dogs, cats, donkeys and badgers. All these species, except badgers, were in immediate contact with pigs or were fed with row pork meat. Previous serological surveys conducted in wild boar in Serbia were limited but suggested a relatively high AD seroprevalence (Lazić et al., 2015; Milićević et al., 2016).

The objective of this study is describing occurrence and clinical symptoms of AD in dog fed with pigs offal with no thermal treatment (internal organs: lungs, spleen, kidneys) and identification of AD virus from the sample material obtained at autopsy. Moreover, the aim is to describe the virus isolation and identification procedures using cell cultures and molecular methods (PCR).

MATERIAL AND METHODS

Tissue samples

The autopsy of the dog died with suspected clinical symptoms for AD, and samples collection for laboratory testing's was performed at the Scientific Veterinary Institute „Novi Sad". Based on the report of the veterinarian, rabies was excluded as the dog has previously been vaccinated several times, according to the Law of RS. At necropsy, focal pulmonary, gastric and renal hemorrhagies were recorded. The splenic pathology was particularly uniform, characterized by numerous dark-red to black, raised, soft, blood-filled areas of various sizes. Samples of brain tissue, lungs and spleen were collected for isolation of *Aujeszky's* disease virus. To the purpose of virus isolation, the samples were homogenized in Phosphate-buffered saline (PBS). Homogenates were centrifuged at 3000 x g for 10 min and supernatants were filtrated through 0.45 μm-pore filters and used for inoculation. One of sample was prepared from pool of organs (lung and spleen) and second sample was the brain tissue.

Virus isolation

The virus isolation was performed on porcine kidney cell line (PK-15, ATCC CCL-33). From a 6-well cell culture plate (Sarstedt, Germany) with a monolayer of 24-hour old PK-15 cell line (of passage No: 164) (ATCC), the cells growing medium has been poured. Supernatants (0.3 mL) of both brain and organ samples (filtrate) were inoculated into two wells of culture plate each. The cells from remaining two wells were saved as a control. The inoculated culture plate was incubated for 60 min at 37±1°C. After incubation, 3 mL-aliquots of cell maintenance medium were added into each well and the

plate was further incubated at 37±1°C. Inoculated cells were observed daily for the occurrence of CPE using inversion microscope.

Identification of isolated virus

Identification of isolated virus has been performed by Polymerase Chain Reaction (PCR). From the supernatant and cell suspension with cytopathogenic effect, the DNA extraction was performed using commercial kit QIAamp DNA Mini Kit according to manufacturer's instruction (Qiagen, Germany).

A total of 5 µl of sample DNA was used to run a PCR and nested PCR methods with "HotStar Taq Master Mix Kit" (Qiagen, Hilden, Germany) in 25µl reaction mixture volumes. The gB gene of AD virus was amplified using previously published oligonucleotide primers (Balasch et al., 1998). The size of the first stage PCR product was 334 bp. The second stage (nested) PCR product size was 195 bp. In both stages, primers were used at a concentration of 0.2 mM, dNTPs at 200 mM and Taq polymerase at 1 U per reaction. The first stage of reaction consisted of 35 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 45 s and extension at 72°C for 30 s. The second stage of reaction (nested PCR) consisted of 30 cycles of denaturation at 95°C for 60 s, annealing at 65°C for 45 s and extension at 72°C for 30 s. PCR products were visualized through UV light in a 2% agarose gel, stained with ethidium bromide.

RESULTS AND DISCUSSION

The clinical signs of Aujeszky's disease

A dog of unknown breed aged about two years was raised in the yard of owner, which fed the dog with piglet offal (lungs, spleen, kidneys) after slaughtering piglets and preparing meat for cooking. The day after consuming animal offal, the dog manifested unusual behaviour, and the owner referred to the veterinarian, who notices clinical signs of ataxia and scratching in the region of head and neck. Veterinarian had a suspicion on Aujeszky's disease and applied symptomatic treatment. The dog died on the same evening and the owner submitted the carcass to the Scientific Veterinary Institute „Novi Sad"on the next morning to excluding rabies as a potential cause of death.

Virus isolation

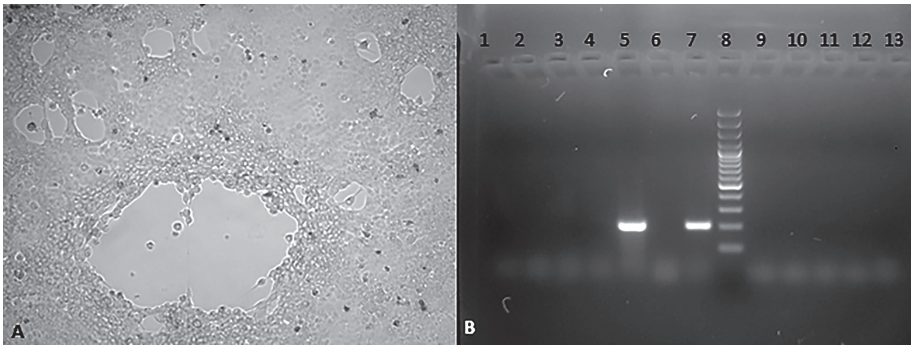
Cytomorphological changes indicating multiplication of *Aujeszky's* disease virus (occurrence of big circular cells with extensions on the margins of the

monolayer where cells were completely destroyed) were observed on porcine kidney cell line (PK15) in both plate wells 48 hours after brain tissue inoculation. Cytomorphological changes on cell cultures inoculated with internal organ samples were observed some 72 hours post inoculation. Throughout next few days, cytomorphological changes have been even more pronounced. On 5th day characteristic cytomorphological changes involved about 90% cells in each well of the inoculation plate. There were no changes on cells in two PK-15 cells control wells.

Identification of isolated virus

Aujeszky's disease virus was identified from the supernatant and suspension of cells with cytopathogenic effect using nested PCR method. Besides afore mentioned sample of PK-15 cells with obvious CPE (inoculated with brain sample of died dog), nine samples of aborted pig foetuses negative for AD virus were also included in the reaction. The obtain nested PCR product of 195 bp was visualized in 1.5 % agarose gel with 0.5 μg/ml ethidium bromide solution (Fig.1).

Figure 1: Cytomorphological changes (cytopathogenic effect) as a result of *Aujeszky's* disease virus replication on cell cultures PK-15 and positive virus finding with nested PCR technique as the method for virus confirmation



A - Cytopathogenic effect: circular cells with cellular extensions on the margins cell-free layer

B – nested PCR products (from left to right): 1 - 4 - tissue samples of negative aborted pig fetuses; 5 - positive brain sample from dog; 6 - negative control (water); 7- positive control; 8 - 100bp DNA ladder; 9-13 negative tissue samples of aborted pig fetuses.

Aujeszky's disease is widely present on pig farms in the Republic of Serbia, and only few of them are AD-free (Pušić et al., 2011; Prodanov-Radulović et al., 2015). Research in this study is in accordance with a range of studies worldwide, which confirmed presence of AD in dogs. The disease was reported in Austria and Germany in 2006 and 2009, respectively. These are the first cases of *Aujeszky's* disease since 1997, when the disease was eradicated in these countries (Thaller et al., 2006; http://www.promedmail.org/post/20100107.0067). Sozzi and collaborators (2012) in Italy, offered an interesting insight into genomic characterization of the strains of *Aujeszky's* disease virus – clear differentiation between strains isolated from hunting dogs, which were similar to those isolated from wild boars and strains isolated from dogs on the farms, which were similar to those affecting domestic pigs. Moreover, in Italy in 2014, a virus was detected in a 7-year old hunting dog that was in contact with blood of wild boar. The AD virus was detected and isolated by real-time PCR and rabbit kidney cell culture (RK13) (Pizzurro et al., 2016). In 2007, in Belgium, *Aujeszky's* disease was confirmed by the methods of virus isolation (PK-15) and real-time PCR in two hunting dogs manifesting characteristic clinical symptoms. The identified strains were highly pathogenic for dogs, and strains that have previously been isolated from wild boars strongly indicated that the virus circulates among the wild boar population even though there were no reports on virus transmission to domestic pigs (Cay and Letellier, 2009). *Aujeszky's* disease was confirmed in 7 dogs in Spain (1995) as well as in 13 dogs in Beijing, China in the period 2011-2013 (Quiroga et al., 1998; Zhang et al., 2015).

CONCLUSIVE REMARKS

Clinical signs of Aujeszky's disease are easily recognizable. Laboratory diagnostic methods like virus isolation and PCR detection of Aujeszky's disease virus, used in this study further facilitate the diagnosis of the disease. Aujeszky's disease virus was isolated on cell culture and identified from the supernatant and suspension of cells with cytopathogenic effect using nested PCR method.

Feeding domestic carnivores with fresh, no thermal treatment pork meat, poses a great risk for the occurrence of AD in such animals. Accordingly, uncontrolled feeding domestic carnivores with fresh pork meat can result in occurrence and spreading of Aujeszky's disease among the population of domestic animals.

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FACILITIES FOR ANIMAL FEED PRODUCTION AS SALMONELLA RESERVOIRS AND SOURCES OF FINAL PRODUCTS CONTAMINATION

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Abstract

Animal feed is the first link in the cycle "farm to fork" and the first potential place for entry of alimentary pathogens into the food chain. Special attention is given to bacteria from the genus Salmonella due to significant health and economic concerns related to salmonellosis in both human and veterinary medicine worldwide. Animal feed can become infected with salmonella through contaminated raw materials of animal and plant origin, but contamination of final products can occur during processing and post-processing in facilities for their production. The life cycle of Salmonella species occurs partly in higher organisms, and partly in the living environment. Their ubiquitous distribution and survival in the environment (outside the host organism) in soil, water, on plant matter as well as on various artificial materials is made possible by the formation of multicellular communities known as the biofilm. Biofilms are multicellular bacterial formations that are irreversibly adhered to surfaces, incorporated into the extracellular substance produced by themselves and which exhibit significantly different properties (biofilm phenotype) in relation to those that grow in the suspension (planktonic phenotype). One of the most important characteristics of biofilm phenotype is the increased bacterial resistance to various stress factors in the environment, including chemical and thermal treatments, and the mechanical cleaning and sanitation. By creating the biofilm, salmonella enables its survival and persistence for months or years on equipment and working surfaces in animal feed production facilities. Due to the ubiquitous distribution of Salmonella species in nature, and therefore on plant matter

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as the primary raw material for the production of animal feed, it is unlikely that Salmonella could be eradicated from the food chain. Control measures should be directed to the prevention of contamination.

Key words: animal feed, Salmonella, biofilm, persistence, control

OBJEKTI ZA PROIZVODNJU HRANE ZA ŽIVOTINJE KAO REZERVOARI *SALMONELLLA* I IZVORI KONTAMINACIJA FINALNIH PROIZVODA

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Kratak sadržaj

Hrana za životinje je prva karika u ciklusu "od njive do trpeze" i prvo potencijalno mesto ulaska alimentarnih patogena u lanac ishrane. Posebna pažnja pridaje se bakterijama iz roda *Salmonella*, zbog velikog zdravstvenog i ekonomskog značaja koji salmoneloze imaju u humanoj i veterinarskoj medicini u zemljama širom sveta. U hranu za životinje salmonele mogu dospeti kontaminiranim sirovinama animalnog i biljnog porekla, ali se kontaminacija finalnih proizvoda može desiti i tokom prerade i postprocesno, u objektima za njihovu proizvodnju. Životni ciklus *Salmonella* vrsta odigrava se delom u višim organizmima, a delom u životnom okruženju. Njihova ubikvitarna rasprostranjenost i preživljavanje u okruženju (izvan organizma domaćina), u zemljištu, vodi, na biljnoj materiji, kao i na različitim veštačkim materijalima, omogućeno je formiranjem višećelijskih zajednica poznatih pod nazivom. Biofilmovi su višećelijske zajednice bakterija koje su ireverzibilno vezane za površine, uklopljene u vanćelijsku supstancu koju same produkuju i koje pokazuju radikalno drugačije osobine (biofilm fenotip) u odnosu na one koje pokazuju dok rastu u suspenziji (planktonski fenotip). Jedna od najvažnijih karakteristika biofilm fenotipa je povećana otpornost bakterija na različite stresogene faktore okruženja, uključujući hemijske i termičke tretmane, kao i procedure mehaničkog čišćenja i sani-

tacije. Rastom u biofilmovima salmonele opstaju mesecima, pa čak i godinama na opremi i radnim površinama u objektima za proizvodnju hrane za životinje. S obzirom na ubikvitarnu rasprostranjenost *Salmonella* vrsta u prirodi, a time i biljnoj materiji kao osnovnoj sirovini za proizvodnju hrane za životinje, malo je verovatno da će *Salmonella* biti iskorenjena iz lanca ishrane. Mere kontrole treba usmeriti na prevenciju kontaminacije.

Ključne reči: hrana za životinje, *Salmonella,* biofilm, perzistencija, kontrola

INTRODUCTION

Bacteria belonging to the genus Salmonella are the causative agents responsible for one of the most important zoonoses and food borne transmissible infections in humans. Food producing animals (poultry, pigs, cattle) are considered reservoirs for many pathogens which can be transmitted by food, including Campylobacter species and non-Typhi serotypes of Salmonella enterica (Crump et al., 2002). The concern about animal feed safety was further raised with the appearance of variants of Creutzfeldt-Jakob disease in humans in the United Kingdom, associated with the feeding cattle with meat and bone meal (Brown et al., 2001). Contamination of animal feed with non-Typhi serotypes of S. enterica can contribute to the burden of human salmonellosis (Crump et al., 2002). Retrospective epidemiological analysis to determine the source of infection includes difficult and demanding processes; however, there is an increasing number of reports on the appearance of salmonellosis in humans where animal feed have been identified as the primary causative source (Pennington et al., 1968; Crump et al., 2002).

Salmonella can reach into the animal feed by multiple ways and during all production stages (Habimana et al., 2010; Berge and Wierup, 2012). Salmonella species successfully persist in soil, aquatic systems, plant matter, equipment and surfaces of artificial materials with which food comes into contact during processing and production. Formation of multicellular communities known as biofilm, represents normal part of the life cycle in most Salmonella species (Jonas et al., 2007; Steenackers et al., 2012). These strains could be a source of contamination of raw materials and final products in food and feedstock production facilities for months or years.

BOTANICAL RAW MATERIALS AS SOURCE OF SALMONELLA

In addition to food of animal origin that are traditionally considered the main source of salmonella for humans, recent epidemiological studies identified food of plant origin as additional source of contamination with Salmonella (Lapidot et al., 2006; Steenackers et al., 2012; Cevallos-Cevallos et al., 2012). Due to the extremely large adaptive ability to diverse environmental conditions and ubiquitous distribution in the living environment, Salmonella species are able to survive on plant matter by internalizing in stems, cracks and fenced areas, or by forming biofilms that are their natural form on the surface of the plants (Srey et al., 2013). Salmonella spp. can reach plants through contaminated irrigation water or using raw fertilizers (Barak and Liang, 2008; Steenackers et al., 2012). Salmonella can be isolated from agricultural crops and soils six months or more after contamination (Teplitski et al., 2009; Barak et al., 2009). Epidemiological studies confirmed that a number of salmonella-related epidemics in humans are associated with the consumption of contaminated plants (seeds, spawns, leaves, root etc.) emphasizing plants as an important vector for transmission of Salmonella spp. (Heaton and Jones, 2008; Berger et al., 2010; Steenackers et al., 2012). Using microscopic techniques for in-situ visualization, it has been found that salmonella has the ability not only to contaminate but also to colonize and actively invade plants (Lapidot and Yaron, 2009; Barak et al., 2009; Kroupitski et al., 2009; Patel and Sharma, 2010). It was experimentally confirmed that strains of S. Newport and S. Enteritidis actively colonize the surface of the alfalfa and form a biofilm (Barak et al., 2009).

SALMONELLA BIOFILM IN ANIMAL FEED PRODUCTION FACILITIES

Salmonella reach animal feed production facilities through contaminated raw materials (Nesse et al., 2003) and utilize similar survival mechanism on inert surfaces, artificial materials, and in the natural environment. Salmonella creates biofilm on the equipment and working surfaces, with which contaminated raw materials come into contact in food production plants. This way, the sources of process and post-process product contamination are established (Nesse et al., 2003; Vestby et al., 2009a). Salmonella spp. could be found in the rooms for raw material receiving, milling facilities and mixing mills, packing machines, conveyor belts, floors, drainage channels (cross-contamination with dust and aerosols), transport vehicles, as well as storage facilities for finished products (Nesse et al., 2003; Vestby et al., 2009a; Habimana et al., 2010; Steenackers et al., 2012; Giaouris et al., 2012).

Biofilm formation is a strategy that Salmonella spp. utilize to persist on various types of materials: stainless steel, plastic, rubber, glass, wood, marble, and granite that are commonly used in animal feed production facilities (Stepanović et al., 2004; Giaouris et al., 2005; Møretrø et al., 2009; Rodrigues et al., 2011; Carrasco et al., 2012; Steenackers et al., 2012; Giaouris et al., 2012). Contaminated equipment and surfaces can be the source of contamination of final products for months and even years, despite the regular implementation of rigorous cleaning and disinfection measures (Nesse et al., 2003; Vestby et al., 2009a; Møretrø et al., 2009).

In industrial plants, the development of bacterial biofilms is commonly related to improper cleaning and disinfection of the equipment (O`Leary et al., 2013; Srey et al., 2013). Bacteria organized in biofilm demonstrate greater levels of resistance to various stress-related factors such as drying and disinfection (Møretrø et al., 2009; Aviles et al., 2013). In general, implementation of effective programs for cleaning and remediation could prevent the generation of new biofilms; however, there are no effective strategies for eradicating already formed biofilms.

Frequent findings of certain Salmonella enterica serotypes such as S. Agona, S. Montevideo, S. Senftenberg, S. Mbandaka, S. Tennessee, S. Typhimurium, S. Livingstone in animal feed facilities are explained by their ability to persist in the form of biofilm on artificial surfaces (Vestby et al., 2009a; Papadopoulou et al., 2009; Habimana et al., 2010). The development of molecular techniques (pulsed-field gel electrophoresis- (PFGE) and plasmid profile typing) provided evidence that the clones of various Salmonella sero-species (the so-called "house strain") can persist for months or years in food production plants (Nesse et al., 2003; Vestby et al., 2009a; Vestby et al., 2009b; Møretrø et al., 2009; Habimana et al., 2010; Prunić et al., 2016).

SUBSEQUENT CONTAMINATION OF ANIMAL FEED WITH *SALMONELLA* SPP.

Potential sources for subsequent contamination of animal feed with Salmonella species are rodents, birds and insects that can transmit or excrete bacteria through feces, urine and feathers. In some production areas, abiotic factors such as condensation caused by temperature variations can provide sufficient moisture levels required to support growth and formation of salmonella or other bacterial related biofilms (Bogvist et al., 2003; Mynt et al., 2007; Habimana et al., 2010; Jones, 2011; Sokolović et al., 2011). Dust is also considered a potential source of contamination in mills for mixing and splitting, worms and

storage facilities (Jones, 2011). Also, leakage, moisture condensation, nutrient retention in individual devices and conveyors, insufficient warming in thermal processes create favorable conditions for the re-development of microorganisms (Habimana et al., 2010; Jones, 2011).

CONTROL AND ERADICATION MEASURES FOR *SALMONELLA* SPP. IN THE PROCESS OF ANIMAL FEED PRODUCTION

Given the broad presence of Salmonella species, it is unlikely that Salmonella will be eradicated from the food chain (Humphrey et al., 2004) but implementation of control measures should reduce the possibility of contamination.

Control measures for Salmonella spp. during the process of animal feed production can be classified into three categories: a) prevention of entry of Salmonella into production facilities; b) prevention of their replication within facilities c) methods for eradicating the already present microorganisms.

Prevention of contamination measures in production facilities involves control of dust, staff movement, use of the equipment, eradication of the rodents, and prevention of access to wild birds, and sanitation of transport vehicles. Reduction or control of Salmonella spp replication in food production facilities involves the detection of favorable niches for their growth and survival, as well as the elimination of the conditions leading to their growth. Elimination of Salmonella spp. refers to thermal treatment (pelleting, extrusion) or chemical treatment (Habimana et al., 2010; Jones, 2011; Berge et al., 2012).

The replication of microorganisms in animal feed is affected by a number of environmental factors such as moisture content or water activity in the food, relative humidity, pH value, oxidation reduction potential, percentage of fat (plant matter), presence of salt and carbohydrates, amount of nutrients, and temperature (Bogvist et al., 2003; Sokolović et al., 2011). In the process of food production control, dust has been identified as a critical point in contamination and re-contamination and control measures for dust levels in production facilities are established (Butcher and Miles, 1995). Dust is mostly present in raw materials, and consequently in reception areas and storage facilities. Furthermore, dust is largely present in milling and mixing mills, rollers, worm conveyors, transport belts, which is why it represents the challenging task for the control of Salmonella in all animal feed production facilities (Jones, 2011).

Animal feed often contains certain percentage of moisture, which can be increased by absorption from environment during long-term storage, condensation or absorption from humid air (Bogvist et al., 2003). Increased moisture content in food can also occur due to roof damages and leaking.

The major problem in controlling Salmonella in animal feed production facilities is the presence of rodents and wild birds. Rodents are a significant source of food contamination for salmonella (Morita et al., 2006), and according to multiple reports, they are estimated as sources of contamination in 47% of reported cases (Meerburg et al., 2007). Salmonella spp. can be permanent inhabitant of the digestive tract in wild birds and could be excreted into the environment. Wild birds get in contact with Salmonella through feeding at landfills, near and in sewage outlets, through feces and corpses, as well as animal feeds close to the food production facilities (Benskin et al., 2009; Jones, 2011). Measures of pest control and control of the access to wild birds must therefore be an integral part of Salmonella control program in animal feed production facilities.

Transportation vehicles are also identified as a potential source of contamination of raw materials and feed ingredients due to difficulties related to thorough cleaning and disinfection between deliveries, as these processes are time consuming an economically not-feasible (Whyte et al., 2003). Staff employed in animal feed facilities could also contribute to the creation of favorable conditions for the growth of microorganisms due to poor training for handling the equipment and improper implementation of hygiene measures in accordance with the principles of good manufacturing practice.

Pelleting and extrusion processes are considered successful in the elimination of microorganisms from the animal feed (Jones, 2011). The pelleting process encompasses three stages: mixing steam with food, exposing food to high pressure (pellet formation) and removing heat and moisture by cooling. The pelleting process reduces number of Salmonella spp at a range from 50% to 93%. (Jones et al., 1991; Veldman et al., 1995; Jones, 2011). The pelleting process involves the use of a large amount of steam for the destruction of microorganisms, which results in an increase in pellet moisture. High temperatures during the pelleting process reduce the number of micro-organisms, but their numbers often increase later again. The reason for that is the potential contamination in the pellet cooling phase through contaminated dust and condensation due to variations in temperature, which ensures the humidity of the microorganism's life cycle (FAO, 2010; Sokolović et al., 2011).

However, heat treatment of animal feed has shown insufficient efficacy in controlling salmonella (Habimana et al., 2010). Studies have shown that Salmonella spp. survive temperature and humidity fluctuations in the feed production facilities by entering viable but nonculturable (VBNC) state (Møretrø et al., 2009; Habimana et al., 2010; Habimana et al., 2014). The impact of stress factors such as temperature changes, pH values, osmolarity, availability of nutrients, etc., induce

the transition of bacteria into a life-long, but not culturally (VBNC) condition. Such VBNC bacteria are metabolically active but do not replicate.

In addition to heat treatment, in order to eliminate Salmonella from raw materials and ready-to-feed foods, animal feed producers use organic acids, mechanical, physical and chemical methods and various treatments for sanitation (Sauli et al., 2005, Papadopoulou et al., 2009; Berge et al., 2012).

Although thermal treatment of food is generally considered to be the most effective method for eliminating pathogens, in some circumstances it is not sufficient and other options are applied. In such cases, the application of chemical methods may offer alternative protection methods. Treatment of food ingredients and mixtures of nutrients with organic acids or formaldehyde at permitted concentrations may be effective in reducing amounts of salmonella spp. and other microorganisms. In order to reduce or eliminate Salmonella spp. in animal feed, organic acids (ant, propionic, vinegar and butter) and formaldehyde are commonly used (Berge et al., 2012). Organic acids are added in an amount of 0.2 -2% to inhibit the growth of Salmonella spp. (Vahl, 1995; Jones, 2011). Acid efficiency is variable and depends on a number of factors such as acid type, chemical form of acid (free acid or acid s), percentage of moisture in food (EFSA, 2008; Jones, 2011). Adding organic acids to animal feed changes its pH value (pH 4.5 and lower) and creates unfavorable conditions for the growth and survival of Salmonella (Dahiya et al., 2006). Application of chemical treatments in order to reduce or eliminate pathogens has its own shortcomings in terms of price, duration (few days), undesirable effect of acid on metal surfaces of equipment (corrosion), change in food taste and reduction in vitamin concentrations (EFSA, 2008; Jones, 2011). The use of formaldehyde is avoided due to evaporation and its toxicity to humans. Other compounds such as chlorine, peroxides, or ammonium compounds are also used, which also have a residual effect and cause changes in the sensory properties of the final products.

In addition to mechanical cleaning, physical sanitation treatments involve the use of ultrasound at different frequencies, magnetic and electric fields and various types of radiation. The use of ultraviolet light (UV) is a good method for disinfection of air and surfaces, including packaging material. Treatment by UV light is a simple, efficient and economical way of sanitation as compared to other technologies. It is also a cold, dry process that does not create chemical residues. Physical treatments, although they provide good results, are often not applicable and unacceptable due to economic reasons (high costs and technical equipment).

In order to avoid the detrimental effects of chemical treatments and expensive physical processes, more efforts were put towards discovering more

effective biological solutions to reduce or eliminate Salmonella spp. and other pathogenic microorganisms in animal feed. Biological approaches include the use of antimicrobial compounds of plant origin such as extracts of essential oils and various spices, antimicrobial compounds produced by microorganisms as well as enzymes (Simões et al., 2010).

Scandinavian countries (Denmark, Norway and Sweden) have developed integrated Hazard Analysis and Critical Control Points (HACCP) for each step in the food chain, ensuring food safety in order to improve food quality, increase safety and accountability (Lević et al., 2009; Sokolović et al., 2011). This multiple control program has shown great success in eliminating Salmonella spp. from facilities for production of animal feed and products of animal origin, which resulted in a decrease in the incidence of salmonella in humans at an annual level (Crump et al., 2002).

Our country does not have developed and integrated monitoring system that includes facilities for production of animal feed, products of animal origin intended for human consumption, and the occurrence of alimentary infections in humans. In Serbia, the provisions of Article 82 of the Veterinary Law imposed from January 1, 2009 (Official Gazette of the Republic of Serbia 91/05) refer to the implementation of the "HACCP" and are based on hazard analysis and critical Control points in production.

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VANADIUM IN POULTRY NUTRITION

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Abstract

Vanadium (V) is essential element for poultry nutrition. Relatively low level of V (< 10 μg/kg of feed) is known to reduce both growth in chicks and Haugh unit value of eggs. The National Research Council (NRC) recommends the presence of very low levels of V in poultry diets, with the maximum tolerance level (MTL) being 10 mg/kg. Excessive vanadium in poultry diets has been shown to be detrimental to egg production, interior quality of eggs (albumen height), body weight and feed consumption. There is little information on the content of V in feedstuffs. Phosphates are known to be the cause of excessive V in various types of poultry diets. The objective of this study was to obtain information about the content of vanadium in phosphates and poultry feed. The samples were prepared by microwave wet digestion. Content of V was determined by the method of coupled plasma with mass spectrometry on the Agilent ICP-MS 7700. The concentrations of vanadium determined in the examined samples were above the minimum recommended levels for poultry feed, still not exceeding the maximum tolerable values.

Keywords: vanadium, phosphates, poultry feed

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VANADIJUM U ISHRANI ŽIVINE

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Kratak sadržaj:

Vanadijum (V) je esencijalni element u ishrani živine. Relativno niski nivoi V (< 10 mg) smanjuju porast pilića i vrednosti Haugh-ovih jedinica jaja. The National Research Council (NRC) preporučuje veoma niske nivoe V u hrani za živinu, pri čemu je za maksimalni nivo tolerancije utvrđena vrednost od 10 mg/kg. Višak vanadijuma u ishrani živine ispoljava štetne efekte u proizvodnji jaja, negativno utiče na unutrašnji kvalitet jaja, telesnu masu živine i efikasnost iskorišćavanja hrane. Podaci o sadržaju V u hrani i hranivima za životinje su oskudni, ali zna se da fosfatna mineralna hraniva često sadrže visoke koncentracije ovog elementa. Cilj ovog istraživanja je bio da se dobiju informacije o sadržaju vanadijuma u hranivima i hrani za živinu. Uzorci hrane za životinje su pripremljeni mikrotalasnom digestijom, a sadržaj V je određen metodom indukovano kuplovane plazme sa masenom spektrometrijom (ICP-MS). Koncentracije vanadijuma u ispitivanim uzorcima bile su iznad minimalnih preporučenih nivoa u ishrani živine, ali nisu prelazile maksimalne tolerantne vrednosti za živinu za ovaj element.

Ključne reči: vanadijum, fosfati, hrana za živinu

INTRODUCTION

Inorganic elements find in the Earth's crust are often referred to as minerals. The essential minerals/elements are those that have well-defined biochemical functions and must be in the diet of verterbrates for optimal health and productivity (NRC, 2005). In spite of relatively small share of minerals in poultry feed their role in normal metabolism is highly important. The deficiency or excess of particular minerals can result in immune system disorders and impairment of overall health status as well as consequent decrease of production performance. Microelement requirements are relatively low and daily

amounts range from a microgram to one milligram. Accumulation of excessive amounts of microelements higher than those required for normal metabolic activity can induce intoxication symptoms. Some minerals are essential for health and productivity of animals and have well-defined nutritional and biochemical roles. Many other minerals naturally occur at trace levels in feed and tissues of animals but they are not typically suspected to play a useful nutritional purpose (NRC, 2005).

Vanadium is an essential element in various enzymes in algae, bacteria, fungi, and lichens (Nielsen, 2000). This mineral has high number of oxidative states (from -1 to +5). This fact makes it multifunctional element in the body and likely contributes to its ability to have effects at relatively low levels (NRC, 2005).

There are conflicting reports as to essentiality of V for animals. Vanadium deficiency has first been described in rats. In chickens fed rations containing less than 10 μg V/kg, poor growth and reduced development of feathers of the tail and wing was observed. Vanadium deficiency in laying hens often results in poorer albumin quality and hatchability loss. Data on natural vanadium deficiency are still scarce; however, it was established that it can occur if vanadium content in feed mixes is below nutritional requirements of poultry. Vanadium plays a role in lipid metabolism; its deficiency in feed can be associated with decreased levels of blood and bone iron, which can result in abnormal bone development (Puls, 1990).

Studies indicate that vanadium is absorbed from digestive tract at an efficiency rate of 1% or less. Absorbed vanadium is excreted by the kidneys with a minor amount excreted in the faeces (NRC, 2005). Vanadium content in chicken liver ranges 0.018-0.038 mg/kg, in kidneys 0.101-0.180 mg/kg, bones 1.3-6.3 mg/kg and in egg yolk 0.002-0.003 mg/kg dry matter. Somewhat lower vanadium levels were established in all organs in ducks.

Relatively high amounts of vanadium are found in fish-based products. Vanadium in high concentrations can be found in phosphates originating from South Africa, Russia, the United States, Finland and China (NRC, 2005).

The toxicity of vanadium has been investigated more intensively in relation to its presence in phosphates at high concentrations, in the form of calcium orthovanadate. Maximum tolerable level for vanadium in poultry is set at 10 mg V/kg (NRC, 1994), whereas toxicity level is 100-800 mg/kg feed. NRC (2005) suggested the maximum tolerable dietary level (MTL) for poultry being 50 mg V/kg. More recent research indicates that poultry can tolerate up to 25 mg V/kg diet and possibly even up to 50 mg V/kg diet without significant decreases in weight gain and health. MTL depends on the valence of vanadium source

(toxicity tends to increase as the valence increases), chemical form of vanadium source, the period of dosing the length of exposure, and size of the dose.

Bones and kidneys are major target organs for vanadium, but oral intake of vanadium leads to its increased content in liver and magnum. Enzyme inhibition and cell damage is considered the most likely mechanism of vanadium toxicity. Vanadium action competes with ions of Ca, Mn, Zn and Fe for ligand-binding sites and interacts with phosphate ions for a range of metabolic processes. Peroxy-form of vanadium often mimic insulin actions in different cell types (Fantus et al., 1989). Selenium given as a selenite can synergistically potentiate vanadyl-induced cell damage (Zwolak, 2015).

The relevant available literature data provide limited information on the content of vanadium in foods, and therefore the aim of this study was to obtain information on the content of vanadium in phosphates and poultry feed.

MATERIAL AND METHODS

In the present study, we examined 10 samples of phosphates (monocalcium phosphate (MCP), monoammonium phosphate (MAP) of domestic producers and imported origin, 5 samples of imported fish meal and 10 samples of complete poultry feed of domestic producers). The samples (1g) were prepared applying the microwave (Ethos, Lab station Microwave, Milestone), digestion method with the use of the mixture H_2O_2/HNO_3 (1:4, v/v). The samples were transferred to 50 ml volumetric flasks and diluted with deionized water. Analyses of vanadium were conducted by ICP-MS 7700 mass spectrometer (Agilent Technologies).

Statistical analysis was performed by the STATISTICA 12 software package, version 16.0. Data were grouped according to tissue and presented as mean ± standard error, minimum and maximum values.

RESULTS AND DISCUSSION

Average values and minimum and maximum values of vanadium obtained in this study are summarized in Table 1. The concentrations of vanadium determined in the examined samples of complete feed for poultry (chickens and layers) were above the minimum recommended levels for poultry feed, still not exceeding the maximum tolerable values (Table 1).

Table 1. Vanadium concentrations (mg/kg) in poultry feed, feed phosphates and fish meal

Sample	Origin	V (mg/kg)	
		Average ± SD	Min-Max
Complete feed for chickens	domestic	0.641 ± 0.103	0.568 - 0.713
Complete feed for layers	domestic	0.484 ± 0.307	0.137 - 0.844
Phosphates	domestic	22.86 ± 26.36	1.650 - 68.6
Phosphates (MCP)	imported	30.37 ± 14.82	18.10 - 46.84
Phosphates (MAP)	imported	19.65 ± 0.212	19.50 - 19.80
Fish meal	imported	0.161 ± 0.076	0.042 - 0.225

Imported monocalcium phosphates were the most V-contaminated feed ingredient (average value 30.37 mgV/kg). Lower levels of V were found in the domestic MCP (22.86 mg/kg), while monoammonium phosphates were the least contaminated (19.65 mgV/kg). The lowest average concentration of V was measured in fish meal (average value 0.161 mg/kg) and the highest level of this element was measured in MCP of domestic origin (68.6 mg/kg).

The results were compared with results from other authors (Table 2). The relevant available literature data provide only limited information on the content of vanadium in feed. Our results for V concentrations are markedly lower as compared with other investigations (Table 2). Since the origin of phosphates was not taken into account during data interpretation in the cited studies (Table 2), the vanadium levels could not be directly compared with our research. Phosphates with high vanadium content usually originate from Rocky Mountains (USA) with vanadium concentrations reaching even over 6000 mg/kg. The use of these phosphates adds 120 mg of vanadium per kg of feed (Henry and Miles, 2001).

Table 2. Vanadium concentrations (mg/kg) according to various authors

V (mg/kg) according to various authors		
V (mg/kg) min - max	Reference	Samples
47 -796	Sullivan et al. (1994)	Monocalcium phosphate
< 20 - 164	Sullivan et al. (1994)	Dicalcium phosphate
45 - 185	Sullivan et al. (1994)	Thermochemically produced defluorinated phosphate
2 - 1210	Limma et al. (1995)	Dicalcium phosphate dyhidrate

The use of phosphates with high vanadium content can occur in conditions of increased price of phosphates on global market, thus less expensive phosphates of poor quality will be brought to the animal feed market causing consequent vanadium-contamination of feed. In conditions of increased price of phosphates, phytase is recommended to the purpose of better use up of plant phosphorus and reducing phosphate supplementation in feed mixes (Miles and Henry, 2004; Živkov Baloš, 2011).

Under the influence of high doses of vanadium, the internal quality of the egg is reduced, probably as a consequence of the weakening of the magnitude of the magnum during the egg formation. Poorer quality of the egg white has been observed in laying hens fed diets containing 6 mg V from DKP/kg feed, whereas a dose of some 28 mg V from DKP/kg feed resulted in a drop of egg production (Sell et al., 1982). Kubena and Philips (1982) reported that 50 mg V from calcium orthovanadate/kg feed did not cause mortality in laying hens after 28-day research period, whereas 100 mg V from calcium orthovanadate/kg feed resulted in an increase of mortality for 56%. According to the results of Miles and Henry (2004) laying hens fed feed-mixes supplemented with 10 mg V/kg feed had poorer albumin quality than birds from the control group (fed diet without vanadium supplementation). The quality of albumen has continued to drop during consequent two days after changing the feed and removing excess vanadium from hens' diet to reach the albumin quality of laying hens from the control group as late as after six days. Odabaşi et al. (2006) reported that feeding hens with diet supplemented with 15 mg V from ammonium-monovanadate/kg feed had adverse effects on eggshell pigmentation.

Detrimental effects of excess vanadium in feed can be alleviated by adding dietary cottonseed meal and vitamin C (Ousterhout and Berg, 1981; Whitehead and Keller, 2003; Odabaşi et al., 2006) or combination of vitamin C, vitamin E or β-carotene (Miles et al., 1997). Henry and Miles (2001) suggested that feed known to contain phosphates with high vanadium content should be supplemented with potentially harmful amounts of copper (400 ppm) and mercury (100 ppm) in order to reduce detrimental impact of excess vanadium. Puls (1990) reported the following: 6 mg V/kg feed negatively affects the albumen quality and growth rate of poultry; levels higher than 40 mg V/kg decrease egg hatchability and body weight of laying hens; levels higher than 80 mg V/kg result in intensive molting, whereas levels above 100 mg V/kg increase the mortality rate.

Phytase can play a role also in the sphere of manure management since feeds with high vanadium content increase faecal moisture for 10%, which may pose problems in manure manipulation on poultry farms especially in

view of controlling the number of flies that correlates with the manure moisture content (Henry and Miles, 2001).

CONCLUSIONS

Based on data presented in this paper the biological role of vanadium in normal metabolism during production of poultry is very important. Essential role of V in poultry nutrition is still under investigation, while toxicity was relatively well established a long time ago. Even though some feeds might represent potential source of harmful amounts of vanadium, the combination of relevant quality control programs in animal feed industry as well as application of good production practices and adequate education of nutritionists can substantially reduce the risks associated with poultry feed contamination. Future studies and additional research are needed to define essentiality and toxicity of vanadium for poultry and possible interaction with other nutrients in the feed.

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Svi podnaslovi se pišu velikim boldiranim slovima. U radu koristiti kratke i jasne rečenice. Tekst treba da bude u duhu srpskog jezika, a sve strane izraze za koje postoje odgovarajuće reči u našem jeziku ne treba koristiti. Za nazive lekova koristiti isključivo njihova internacionalna nezaštićena imena (tj. generička imena) i pisati ih onako kako se izgovaraju (ne na latinskom ili engleskom jeziku). Ukoliko se, pak, želi ipak istaći ime nekog preparata, onda se njegovo ime (zajedno sa imenom proizvođača) stavlja u zagradu iza naziva aktivne supstancije. Uređaji ili aparati se takođe označavaju njihovim trgovačkim nazivima, s tim što se i ovde u zagradi mora navesti ime i mesto proizvođača. Za svaku skraćenicu, koja se prvi put javlja u tekstu treba navesti i pun naziv. Skraćenice nikako ne koristiti u naslovu, a u kratkom sadržaju ih takođe treba izbegavati. Decimalne brojeve pisati sa zarezom i bar još jednom nulom. Obim rukopisa bez priloga, ne treba da bude veći od 8 stranica kucanog teksta. Izbegavati veliki broj priloga.

Tabele se označavaju arapskim brojevima (iznad tabela) po redosledu navođenja u tekstu, sa nazivom na srpskom jeziku. Koristiti font Times New Roman, veličina slova 12 pt, sa jednostrukim proredom i bez uvlačenja. Ukoliko se u tabeli koriste skraćenice treba ih objasniti u legendi ispod tabele.

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Sheme (crteži) se označavaju arapskim brojevima (ispod shema) po redosledu navođenja u tekstu, sa nazivom na srpskom jeziku. Koristiti font Times

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Poglavlja rada su: **Uvod, Materijal i metode rada, Rezultati, Diskusija (ili Rezultati i diskusija zajedno), Zaključak i Literatura.**

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Rezultati. Rezultate prikazati pregledno uz pomoć tabela ili grafikona. Svuda treba da stoji redni broj i tekst, koji opisuje šta određena slika, tabela, grafikon prikazuje. Redni broj sa tekstom se stavlja iznad tabela, a kod svih ostalih prezentacija ispod.

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2. **Knjige i druge monografije:**
Qinn P.: Clinical Veterinary Microbiology. London, Mosby, 1998

3. Poglavlje u knjizi:
Vidić B., Boboš S., Lako B., Lončarević A.: Dijagnostika bruceloze. U: Aleksandar Lončarević, Bruceloza svinja, Beograd: Poljoprivredni fakultet, 2000, str. 47-49.

4. **Članak u zborniku radova sa naučno-stručnog skupa:**
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3. **Chapters in books:**

Vidić B., Boboš S., Lako B., Lončarević A.: Dijagnostika bruceloze. U: Aleksandar Lončarević, Bruceloza svinja, Beograd: Poljoprivredni fakultet, 2000, str.47-49

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