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EMERGING VIRAL DISEASES OF CYPRINIDS

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Abstract

The intensive aquaculture remains the world's fastest growing sector producing food of animal origin. In fact, it is the only animal food-producing sector growing faster than the human population, and provides an acceptable supplement to and substitute for wild fish. A number of cyprinid diseases have emerged globally and their study has become increasingly important. The expansion of aquaculture, which has relied heavily on the movement of animals and farming species new to aquaculture, has been paralleled with disease emergence. In the last few years several emerging or re-emerging fish diseases have been detected in cyprinid fish populations in Serbia. In this paper, the authors overview the major viral threats for cyprinid fishes in Serbia.

Keywords: cyprinids, fish viruses, emerging diseases

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NOVE I PRETEĆE VIRUSNE BOLESTI CIPRINIDA

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Kratak sadržaj

Na svetskom nivou, intenzivna akvakultura je najbrže rastući sektor za proizvodnju hrane životinjskog porekla. Šta više, to je jedini sektor koji raste brže od ljudske populacije i obezbeđuje značajnu dopunu i zamenu izlovljavanju riba iz otvorenih voda. Na žalost ovaj trend je praćen pojavom brojnih bolesti ciprinida na globalnom nivou. Ekspanzija akvakulture, koja se u velikoj meri oslanja na trgovinu i promet riba, uključujući i vrste koje ranije nisu korišćenje u akvakulturi je dovela i do istovremenog širenja patogena. U poslednjih nekoliko godina u populaciji ciprinidnih riba u Srbiji otkriveno je nekoliko novih bolesti, a uočena je i ponovna pojava ranije prisutnih bolesti. U ovom radu autori prezentuju najznačajnije nove i preteće bolesti ciprinida u Srbiji.

Ključne reči: ciprinidi, virusi riba, nove i preteće bolesti

INTRODUCTION

Diagnostic testing of cyprinids often results in the occurrence of the cytopathic effect on cell culture and virus isolation, but fortunately, they are mostly benign. For a long time, spring viremia of carp was single viral disease of cyprinids listed by the OIE. In 2007 koi herpesvirus disease (KHVD), caused by the Cyprinid herpesvirus 3 (CyHV-3), was listed by the World Organization of Animal Health (OIE) and listed as a non-exotic disease in the European Union, related to the Directive 2006/88/EC. Recently, concern has been shown about diseases caused by other viruses, primarily herpes and pox viruses. Goal of this paper is to present an overview of current viral threats for cyprinid fish in Serbia.

Carp Edema Virus (CEV)

Although known for a long time as a cause of disease in koi carp in Japan, carp edema virus (CEV) has recently been recognized as a global threat to the koi and the common carp aquaculture. Carp Edema Virus (CEV) is the etiological agent of Carp edema virus disease (CEVD), firstly described in Japan in the 1970's as a viral edema of juvenile color *Cyprinus carpio* koi and later morphologically identified as a poxvirus. In koi, diseased fish exhibit erosive or hemorrhagic skin lesions with edema of the underlying tissues, thus the disease was originally named "viral edema of carp" (Oyamatsu et al., 1997). The disease has also been referred to as "koi sleepy disease" (KSD) because infected koi become lethargic and unresponsive (Miyazaki et al., 2005). After Japan, disease outbreaks in koi at import sites and in hobby ponds has been detected in USA since 1996.

In Europe, outbreaks of KSD and PCR detections of CEV-like virus were reported since 2009. In 2009 CEV first detection in Europe was in England in imported diseased koi. Low levels of CEV-like virus were also detected in healthy koi imports from Israel and Japan at ornamental fish wholesalers during 2013 in the UK (Way and Stone, 2013). In 2016, the disease outbreaks in koi are recorded in India and in China (Swaminathan et al., 2016; Zhang et al., 2017)

In koi carp, the virus causes severe damage to gill lamellae, leading to hypoxia and lethargy, which manifests as sleepy behaviour, and mortality can reach 80-100% (Ono et al., 1986; Lewisch and Gorgoglione et al., 2015).

In common carp, carp edema virus has been initially detected in the United Kingdom and in the Netherlands in 2012 (Way and Stone, 2013; Haenen et al., 2014). After those initial detections, the virus has been detected in Czech Republic and Poland in 2013 (Vesely et al., 2015; Matras et al, 2017), followed by Austria and Italy in 2014 (Lewisch et al., 2015; Pretto et al., 2015), and more recently in Hungary in 2016 (Adamek et al., 2018a). The virus has been detected in Lithuania and Croatia in 2018 (Adamek et al., 2018b).

In common carp, during outbreak of the disease, fish congregate lethargically under the water surface or lie at the bottom of the tank and die in the following 2 weeks. Gross changes with enophthalmos, gill hyperplasia, and anal ulcerative inflammation are evident in the infected fish (Jung-Schroers et al., 2015; Lewisch et al., 2015).

In Serbia, episodes of disease characterized by a typical sleepy behaviour, enophthalmia, generalized oedematous condition and gill necrosis, leading to hypoxia and mortality of up to 20% were observed during 2015 and 2016

in spring time on many carp farms, but causative agent was detected in 2017 (Radosavljević et al., 2018). During April and May 2017 unusual mortalities occurred in common carp at water temperatures between 9°C and 15°C. Diseased fish showed similar clinical signs and experienced about 20 percent mortality. Fish were lethargic, congregated around pond margins or at the water surface and became increasingly unresponsive.

Herpesviral hematopoietic necrosis (HVHN)

Cyprinid herpesvirus 2 (CyHV-2, Goldfish herpesvirus, GHV) is the causative agent of herpesviral haematopoietic necrosis (HVHN) in goldfish (*Carassius auratus).* The disease was first identified in Japan in 1992 and 1993 in goldfish (*Carassius auratus*) (Jung and Miyazaki, 1995). Until recently, Cyprinid herpesvirus 2 (CyHV-2, Goldfish herpesvirus, GHV) was described as the etiological agent of a disease named Herpesviral hematopoietic necrosis (HVHN)**,** which occurred only in goldfish (*Carassius auratus*). This disease was first described in western Japan in the 1990s, causing high mortality in goldfish and occurring during the spring and autumn (Jung and Miyazaki, 1995). Since then, CyHV-2 has been reported in North America (Goodwin et al, 2006) as well as Taiwan (Chang et al, 1999) and Australia (Stephens et al., 2004). In Europe, the virus was first detected in the United Kingdom in 2006 **(**Philbey**,** 2006), followed by Switzerland in 2010 (Giovannini et al., 2015), Italy in 2013 (Fichi et al., 2013), France (Boitard et al., 2016) and Germany (Haenen et al., 2016). Clinically, affected goldfish display signs such as apathy, pale gills, increased respiratory rate, and ascites. Histopathological consistent finding in CyHV-2-infected goldfish is necrosis of the hematopoietic tissue in kidney and spleen. The disease in goldfish was not detected in Serbia, but outbreak of a disease caused with CyHV-2 was recorded in Prussian carp (*Carassius gibelio)* in 2017.

Recently, the virus has been detected in Prussian carp (*C. gibelio)* in China (Wang et al., 2012), and also in Czech Republic (Daněk et al., 2012), the Netherlands (Haenen et al., 2016) and in Serbia (Radosavljević et al., 2018). In Serbia, the outbreak of herpesviral hematopoietic necrosis in Prussian carp lasted for 1 week, at a water temperature of 26°C, and did not affect other fish species. Some fish showed a whitish slime layer over their eyes and an erythema of their skin, sometimes with haemorrhagic scales. Since the reports suggest that this virus has been spreading, and also CyHV-2 has the potential to infect other species of the genus *Carassius* as a host, understanding the properties and the preventive measures of this disease has become important.

CONCLUSIONS

Understanding of the emerging cyprinid viruses is still evolving. It is very probable that more cyprinid viruses will be detected as improved diagnostic methods come into general use. However, for detection of different emerging viruses of cyprinids, advanced diagnostic techniques have had to be used. The present findings indicate that the prevalence and spread of CEV and HVHN must be closely monitored in our country to avoid potential economic losses.

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ANIMAL WELFARE STANDARDS IN RED DEER (CERVUS ELAPHUS) FARMING

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Abstract

In the last decades, significant interest of consumers in game meat and products has reflected in the worldwide development of wild animal farming. Among the different farmed species, the Red deer (*Cervus elaphus*) takes an important place, being farmed in several different countries around the world and in most countries within the European Union. In this regard, the European Union has promulgated laws and regulations aimed at defining and organizing this business as well as ensuring the welfare of the animals. So far, relevant data on this topic are not available in Serbia probably due to the lack of deer farms across the country. In this paper, we will review the major issues related to Red deer keeping (i.e., housing, feeding and watering practices, management and handling, transport and velvet harvest) and give the proposals as a basic guideline principles for future Red deer farming in Serbia.

Key words: EU legislation, wildlife well-being, farm

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STANDARDI DOBROBITI ŽIVOTINJA NA FARMAMA EVROPSKOG JELENA (CERVUS ELAPHUS)

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Kratak sadržaj

Poslednjih decenija uočava se povećan interes potrošača za meso i proizvode od divljači, što je dovelo do razvoja farmi za uzgoj divljih životinja širom sveta. U odnosu na različite vrste farmskih životinja, Evropski jelen (*Cervus elaphus*) zauzima značajno mesto, obzirom da se uzgaja na farmama u velikom broju država u svetu i u većini članica Evropske Unije. U tom smislu, Evropska Unija je donela zakone i propise u nameri da definiše i uredi ovaj oblik poslovanja, kao i da osigura dobrobit životinja. Utisak je da u Srbiji nema informacija u odnosu na ovu specifičnu materiju, verovatno i zato što na njenoj teritoriji nema farmi jelena. U ovom radu, mi opisujemo najvažnije teme vezano za gajenje Evropskog jelena (npr. držanje, obezbeđenje hrane i vode, upravljanje i manipulacija sa životinjama, transport i seča rogova) i dajemo predloge kao vodič o osnovnim principima za buduće farme Evropskog jelena u Srbiji.

Ključne reči: EU zakonodavstvo, blagostanje divljih životinja, farme

INTRODUCTION

The development of deer farming in the world

The Red deer (*Cervus elaphus*) population has significantly increased in Europe during the last decades, both in population density and in areas occu-

pied (Milner et al., 2006). The increasing interest of consumers in the so-called "free-range products" is particularly reflected in the worldwide development of wild animal farming (Piasentier et al., 2005). According to the last reports (Kotrba, 2014), more than 10,000 deer-farming operations exist in several countries of the EU (European Union), counting about 300.000 animals. Apart from the reindeer (*Rangifer tarandus*), which has been the most widely bred deer species in many Northern countries for the last two thousand years (Clutton-Brock, 1987), the most common species housed in modern deer farms is surely the Red deer (Reinken, 1990; Bartos and Siler, 1993). Although currently there are no registered deer farms in Serbia, it is important to lay the foundation for the future and get prepared for the establishment of this new agricultural option. Thus, it would be useful to design specific animal welfare policies pertaining to the following topics: I) Accommodation and housing II) Provision of feed and water III) Management and handling IV) Transport and V) Velvet harvest. Therefore, we hereby present a scientific investigation and short analysis of the existing legislation in two countries of the European Union, making a summary of the available regulation and research documents concerning the establishment, functioning and management of deer farms.

I. Accommodation and housing

Basic technical requirements of a Red deer farm pertain to the minimum size of the pen and stocking density. The criteria regarding the optimal density of animals for enclosed area are defined in the First Animal Keeping Ordinance and shown in Table 1. (Austrian regulation, 2004).

Table 1. Minimum requirements for keeping of Red deer on the farm

Species	Minimum pen size	Maximum stocking density	Minimum area of weather protection
Red deer	2.0 ha	10 adult animals*/ ha	4.0 m² / one adult animal*

*Explanation:

1. Two animals up to 18 months of age correspond to one adult;
2. Three animals up to 12 months of age correspond to one adult;
3. Animals from 6 to 12 months of age are equivalent to one adult;

4. Newborns up to 6 months of age are not to be considered in the stocking density.

These stocking rates are set in order to prevent detrimental effects on production parameters deriving from overcrowded or inadequate farming conditions.

Keeping of male and/or female animals

According to the biology of the species, a breeding group must consist of at least one breeding male (stag) and three breeding females (hinds) (Austrian regulation, 2004). Among the species of Cervids currently existing, the Red deer shows one of the highest levels of sociality (Mattiello and Mazzarone, 2010). Indeed, the young males will aggregate in so called "bachelor groups", whereas the females will keep together with other females and the calves of the year. Older males are rather solitary and join the females only just before the rut, starting to wander again right after (Clutton-Brock et al., 1982). Due to this behavioral peculiarity of the species and where the clinical state of the animals will not require a period of quarantine, it will be consequently necessary to house the animals in groups. It is required to form a unit of at least three individuals to avoid unnecessary stress that may negatively affect the reproductive cycle of the animal, as well as its social and sexual behavior.

Provision of shelter

Keeping of the deer must be managed in paddocks that can be arranged so that at least 5% of the surface of the whole pen is covered in shrubs or trees or otherwise shielded. If this is not the case, an additional weather protection must be available. This must be composed of at least two walls and covered with a roof. Its size must be sufficient to provide shelter to all the animals simultaneously (Austrian regulation, 2004). As deer are not well insulated, protection and shelter from climatic extremes should be provided (British regulation, 2006). The provision of sheltered areas is also particularly important during the calving season in paddocks where parturitions occur, as the Red deer is a "hider" species (Putman, 1988).

Design of the fence

Fences are one of the most important features of a deer farm. The fence must be designed to make it impossible for the animals to jump it over or break

it through. Moreover, it should be designed to prevent the animals from being injured. The contours of the fence must not have corners with acute angles or form a funnel (Austrian regulation, 2004) and its condition, together with the gates', must be checked daily in order to assess potential damages (e.g. fallen trees, willful damage, etc.). Besides preventing the animals from escaping, the role of the fence is to guarantee adequate protection to the stock from predators. Therefore, they need to be of suitable height (minimum 2.5 meters) and well buried into the ground (Austrian regulation, 2004). Barbed wire has to be avoided, as it might cause severe injuries in the case of frightened deer trying to jump out of the fenced area (Kilgour and Dalton, 1984). Electric fences have been successfully used in some cases and they have been recommended by several authors (Kilgour and Dalton, 1984; Reinken, 1990). .

The wallowing

Another behavioral need that must be satisfied is the "wallowing" (i.e., the action of bathing and rolling in the mud) (Kilgour and Dalton, 1984). It requires the existence of wet depressions in the ground big enough to host more animals at the same time and deep enough to allow the animals to be completely covered in mud up to the withers. Wallowing is believed to have a controlling actions on the ecto-parasites (e.g., ticks and lice), as well as to reduce the body temperature of the animals during particularly hot days. Even if it is included in the behavioral repertoire of both sexes, in the Red deer the stags use wallowing for marking their territory during the rut, urinating in the mud pools before rolling in it in order to enhance their scent (Clutton-Brock et al., 1982).

II. Feeding and water supply

Stocking rates must be managed so that every individual will have the chance to forage when grazing on the pasture and consequently maintain an adequate body condition in winter. The facilities for additional food (i.e., hayracks) must be also roofed and raised from the ground level in order to avoid the contamination of the supplies by means of feces and urine. The distance between the boards of the rack should be wide enough to allow the animals to feed comfortably avoiding the risk of being stuck with the head between the planks. Both if the animals feed on pasture or on hay, their diet may be occasionally enriched with potatoes, beets, apples, pears, soy, barley and oats (Dusek et al., 2007) and by salt licks. As regards the water provision, deer must

have continuous access to a plentiful supply of fresh, clean water. The water troughs must be regularly cleaned and checked as the water must not become stagnant and each water bowl must be systematically checked to ensure that they are functioning correctly (BDFPA, 2016).

III. Management and handling

The handling facility

The design of facilities for the capture, handling and manipulation of the animals must be done by keeping in mind the behavior of the species and taking into account the perception that deer have of the surrounding environment and of human beings (Blackshaw, 2003). As the Red deer is a social species (Putman, 1988), isolation is a stressful event for them that could lead to panic (Blackshaw, 2003). Therefore, it is not recommended to confine them alone for long periods, except for quarantine or specific management purposes (Austrian regulation, 2004). A pen book with all the information about the stock (input and output of animals, treatments, findings, deaths, etc.) should also be kept (Austrian regulation, 2004).

Veterinary control in general

The Farm Animal Welfare Committee (British regulation, 2013) marks some handy guidelines regarding the veterinary controls of farmed animals. Medical checks are necessary for all the deer farms. The animals have to be handled (up to 5 times a year) for routine husbandry procedures, as well as for occasional veterinary procedures. The handling can be stressful and physically demanding for the animals, but this factor can be minimized by using well-designed facilities. Veterinaries must also perform an "ante-mortem" inspection in case of deer killing or slaughtering (British regulation, 2013).

IV. Transport

Transport of live animals

In 2005, the Council Regulation (EC) No 1/2005 (Council Regulation, 2005) on the protection of animals during transport and related operations defined the roles and the responsibilities of everybody involved in the transport chain in order to ensure the safety and the wellbeing of the animals. For this

purpose, the following four mandatory organizational steps have been codified:

i) Control of the conditions of animals and their fitness for travelling.

For all mammals, the following animal categories are considered not eligible for transport: animals that are too young, sick or injured, newborns whose navel is not completely healing, heavily pregnant females and females who have given birth in the previous week before the transportation. According to a specific rule for Cervine species, animals in velvet are not allowed to be transported.

ii) Plan the journey assuring that travelling time is kept to the minimum and the conditions of animals can be periodically checked. For any type of transport, the drivers must hold a relevant certificate of competence, and drivers or handlers need to receive a specific training. There are three different classes of travel: a) Journeys less than 65 kilometers (training without formal qualifications required) b) Journeys over 65 kilometers (relevant certificate of competence required) c) Long travels (>8 hours). In order to receive permits for this class of travel, vehicles inspection and approval are legal requirements. A journey log is also mandatory.

iii) Ensure that the mean of transport and all the loading and unloading facilities are well designed and in good conditions. The transportation vehicles must be non-slip, easy to clean and disinfect, provide weather protection and sufficient air supply, prevent breakout, and provide to the animals enough space to lie down. The marking of the vehicles or the means of transport must be carried out with a symbol for living animals in an upright position (Austrian Regulation, 2004).

iv) Ensure that the animals can receive food and water every time it is needed, rest is possible and enough space is given. In case of extreme weather conditions, it is necessary to take special measures. In case of particularly hot weather, for example, animals must easily access to shade and water, and have to be regularly inspected. People responsible for the transport should have emergency contingency plans in place for every journey (Council Regulation, 2005).

V. Velvet harvest

In many EU countries, the removal of any part of the growing antlers (i.e., before the natural shedding of the velvet) for commercial purposes is forbidden (British regulation, 1980; Austrian regulation, 2014). Antlers removal can be carried out legally only when they are fully calcified and the velvet has been shed (British regulation, 2013). There should be no pain associated with the operation and any accessory cause of stress must be minimized.

DISCUSSION

The main welfare issues in deer farming are related to accommodation, housing, management, handling, transport, and velvet harvest (Burton, 1993). As it is inferred from above mentioned examples, some of the countries of the European Union (e.g., Austria and United Kingdom) have a detailed regulation about the deer farming. This refers to the aspects of health, keeping (area, buildings and equipment), protection and welfare of the animals. We expect that the above mentioned list of requirements will meet the needs in Serbia as it had already happened in other European countries (i.e. Austria, United Kingdom) and it will result in proposing the appropriate guidelines for the future Serbian deer farmers. Obviously, it will be necessary to adapt the existing provisions to the Serbian agricultural environment, keeping in mind that deer farms still do not exist in our country. A detailed analysis and feasibility study would represent another important step that has to be taken before the establishment of deer farms in Serbia. After that, it will be necessary to plan a specific strategy for developing of this typology of agribusiness (BDFPA, 2016). Finally, correspondingly with the common practice in many EU countries, it would be very important to establish a regional association of deer farmers as soon as the first farms arise.

CONCLUSION

According to the BDFPA (2016), deer that have been farmed and are accustomed to human presence (winter housing, regular handling, etc.) behave similarly to cattle and sheep and, with appropriate care and facilities, they can be housed and transported with minimal stress. The low labor regime for deer farming means that it can easily compare to other livestock and arable enterprises.

In this way, the above mentioned regulations could be a good starting point for drafting basic guideline principles for Red deer farming in Serbia. Additionally, considering the lack of information on the topic, it is recommended to perform an extensive scientific research on the status of existing deer parks in Serbia. This project would best be carried out in partnership with a competent institution of the European Union. Moreover, raising awareness about the importance of new regulations and measures for ensuring and monitoring wildlife health should be a prerequisite in the education of all the professional figures involved (deer farmers, technicians, veterinarians, etc.). According to the experiences of the countries of the European Union with

well-established deer farms (i.e., Austria, United Kingdom, Germany) we can make some estimations in terms of future market trends. Specifically, because of the substantial demand for game meat on the markets in whole Europe, we expect an increase of deer farming production in this part of the world. An additional benefit of deer farming is the production of trophies, highly valuable and particularly requested by Chinese companies.

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AVIAN PATHOGENIC *ESCHERICHIA COLI*: DIAGNOSIS, VIRULENCE AND PREVENTION

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Abstract

Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis within poultry flocks all around the world. There is a number of virulence mechanisms involved in the disease process in poultry and determination of some of the responsible genes is important for diagnosis of colibacillosis. In this work, research data regarding diagnostics of APEC and how certain clonal lineages could cause infection in different hosts is presented. In order to determine virulence genotype of APEC, multiplex polymerase chain reaction, based on a published sequence of seven pairs of primers (*iroN*, *ompT*, *hlyF*, *iss*, *iutA*, *elitC* and *cvaC*), was used in our laboratory. It was established in the research of other scientists that isolates with two or more of these genes can develop pathogenic phenotype, while isolates with one or none of the genes are mostly commensal *E. coli*. Additionally, virulence mechanisms in APEC were also briefly described. It was emphasized that resistance genes and virulence genes are sometimes co-located on the same plasmid and that such plasmids could be shared among related or unrelated bacteria species. Since APEC often confers resistance to antibiotics, the therapy is less effective in poultry with multidrug resistant strains. It was concluded that good management practice, treatment with probiotics and/or vaccination are necessary to reduce colibacillosis outbreaks. This approach is even more pronounced since APEC resides in intestine of healthy poultry and could cause disease if poultry is exposed to various stressors.

Keywords: *Escherichia coli*, antibiotic resistance, virulence mechanisms, diagnostic, prevention

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AVIJARNA PATOGENA *ESCHERICHIA COLI*: DIJAGNOZA, VIRULENCIJA I PREVENCIJA

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Kratak sadržaj

Avijarna patogena *Escherichia coli* (APEC) je uzročnik kolibaciloze u jatima živine širom sveta. Brojni mehanizmi virulencije uzročnika su uključeni u patogenezu oboljenja, zbog čega je determinacija gena virulencije važna u dijagnostici kolibaciloze. U ovom radu prezentujemo rezultate istraživanja koji se odnose na dijagnostiku APEC-a i pojavu infekcija izazvanih određenim klonovima kod različitih domaćina. Za određivanje virulentnog genotipa izolata APEC-a, primenili smo prethodno opisanu metodu multipleks PCR sa sedam parova prajmera (*iroN*, *ompT*, *hlyF*, *iss*, *iutA*, *elitC* and *cvaC*). Rezultati ranijih istraživanja pokazuju da izolati APEC koji imaju dva ili više gena virulencije mogu da razviju patogeni fenotip, dok su izolati bez ili sa jednim genom, uobičajeno komensali. Kod izolata APEC-a često se ustanovljava rezistencija na antibiotike, zbog čega je terapija manje uspešna u tretmanu infekcija izazvanih sojevima sa multiplom rezistencijom. Uz prikaz mehanizama virulencije APEC-a, u radu iznosimo i literaturne podatke koji ukazuju da su geni za virulenciju i rezistenciju na antibiotike ponekad locirani na istim plazmidima koji lako mogu da se prenesu na druge srodne i nesrodne vrste bakterija. Zbog činjenice da se APEC može naći u intestinalnom traktu i zdravih životinja, u zaključku istraživanja ukazujemo na značaj primene prakse dobrog menadžmenta, upotrebe probiotika i/ili vakcinacije u cilju smanjenja pojava kolibaciloza na farmama živine.

Ključne reči: *Escherichia coli*, rezistencija na antibiotike, mehanizmi virulencije, dijagnostika i prevencija

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) causes outbreaks of colibacillosis in poultry all around the world. The disease is often provoked if poultry is exposed to various stressors. Infections with viruses causing acute disease and immunosuppression (such as Infectious bursal disease virus) or those which affect the respiratory tract of chickens (such as Infectious bronchitis virus) are often followed by the "episodes" of colisepsis. Sometimes APEC induces higher mortality rate in poultry flocks than the viruses themselves. However, many other non-infectious factors may influence disease symptoms caused by APEC. Those non-infectious factors are often overcrowding, ammonia, heat during summer months, poor sanitation etc. (Barnes et al., 2008). Besides mortality, APEC can also cause decreased body weight in chickens and condemnation of carcasses at the processing plants, which lead to economic losses for the farmers. It is also important to note that many APEC isolates are multidrug resistant, which reduces the effectiveness of antibiotic treatment. As APEC is an invasive bacterial pathogen, it is not possible to completely eliminate the clinical symptoms in poultry flocks, even if farmers substantially improve management to decrease the risks of colibacillosis.

DIAGNOSIS

The basic diagnostic criterion for identification of *E. coli* is its characteristic growth on McConkey agar (bright pink colonies surrounded by precipitate). The colonies may also appear as mucoid, large and sticky. Brief biochemical characterization include indol (+), methyl red (+), Voges Proskauer (-) and citrate (-). Automatic systems are also utilized for the identification of *E. coli,* mostly by VITEK and MALDI TOFF mass spectrometry. The polymerase chain reaction (PCR) is also suitable for the detection of the specific genes such as *gadA*/*B* genes that encode enzyme glutamate decarboxylase in *E. coli* (McDaniels et al., 1996). In the case of coli sepsis, *E. coli* is isolated from parenchymatous organs of the chicken and/or from the bone marrow. However, there are more localized pathological changes, such as coliform omphalitis, coliform-cellulitis, swollen head syndrome, coliform orchitis, veneral colibacillosis, coliform salpingitis, diarrheal disease, which require specific diagnostic methods. Colisepticemia develops when APEC enters the bloodstream, often when the infection of the respiratory tract is already established. It is important in diagnostics to take into account that other bacteria, such as pasteurella, salmonella, streptococci and chlamidiophila, also cause acute septice-

mic disease and their identification must be included in differential diagnosis (Barnes et al., 2008).

In the research work of Johnson et al., (2006) and Johnson et al., (2008a) it was shown that *iroN*, *ompT*, *hlyF*, *iss*, *iutA*, virulence genes are found in more than 70% of APEC isolates. These genes are located on a large virulence plasmid, called ColV plasmid (Johnson et al., 2008a). *E. coli* isolates with two or more of the aforementioned virulence genes could develop pathogenic phenotype for day old chickens, while isolates with one or none of the virulence genes (*iroN*, *ompT*, *hlyF*, *iss*, *iutA*) are usually commensal strains (Johnson et al 2008a, De Oliveira et al., 2015). Therefore, molecular detection of virulence genes in presumptive APEC isolates is useful in order to improve diagnostic protocol.

PHYLOGENY AND VIRULENCE OF *Escherichia coli*

Phylogenetic characterization of *E. coli* is an important molecular typing method. Based on PCR detection of two genes and a DNA fragment (with primers chuA1/2, yjaA.1/2 and TspE4C2.1/2.2), *E. coli* is assigned to four main phylogenetic groups: A, B1, D, B2 (Clermont et al., 2000), as it is shown in Figure 1. This scheme is used especially for PCR typing of extraintestinal *E. coli* isolates which cause infections of the urinary tract (UTI) in humans and animals. Regardless of the origin of the isolates (human or animal), these extraintestinal pathogenic *E. coli* (ExPEC) belong mainly to phylogenetic group B2.

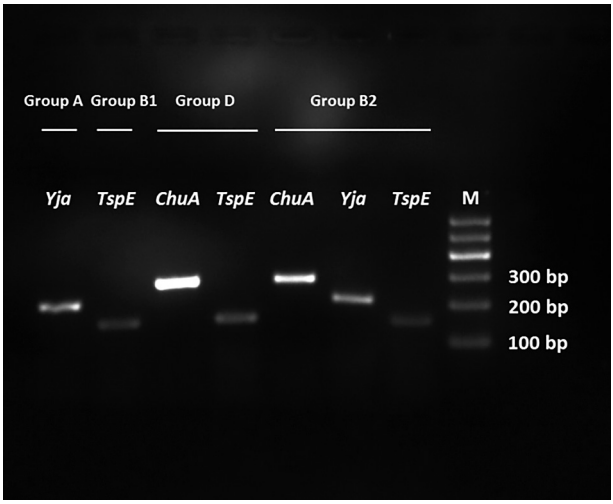


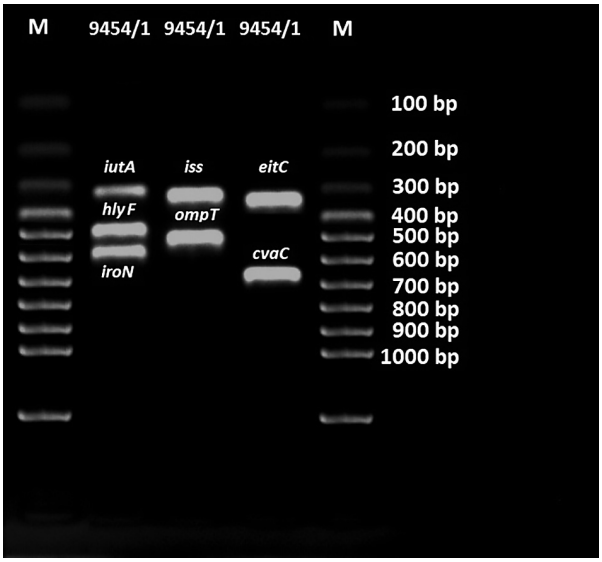
Figure 1. Phylogenetic characterization of *E. coli* isolates by PCR, with three set of primers (Figure prepared by Dalibor Todorović, Scientific Veterinary Institute "Novi Sad")

Such close relationship among the ExPEC isolates is associated to their virulence plasmids. In the research work of Johnson et al., (2008a) and Johnson et al., (2009), APEC isolates from poultry lesion due to colibacillosis belong to phylogenetic groups A, B1, D and to group B2. Moreover, the link between phylogenetic type, multi-locus sequence type and virulence type was found between human ExPEC and APEC isolates in the research work of Moulin-Schouleur et al., (2007). It was documented that ExPEC and APEC of serotypes O18:K1, O2:K1 and O1:K1, that belong to subcluster B2-1, are highly pathogenic for day old chickens and, in addition, human ExPEC-B2-1 isolates have caused avian colibacillosis in 3.5 week old birds which was similar to diseases caused by APEC. High genetic similarity between those isolates and their pathogenicity indicate that there is no strict host specificity for some of the ExPEC and APEC isolates. APEC isolates belonging to serotype O78 belong to phylogenetic group B1 and D and, according to their MLST type and common virulence patterns, they present a different phylogenetic cluster comparing to group B2-1 (Moulin-Schouleur et al., 2007). It is worth mentioning that some of the *E. coli* isolates from retail products were found to contain virulence genes from PAI (Pathogenicity islands). In the same research, the gizzard content was assumed to be the source of contamination and significant number of *E. coli* isolates from retail belonged to phylogenetic group B1 (Johnson et al., 2009).

In theory, APEC poses a threat to human health, but the concrete experimental evidence for this assumption is still lacking. Therefore, to evaluate the ability of *E. coli* of animal origin to cause disease in humans, animal infection models are utilized by inducing sepsis in mouse and meningitis in neonatal rats. Such animal experiments are used to study the ability of APEC to establish infection in different hosts (Johnson et al., 2012). In an example in the research of Skyberg et al., (2006) it was shown that commensal *E. coli*, which had conjugative virulence plasmid, significantly increased the ability of *E. coli* to kill chicken embryo, its ability to grow in human urine and colonize the murine kidney. The host specificity of pathogenic *E. coli* is also determined by a set of specific adhesins or other surface associated or secreted molecules which promote bacteria colonization and dissemination (Clermont et al., 2011, Pan et al., 2014). Important mechanisms of the host specificity are also the immune evasion and nutrient acquisition systems in bacteria (Pan et al., 2014).

In our research, we used a multiplex PCR method for detection of virulence genes that are often found on conjugative virulence plasmids in APEC strains (Rodriguez-Siek et al., 2005, Johnson et al., 2008a) (Figure 2). The following primes were included in the master mix: *iroN*, *ompT*, *hlyF*, *iss*, *iutA*,

elitC and *cvaC*. The *iroN* gene encodes outer membrane siderophore receptor IroN which is participating in the transport of ferric siderophores in Gram negative bacteria (siderophores are complex molecules that enable bacteria to uptake the iron in iron poor environments, such as urine). Iron is important for metabolic functions of bacteria and for their survival in the host (Hantke et al., 2003). The *ompT* gene encodes outer membrane protease (OmpT) which catalyzes the activation of plasminogen to plasmin, inactivates antibiotic peptides and colicin. In addition, it has an important function in degradation and proteolysis of proteins (McCarter et al., 2004). The *hlyF* gene is frequently found in APEC isolates and on ColV plasmids. The HlyF (putative avian hemolysin) is directly involved in the production of outer membrane vesicles (OMVs). These vesicles are transporting virulence factors of bacteria promoting pathology process in the infected host (Murase et al., 2016). Similar to *hlyF*, increased serum survival gene (*iss*) is also found in ColV plasmid, but it could also be found in a bacterial chromosome. The function of Iss protein is to protect bacteria from the killing effect of the complement (Johnson et al., 2006, Johnson et al., 2008b). The outer membrane receptor gene for ferric aerobactin is *iutA* and that gene is also very frequently detected in APEC isolates compared to commensal *E. coli* (Johnson et al., 2006, Johnson et al., 2008a). All of these genes are often located on pathogenicity island-PAI on host chromosome or plasmids. In addition, several plasmid borne genes (*cvaA*, *cvaB*, *cvaC* and *cvi*) participate in the synthesis, export and immunity of a peptide antibiotic Colicin V. These molecules are involved in killing competitor sensitive bacteria in order to uptake essential nutrients and they have been discovered in *Enterobacteriaceae* (Gérard et al., 2005).



iutA-302 bp
hlyF-450 bp
iroN-553 bp
iss-323 bp
ompT-496 bp
eitC-980 bp
cvaC-678 bp

Figure 2: Virulence gene detection in *E. coli* isolate (APEC strain 9454/1) (Figure prepared by Dalibor Todorović, Scientific Veterinary Institute "Novi Sad")

COEXISTENCE OF VIRULENCE GENES AND GENES ENCODING RESISTANCE TO ANTIBIOTICS

E. coli are versatile bacteria which have the ability to survive in various hostile environments. There are a number of genetic mechanisms *E. coli* uses to survive in nature and different hosts. Most important are the mechanisms of virulence and resistance to antibiotics, heavy metals, disinfectants, detergents and many other toxic substances. *E. coli* share their genetic material within closely related or even unrelated population of bacteria via horizontal gene transfer. For example, *Salmonella* Kentucky isolates from poultry flocks in the USA have been found to contain virulence plasmid ColV from APEC. Therefore, *S*. Kentucky manage to persist on poultry farms even in the presence of other highly competitive *Salmonella* strains, mainly because they acquire virulence plasmids (Johnson et al., 2010a). Healthy poultry may also harbor extraintestinal pathogenic *E. coli* in their intestinal tract. In fact, it was shown that number of fecal isolates from poultry possess virulence genes and can cause disease in animal models of infection (Stromberg et al., 2017). Therefore, it is difficult to prevent infection of chickens with APEC, and management and prophylaxis are still the only ways to cope with colibacillosis worldwide.

The growing problem is resistance to antibiotics, especially to fluoroquinolones and beta lactam antibiotics in *E. coli* strains (Su et al., 2016). The research work conducted in Serbia has shown high resistance to fluoroquinolones in *E. coli* isolates from clinical bovine mastitis, pigs and wildlife (Todorović et al., 2018; Velhner et al., 2018). One isolate from a pig had multiple mutations on topoisomerase genes, conferring resistance to ciprofloxacin and also carried $bla_{\text{CTX-M-1}}$ gene (Todorović et al., 2018). However, in poultry industry in Serbia third generation cephalosporins are not frequently used, or not at all, but *E. coli* can gain resistance to these antibiotics by conjugative transfer of multidrug resistant plasmids. The important trait is when bacteria possess antibiotic resistance genes and virulence genes. For instance, genetic analysis of the ColBM plasmid from *E. coli* isolate (ExPEC 408) that have caused peritonitis in commercial laying hen was sequenced and studied in detail. It was documented that ColBM is a complex plasmid with resistance genes and virulence associated genes and that *E. coli* with ColBM plasmid caused colibacillosis in day old chickens and induced bacteremia and meningitis in rat model of infection (Johnson et al., 2010b). It is also important to note that virulence genes were found on a $bla_{\text{CMY-2}}$ conjugative plasmid (IncF) from the APEC strain that caused colibacillosis in broiler chicken (Touzain et al., 2018). Therefore, bacteria with plasmids conferring virulence and resistance to antibiotics may have increased fitness and consequently their ability to survive in host and environment will increase as well (Schroeder et al., 2017).

PREVENTION

Proper management practice and vaccination are important to prevent colibacillosis. However, there are a number of serotypes and virulence types of *E. coli* and it is difficult to develop universal vaccines against APEC. It is yet to be learned what the benefits of free range breeding system would be to the poultry industry. More knowledge about *E. coli* pathogenesis is necessary in order to design more efficient vaccines or drugs. Meanwhile, the best way to prevent viral and bacterial diseases in poultry industry and to decrease outbreaks caused by infectious agents, is to implement strict farm biosecurity measures and animal welfare systems.

In conclusion, it is important to identify virulence genes in *E.coli* isolates from poultry and to recommend effective management, probiotic treatment and/or vaccination if needed. Such approach will contribute to the reduction of outbreaks caused by APEC and will decrease the dissemination of virulent and multidrug resistant bacteria in the entire food chain.

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LABORATORY DIAGNOSIS OF *BORDATELLA BRONCHISEPTICA* TRACHEOBRONCHITIS IN DOG

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Abstract

In the present paper the laboratory isolation and identification of *Bordatella bronchiseptica,* the causative agent of canine tracheobronhitis, is described. A dog which suffered persistent cough, loss of appetite and fever was previously unsucceffully treated with antibiotics, which is why nasal swabs were taken and sent for microbiological assessment. The isolation of the causative agent was performed on routinely used standard solid growth mediums. The final identification of the isolate was done with MALDI-TOF (matrix-assisted laser desorption/ionization - time of flight) and real-time PCR (polymerase chain reaction) assays. Therapy based on the results of the antibiogram lead to successful recovery. The necessity of cooperation of veterinary clinicians and veterinary microbiologists for timely and reliable identification of the microbe(s) and selection of antimicrobials based on the results of the susceptibility testing is emphasized. The significance of the collaboration between microbiological veterinary laboratories and those dealing with human material is underlined. These can provide precise identification of zoonotic agents.

Key words: *Bordatella bronchiseptica*, dog, tracheobronchitis, MALDI TOF, real-time PCR

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LABORATORIJSKA DIJAGNOZA TRACHEOBRONHITISA PSA ČIJI JE UZROČNIK *BORDATELLA BRONCHISEPTICA*

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Kratak sadržaj

U ovom radu prikazujemo laboratorijsku izolaciju i identifikaciju *Bordatella bronchiseptica,* uzročnika traheobronhitisa psa. Pas koji je imao uporan kašalj, ispoljavao gubitak apetita i imao hipertermiju, prethodno je bez uspeha bio lečen antibioticima, zbog čega su brisevi nosa poslati na mikrobiološki pregled. Izolacija uzročnika izvedena je na podlogama koje se uobičajeno koriste u mikrobiološkim laboratorijama. Identifikacija izolata do vrste izvedena je primenom MALDI-TOF (matrix-assisted laser desorption/ionization - time of flight) i real-time PCR (polymerase chain reaction) metoda. Posle primene terapije na osnovu antibiograma, pas je uspešno izlečen. Istaknuta je neophodnost saradnje veterinara kliničara i veterinarskih mikrobioloških laboratorija u cilju blagovremene i pouzdane identifikacije uzročnika i odabira terapije na osnovu rezultata ispitivanja osetljivosti izolata na antibiotike. Dodatno ukazujemo na značaj povezanosti mikrobioloških laboratorija humane i veterinarske medicine u preciznoj identifikaciji zoonotskih agenasa.

Ključne reči: *Bordatella bronchiseptica*, pas, traheobronhitis, MALDI TOF, real-time PCR

INTRODUCTION

Bacterial species of the *Bordetella* genus are inhabitants of the respiratory system in both healthy and diseased animals and humans (Markey et al., 2013). The most infamous among these is *B. pertussis*, the primary aetiological agent of whooping cough, which can also be caused by *B. parapertussis*. A similar ailment in dogs is caused by *B. bronchiseptica*, and results in infectious tracheobronchitis, which may attack any dog breed at any age (Ford, 2006). In laymen it is referred to as kennel or canine cough and among professionals as canine croup. The etymology originates from the idea that it primarily affects dogs dwelling in kennels (but also other places where there are many

animals in limited areas), and that it resembles the synonymous childhood diseases. The pathogen is transmitted by direct contact or via Flügge droplets (Vieson et al., 2012), but contaminated fomites may also serve as a source of infection (Datz, 2003). Following an incubation which lasts generally from two days to two weeks, the infected dogs start coughing; in the beginning the cough is a dry, paroxismal cough, but later transforms into productive, with nasal discharge, conjunctivitis and fever (Shelton et al., 1977). The most severe complications develop in young dogs due to the immaturity of their immune systems. Aged animals, those with impaired immunity and pregnant bitches are at higher risk. The disease may lead to tracheal collapse (Oskouizadeh et al., 2011). *B. bronchiseptica* has been identified in rabbits with bronchopneumonia, causing even septicaemia, in cats, horses, guinea pigs and rats suffering from respiratory ailments, and as an opportunistic agent contributing to atrophic rhinitis in pigs (Pittman, 1984; Datz, 2003). Although relatively rare in humans, it was found in people with endocarditis, peritonitis, meningitis and infected wounds, as well as in immunocompromised persons suffering from respiratory infections (Hadley et al., 2009; Woolfrey and Moody, 1991).

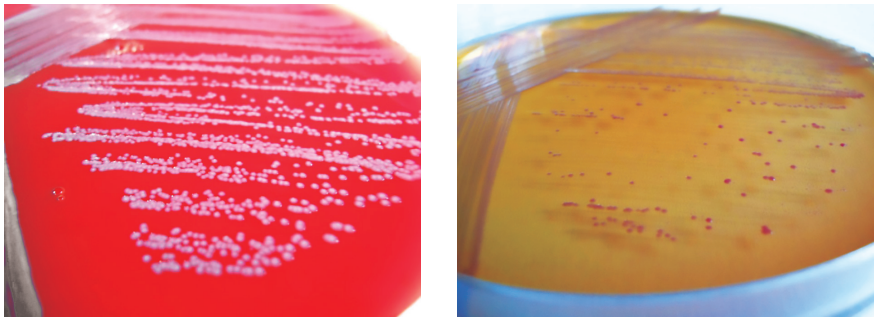
Several virulence factors play role in the pathogenesis of respiratory infections which develop owing to *B. bronchisepica.* For example, fimbriae (FIM), filamentous haemagglutinin (FHA) and pertactin (PTN) mediate the attachment to specific receptors in the respiratory system (Datz, 2003). Since adhesion is a prerequisite for invasion, flagella also may contribute to the adherence to eukaryotic cells (Savelkoul et al., 1996). *B. bronchiseptica* (and *B. avium*) are motile by peritrichous flagella (*B. pertussis* and *B. parapertussis* are non-motile) (Markey et al., 2013).

Not unlike other gram-negative bacteria, the outer membrane of *Bordetella* species contains a lipopolysaccharide endotoxin (Woolfrey and Moody, 1991). In addition, they produce several toxins: tracheal cytotoxin (TCT), dermonecrotic toxin (DNT), osteotoxin, and adenylate cyclase toxin (ACT) (Markey et al., 2013). TCT disrupts ciliated cells (Cookson et al., 1989), DNT is capable of damaging tissues and suppresses both humoral and cellular immunity (Magyar et al., 2000), ACT inhibits the phagocytic function of neutrophils and macrophages (Datz, 2003) and ACT interferes with the activities of epithelial cells (Woolfrey and Moody, 1991). Pertussis toxin (PTX) is an exoprotein produced only by *B. pertussis*, although the corresponding genes are found also in *B. parapertussis* and *B. bronchiseptica* (Masin et al., 2015).

Isolation and identification of *B. bronchiseptica*.

From a four-month-old dog with symptoms of respiratory disease samples of nasal discharge were taken with two flexible nasal swabs. One sample was sent to the laboratory of the Scientific Veterinary Institute "Novi Sad" for the isolation of the microbes and the other was delivered to the Institute of Public Health of Vojvodina to be subjected to real time-PCR (polymerase chain reaction) assay.

On arrival, the nasal swab was streaked on to Columbia blood agar base (CM0331, Oxoid, UK) with 5% defibrinated ovine blood and MacConkey agar (CM0007, Oxoid, UK). The plates were incubated at 37°C in aerobic conditions. After 24h of incubation, the blood agar was covered in very small (0.5-1 mm in diameter), convex, smooth, non-haemolytic colonies, which turned to opaque in the next 24h (Figure 1. A). On the MacConkey agar plate (Figure 1. B) the isolate gave rise to minute, pale colonies.



A. **B.**

Figure 1. Colonies of isolate on blood (A) and MacConkey agar (B) after 48h incubation at 37°C.

The isolate did not ferment carbohydrates (glucose, sucrose and arabinose), but was positive for catalase, urease and oxidase production, and citrate utilization. When the slides were stained with Gram, small Gram-negative coccobacilli were revealed with light microscopy. Based on these characters, the isolate was identified as *B. bronchiseptica*, and was sent to the Institute of Public Health for confirmation by MALDI TOF (matrix-assisted laser desorption/ ionization - time of flight).

The isolate was prepared using the standard Bruker's direct transfer sample preparation procedure for MALDI-TOF MS. A single bacterial colony was spot-

ted directly onto a MALDI target plate (Bruker Daltonics, Germany), allowed to dry and overlaid with 1.0 µL of matrix solution (Bruker Matrix HCCA; α-Cyano-4-hydroxycinnamic acid). MALDI-TOF mass spectrometry was performed on Microflex LT/SH Biotyper system (Bruker Daltonics, Germany) under the control of flexControl software ver. 3.4 (Bruker Daltonics, Germany) (Fig 2). Spectra in the mass range of 2 to 20 kDa were collected by accumulating 240 laser shots (laser frequency, 60 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.15 kV; lens voltage, 6 kV) at 30–40% of maximum laser power.

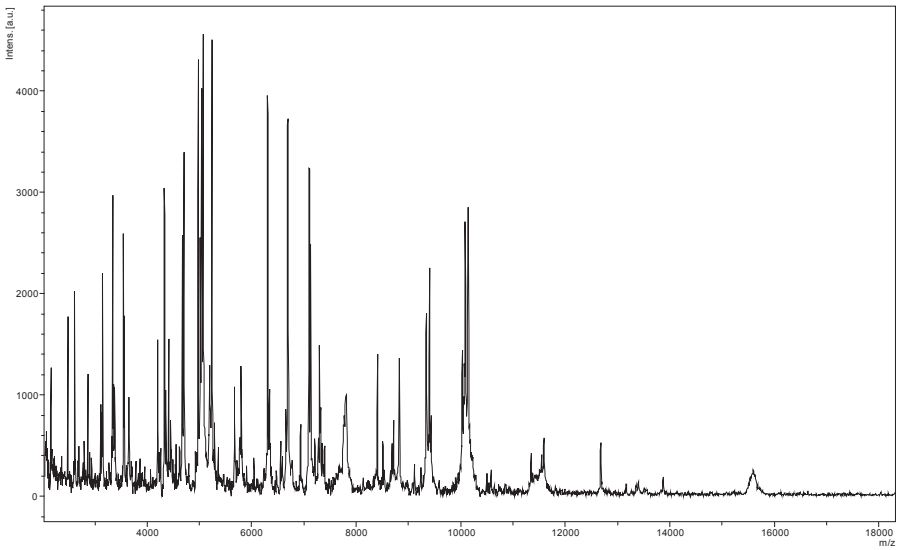


Figure 2. Spectra of *Bordatella bronchiseptica* isolate generated by MALDI-TOF Bruker flexControl software.

RT-PCR. *Bordetella* species determination in the PCR with hybridization fluorescent detection includes three stages: DNA extraction from the samples, PCR amplification of pathogen genome specific region and real-time hybridization fluorescent detection. In real-time PCR, the amplified product is detected with fluorescent dyes, linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. DNA was extracted with the DNA-Sorb-A kit (Sacace, Italy). Real-time PCR was done with a commercial kit on SaCycler-96 system (Sacace, Italy). *Bordetella pertussis /B. parapertussis/ B. bronchiseptica* Real-TM PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of these three species. It can be used for both clinical materials (nasal and oropharyngeal swabs) and mi-

crobial cultures with real-time hybridization fluorescence detection. During the amplification stage, three simultaneous reactions take place – amplification of the conservative region of *ptxA* gene that codes pertussistoxin located in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* genomes; identification of specific regions in genomes of *B. pertussis* and *B. bronchiseptica*, as well as amplification of nucleic acid sequence of the Internal Control (IC) sample. The target regions are detected by different detection channels (FAM for IC, JOE/HEX for *ptxA*, ROX for *B. pertussis* and Cy5 for *B. bronchiseptica*). Sample was tested positive for *B. bronchiseptica*.

The susceptibility of *B. bronchiseptica* isolates to antibiotics was assessed with the standard disc-diffusion test on Müller-Hinton agar medium and presented in Table 1.

Table 1. A*ntibiotic susceptibility* of *B. bronchiseptica* isolate

Antibiotics (abbreviation & dose)	**Inhibition zone (mm)**
Penicillin (P 10 U)	0
Amoxicillin (AX 25 mg)	0
Ampicillin (AM 10mg)	25
Amoxicillin/clavulanic acid (AMC 30mg)	30
Cefpodoxime (CPD 10mg)	0
Ceftazidime (CAZ 30mg)	32
Cefotaxime (CTX 30mg)	25
Ceftriaxone (CRO 30mg)	30
Erythromycin (E 15mg)	25
Tetracycline (TE 30 mg)	35
Streptomycin (S10 mg)	0
Trimethoprim/sulphamethoxazole (SXT 1.25/23.75 mg)	17
Neomycin (N 30mg)	12
Gentamicin (CN 10mg)	24
Enrofloxacin (ENR5mg)	28
Ciprofloxacin (CIP 5mg)	35
Nalidixic acid (NAL 30mg)	34

No breakpoints have been set for any of the antimicrobials tested for *B. bronchiseptica* susceptibility (Morrissey et al., 2016), which is why it is impossible to catagorize the isolates even based on their MIC values (Kadlec et al., 2006).

COMMENT

Besides parainfluenza virus and canine adenovirus type 2, *B. bronchiseptica* is the most frequent causative agent of canine respiratory diseases (Datz, 2003; Vieson et al., 2012). Although bordetella infection in the dog is usually mild and results in a self-limiting disease, it can be fatal for young animals. In this case, a four-month-old dog of mixed breed was treated empirically, with the combination of antibiotics: penicillin and streptomycin. Both were administered i.m., on five consecutive days: benzyl penicillin 20,000 IU/kg and streptomycin 150 mg/kg. The laboratory testing was required due to the absence of the response to the therapy, but not before two weeks had passed from the onset of the symptoms. The results of *in vitro* investigation of the nasal swabs confirmed the clinical suspicion of antibiotic resistance, which is understandable. Streptomycin seems generally ineffective against *B. bronchiseptica in vitro* (Woolfrey and Moody, 1991). Despite the wide use of penicillin, ampicillin and amoxicillin for canine respiratory infections, they have been proven ineffective against *B. bronchiseptica*, except when the latter is combined with clavulanate (Lappin et al., 2017). Resistance to penicillin has been reported in canine isolates (Markey et al., 2013). In addition, penicillin does not penetrate well into bronchial secretions, which impairs its efficacy. The susceptibility of *B. bronchiseptica* is intrinsically low to some β-lactams (e.g. penicillins and first-generation cephalosporines) owing to the production of β-lactamase and/or low membrane permeability to cephalosporines (Prüller et al., 2015; Morrissey et al., 2016). By contrast, aminoglycosides appear to be highly effective against *B. bronchiseptica*: in severe infections when animals do not respond to parenteral therapy, aerosolized gentamicin may be helpful (Vieson et al., 2012). The most commonly used antibiotics are amoxicillin/clavulanic acid and cephalexin (Vieson et al., 2012). Tetracyclines are also highly efficacious in treating bordetellosis.

To conclude, antibiotics should be selected based on culture and sensitivity tests. Definitive diagnosis of *B. bronchiseptica* infection in dogs should be confirmed by microbiological findings in nasal or pharyngeal swabs. History and clinical signs can only imply that it is infectious tracheobronchitis caused by *B. bronchiseptica* we are dealing with. Frequently, it is necessary to cooperate with public health diagnostic laboratories, which are provided with more sophisticated equipment and are capable of performing more precise diagnostic procedures, as it was in this case. In addition, this collaboration can result in better insight into the epidemiology of zoonoses. The initiation of therapy should be guided by the clinical signs: in critically ill animals, empiric therapy

should start on the collection of swabs, but in those more stable, antimicrobial therapy should be postponed until the results of the antibiogram arrive, which takes two to three days. Treatment should last about two weeks, or seven days beyond the resolution of health problems (Leekha et al., 2011). When deciding on antibiotic therapy, veterinary surgeons must always have in mind the threatening possibility of resistance development and by giving adequate therapy not contribute to its emergence and spread.

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FUMONISINS IN PIG FEED

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Abstract

Fumonisins are mycotoxins belonging to a group of potential carcinogens for humans. It is well known that they also have potentially harmful effects on various species of animals. After consumption, in the horse they may cause leukoencefalomalation, and in pig pulmonary edema. The legal regulations in Serbia do not prescribe the maximum allowed levels (ML) of fumonisins in feed. As products of *Fusarium*, fumonisins are often detected in corn, and their presence in wheat is also proven. However, there is not enough data on the occurrence and levels of these mycotoxins in feed. Considering the harmful effect of fumonisin on pig health and data on corn contamination, the aim of this paper is to examine the concentration of fumonisins in feed for pigs. A sum of fumonisins (B_1, B_2 and B_3) in a total of 30 samples of feed for different categories of pigs was tested. Fumonisins were found in 83% of the examined samples. Although no sample contained more than 5 mg/kg (the maximum recommended level according to EU regulation), studies on the effect of low-dose toxin on pig health and the frequent grain contamination with multiple mycotoxins pointed out the necessity of monitoring of fumonisins levels in pig feed.

Key words: fumonisins, feed, pig, ELISA

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FUMONIZINI U HRANI ZA SVINJE

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Kratak sadržaj

Fumonizini su mikotoksini koji pripadaju grupi potencijalnih karcinogena za ljude. Takođe, veoma su toksični za brojne životinjske vrste. Kod konja nakon unošenja mogu uzrokovati pojavu leukoencefalomalacije, a kod svinja izazivaju pulmonalni edem. Zakonskom regulativom u Srbiji nisu propisane maksimalno dozvoljene količine fumonizina u hrani za životinje. Kao produkti plesni roda *Fusarium*, fumonizini su često detektovani u kukuruzu, a dokazano je njihovo prisustvo i u pšenici. Međutim, nema dovoljno podataka o rasprostranjenosti i nivou koncentracije ovih mikotoksina u smešama za ishranu životinja. Ukupni fumonizini su ispitani u ukupno 30 uzoraka potpunih smeša za različite kategorije svinja. U 83% analiziranih uzoraka je utvrđeno prisustvo fumonizina. Iako nijedan uzorak nije sadržao više od 5 mg/kg (maksimalna preporučena količina saglasno EU regulativi), studije o uticaju niskih doza toksina na zdravlje svinja i čestoj kontaminaciji žitarica sa više mikotoksina upozoravaju na neophodnost praćenja koncentracije fumonizina u hrani za svinje.

Ključne reči: fumonizin, hrana za svinje, ELISA

INTRODUCTION

The health issues in different pig categories on pig farms are frequently caused directly or indirectly by the present mycotoxins in complete feed (Prodanov-Radulović et al., 2016). In the absence of regular control of feed, various health disorders that indicate mycotoxicosis in different types of pig production systems may occur. *Fusarium* mycotoxins such as deoxynivalenol and fumonisins are the most common cereal (especially corn) contaminants (Jakšić et al., 2011).

Fumonisins are a large group of mycotoxins, but most abundant and the most toxic are fumonisins from B group, that is, fumonisin B_1. In addition to being potentially carcinogenic to humans (IARC, 2002), its toxicity to animals has been confirmed. Given the high percentage of grains in pig feed, and the fact that pigs are monogastric livestock with lack a rumen with a microbiota able to degrade mycotoxins, these animals are practically most vulnerable to fumonisins.

The mechanism of the fumonisin B_1 toxicity is related to the structure of this toxin. Namely, it has an unsubstituted primary amino group at C2 and competitively inhibits ceramide synthase. This results in accumulation of the enzyme's substrates sphinganine (Sa) and sphingosine (So) in tissues, serum, and urine (Dilkin et al., 2010). Fumonisin-induced Sa accumulation is in correlation with onset of apoptosis and mitosis in the liver and kidney of several species including pig (Dilkin et al., 2010).

Consumption of high doses of fumonisins in pigs can induce a specific syndrome known as pulmonary porcine edema. First report of pulmonary edema in pig was noted in 1981 (Kriek et al., 1981). Field cases of pig pulmonary edema and hydrothorax occurred in USA during the 1989 corn harvest, and all cases were in relation with corn contaminated with fumonisin B_1 (Ross et al., 1991). Pulmonary porcine edema is usually lethal and is characterized by fluid accumulation in the lungs, which has been hypothesized to be due to left-side heart failure mediated by altered sphingolipid biosynthesis (Haschek et al., 2001). The clinical symptoms in addition to pulmonary porcine edema include lower weight gain, poor feed conversion, the clinical signs of cardiovascular, hepatic, and intestinal dysfunction (Haschek et al., 2001; Pierron et al., 2016). It is well known that enteric disease of suckling piglets could be provoked by the feed quality, i.e. the presence of mycotoxins in the feed for lactating sows and in the piglets first feed (Prodanov-Radulović et al., 2014). In conclusion, organs that may be affected by fumonisins in this species are the lungs, liver, heart, and pancreas (Dilkin at al., 2010). Similar as other mycotoxins, fumonisins also impair the immune response and potentially increase susceptibility to various infections (Halloy et al., 2005). Studies showed that fumonisin B_1 had combined or cummulative effect on animal health with some other toxins, and in combination with α-zearalenol, they may notably impair pig reproductive function activity (Cortinovis et al., 2014).

In animal feed, the European Commission provides recommendations of maximum levels of fumonisin in raw materials and in complete feed for pigs (EC, 2006a; Table 1). Food and Drug Administration in USA also established guidance levels for fumonisins in corn and pig feed (FDA, 2011).

Table 1. Regulated maximum levels of fumonisins in feed for pigs

	Total Fumonisins (mg/kg)	Reference
Maize for feed	60	EC, 2006a
Complementary and complete feed for pigs (Guidance value relative to a feed with a moisture content of 12 %)	5	
Corn and corn by products not to exceed 50% of the diet for pig	20	FDA, 2011
Complete diet for pig	10	

Fusarium moulds are often isolated from the feed materials produced in Serbia, and fumonisins are detected as an inevitable metabolite, especially on corn (Jakšić et al., 2011). However, the legal regulations in Serbia do not prescribe the ML of fumonisins in feed, and also there are no recommendations on their control and maximum levels. Considering the harmful effect of fumonisins on pig health and data about corn contamination, the aim of this paper was to examine the concentration of fumonisins in complete feed for different categories of pigs.

MATERIAL AND METHODS

In the period from January to June 2018, in the laboratory of Scientific Veterinary Institute „Novi Sad", different pig feed samples were analyzed for the content of the total fumonisins (B_1, B_2 and B_3). Feed samples were collected from feed producers and directly from different pig farms. Immediately upon sampling, 1000 g of each sample was prepared by grinding in a laboratory mill in such a way that >93% passes through a 0.8 mm sieve. The samples were than homogenized by mixing, packed in plastic bags and stored at -20°C in a freezer. The samples were allowed to reach room temperature prior to analysis.

Determination of fumonisins level using Enzyme Linked Immunosorbent Assay (ELISA) method was performed applying ELISA Veratox for Fumonisin 5/10 test kit (Neogen, USA). The analytical quality of the ELISA method was assured by the use of certified reference materials as well as by participation in interlaboratory studies. For validation and analytical quality of the ELISA method, there was used naturally contaminated maize reference material with certified fumonisins content of 2.1±0.1 mg/kg (TR-F100, lot #F-C-439, Tril-

ogy Analytical Laboratory, Washington, USA). The validation parameters were calculated and expressed using procedure according to Council Directive 96/23/EC (EC, 2002), and the obtained values were in accordance with recommendations given in EU Regulation 2006/401 (EC, 2006b). Laboratory limit of detection was 0.2 mg/kg, and limit of determination 0.5 mg/kg. Recovery was 108%. Furthermore, the analytical quality of the ELISA method was assured by participation in proficiency testing schemes (MPZ UKZUZ, 2014 and 2018).

RESULTS AND DISCUSSION

The results of fumonisins content in pig feed samples are presented in Table 2.

Table 2. Contents of fumonisins in pig feed samples from Serbia in 2018

Complete pig feed	Positive / total no. of samples	Positive samples (%)	Min (mg/kg)	Max (mg/kg)	Average (mg/kg)	Median (mg/kg)
Piglets	7/10	70	0.234	3.54	1.02	0.672
Fattening pigs	9/10	90	0.263	1.79	0.770	0.659
Sows	5/5	100	0.845	2.15	1.15	1.05
Boars	4/5	80	0.218	0.729	0.550	0.703
Total	25/30	83	0.218	3.54	0.893	0.685

As it can be seen from obtained results, fumonisins are frequent contaminants of feed for all pig categories. In 83% of analysed samples, fumonisins are detected by applied method in average concentration of 0.893 mg/kg. The highest mean value of fumonisins concentration is determined in samples intended for sows feeding (1.15 mg/kg), and in this type of samples the most frequent contamination with fumonisins was noted (100%). Although the measured concentrations in all investigated samples did not exceed the maximum recommended level according to EU regulation (5 mg/kg), the fact that the highest concentration (3.54 mg/kg) was recorded in the feed sample for a category whose health is important for long-term farm production is worrying. To the best of our knowledge, in the literature there is almost no data on fumonisins in pig feed to compare with obtained results. Data from our previous investigation are comparable with the obtained results (Jakšić et al., 2015). In 2014, slightly smaller number of samples was analyzed, all of them were positive for fumonisins, with a lower maximum concentration (2.15 mg/kg), and higher average value (1.42 mg/kg), if compared with this research.

Still, the question is: could these concentrations yet cause health problems

in pigs? As it was said in the introduction, the fumonisin toxicity in pigs is well proven and it varies according to several parameters such as the dose, the duration of exposure, the age, and the sex of the animal, with the greater effects on performance in males and younger pig categories (Andretta et al., 2012). Exposure to an average concentration of fumonisins in naturally contaminated feed had effect on the digestive microbiota balance, with *Salmonella* exposure amplifying this phenomenon (Burel et al., 2013). In growing pigs, the presence of fumonisin B_1 at concentrations of more than 0.1 mg/kg resulted in a lower weight gain, while in the final fattening concentrations from 1 mg/kg of toxin can have a negative effect on the quality of meat (increased fat and reduced meat percentage) and cause economic losses to producers (Rotter et al., 1994).

The important question is: it is possible to spot and prevent health problems of low fumonisin concentrations in feed? The first clinical symptoms of intoxication are nonspecific and can easily be unnoticed and mistaken for other diseases. Oral administration of fumonisins at level of 5 mg/kg body weight to pig, induced clinical alterations compatible to pulmonary porcine edema (Dilkin et al., 2010). However, low doses of fumonisins can induce various hematological changes and pathological signs that are not specific and not sufficient to diagnose fumonisin intoxication, although biochemical and clinical alterations might be in some cases indicative. Liver alterations are detected through increase in certain enzymes, such as alkaline phosphatase, sorbitol dehydrogenase, aspartate aminotransferase, and gamma-glutamyl transpeptidase (Riley et al., 1993). In some cases, serum cholesterol and biliary acid concentrations also show significant increases (Casteel et al., 1994). Free sphinganine (Sa) and sphinganine to sphingosine ratio (Sa/So) could be used as a sensitive marker for fumonisin exposure, since the highest Sa and Sa/So ratios were obtained at 12 and 48 h after toxin administration (Dilkin et al., 2010). Fumonisins are poorly absorbed from the gastrointestinal tract (Pierron et al., 2016). They have a low elimination rate in urine (0.93%), while larger amounts of fumonisins can be detected in feces (76.5%; Dilkin et al., 2010). Accumulation in tissues is greatest in the liver and kidneys (Prelusky et al., 1996). There is also possibility to evaluate hair as a biomarker to assess the dietary exposure of pigs to fumonisins (Souto et al., 2017).

It was found that fumonisin B_1 has immunosuppressive effect on humoral immune response in pigs, and therefore can provoke some secondary bacterial (*Escherichia coli*, *Pasteurella multocida*) (Halloy et al., 2005) or complications with the viral infections (porcine reproductive and respiratory syndrome) (Ramos et al., 2010), which can arise in immunocompromised animals.

CONCLUSSION

There is a factual risk of pig feed contamination with fumonisins in Serbia, especially after climatic changes during the last decade. Although measured concentrations of toxin levels were lower than recommended, it should be considered the effects of chronic exposure to such low dietary levels of fumonisin B_1 in pigs, which were found under natural conditions. Avoiding economic losses due to presence of fumonisins in pig feed, it is necessary to control these mycotoxins in corn. In order to ensure safe feed, it is necessary to take into account both the presence of fumonisins themselves and other mycotoxins due to the possible additive and synergistic negative effects of the mycotoxin mixture.

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FLAVIVIRUSES AT THE TERRITORY OF SERBIA – PRESENT SITUATION AND CHALLENGES

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Abstract

The aim of this study is to summarize the data on the occurrence, presence and prevalence of some zoonotic flaviviruses, which have been actively circulating in the territory of the Republic of Serbia during past decade. The genus *Flavivirus,* family *Flaviviridae,*encompasses vector-transmitted viruses with zoonotic potential. The genus is comprised of more than 70 viruses transmitted to humans by mosquitoes and ticks. Some of those are associated only with human diseases (such as yellow fever and dengue fever), whereas others can cause diseases in both humans and animals. Animals are major reservoirs and primary hosts for the latter group. The virus transmission to other animal species or humans occurs via diverse vectors – mostly mosquitoes and ticks. A range of these virus species is widely distributed worldwide, especially in tropical and sub-tropical climatic zones. Some representatives of these species have only recently been identified at the territory of Republic of Serbia. This paper gives an evidence on the presence and

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distribution of West-Nile virus, tick-borne encephalitis (TBE) virus and Usutu virus that were conducted within the study in Serbia. The research pointed out the presence of recently detected West-Nile virus and Usutu virus as well as the current circulation of tick-borne encephalitis virus, for which only limited serological evidence on the presence of virus-specific antibodies in humans was available so far. In the aspect of public health, the full understanding of the importance of identification and prevalence of different flaviviruses still require further comprehensive entomological-acarological, seroepidemiological, clinical and virological research.

Key words: flaviviruses, West-Nile virus, tick-borne encephalitis virus, Usutu virus, Serbia

FLAVIVIRUSI NA PODRUČJU SRBIJE – TRENUTNO STANJE I IZAZOVI

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Kratak sadržaj

Cilj ovoga rada je da sumarno prikaže podatke o pojavi, prisustvu i raširenosti nekih zoonotskih flavivirusa čija cirkulacija je utvrđena na području Republike Srbije tokom poslednjih desetak godina. U rod *Flavivirus,* familije *Flaviviridae*, spadaju vektorski prenosivi virusi zoonotskog

potencijala. Rod obuhvata više od 70 vrsta virusa koji se preko komaraca i krpelja prenose na ljude. Neki od ovih virusa izazivaju oboljenja samo ljudi (kao što su žuta groznica i Denga), dok drugi izazivaju oboljenja životinja i ljudi. Rezervoari i prirodni domaćini za ove drugo navedene viruse su pre svega životinje a virusi se putem različitih vektora, najčešće komaraca i krpelja mogu preneti i na druge vrste životinja, kao i na ljude. Veći broj vrsta ovih virusa je široko rasprostranjen u svetu, posebno u tropskim i suptropskim klimatskim pojasevima. Pojedini predstavnici ovoga roda su utvrđeni na području Republike Srbije tek u skorašnjem periodu. U radu su prikazani podaci istraživanja koja su sprovedena na području Republike Srbije, a odnose se na utvrđivanje prisustva i raširenosti virusa Zapadnog Nila, virusa krpeljskog encefalitisa i Usutu virusa. Pomenuta istraživanja su ukazala, kako na prisustvo skorije detektovanih virusa Zapadnog Nila i Usutu virusa, tako i na trenutnu cirkulaciju virusa krpeljskog encefalitisa za koji su ranije postojali ograničeni serološki podaci o prisustvu antitela kod ljudi. Za razumevanje istinskog značaja nalaza i prevalencije različitih flavivirusa za javno zdravlje u Republici Srbiji, neophodno je sprovesti detaljnija entomološko-akarološka, sero-epidemiološka, klinička i virusološka istraživanja.

Ključne reči: flavivirusi, virus Zapadnog Nila, virus krpeljskog encefalitisa, Usutu virus, Srbija

PRESENCE AND PREVALENCE OF WEST NILE VIRUS (WNV)

West Nile virus (WNV) is a neurovirulent mosquito-borne flavivirus with zoonotic potential. The virus is maintained in nature in an enzootic transmission cycle between avian hosts and mosquito vectors, mainly from the genus *Culex* but also some other mosquito species. The virus occasionally infects other vertebrates, including humans and horses, in which it may cause sporadic disease outbreaks that could result in fatal outcomes (Martin-Acebes et al., 2012). WNV was first isolated in 1937 (Smithburn et al., 1940) from samples originating from highly febrile woman in Uganda (*West Nile* district). This is how the infection got its name West Nile Fever (WNF). Nowadays, it is considered second, after Dengue virus, most widespread flavivirus in the world. WNV is endemic in the regions of Africa, Asia, Europe, Middle East, Australia and Americas (Komar et al., 2003; Hrnjaković-Cvjetković et al., 2009; Calistri et al., 2010; Weissenböck et al., 2010 and Papa et al., 2011). In Europe, mainly in the countries of Mediterranean Basin, only lineage 1 strains circulated until

2004, when lineage 2 strain was (for the first time in Europe) isolated in goshawks in Hungary. Ever since, the lineage 2 WNV strains have been identified in a range of wild bird species and mosquitoes with established vector capacity as well as in humans in Hungary, Austria, Russia, Italy, Greece and more recently in Serbia (Bakonyi et al., 2006; Valiakos et al., 2011; Savini et l., 2012; Papa et al., 2011; Erdély et al., 2007; Platonov et al.,2008; Wodak et al., 2011; Bagnarelli et al., 2011; Petrović et al., 2013).

In the aspect of human and animal health, wild birds are particularly important due to migration across national and intercontinental borders they contribute to a long-range virus spread (Linke et al., 2007). Many bird species, though not all that are susceptible to infection, produce levels of viremia that are sufficient for transmitting the virus to mosquitoes (Komar et al., 2003). Humans and mammals, especially horses, are incidental "dead-end" hosts and play limited roles in the natural cycle because viremia after infection is generally too low to allow further virus transmission to the mosquitoes at the moment of bite (Hrnjaković-Cvjetković et al., 2009; Valiakos et al., 2010). However, in both humans and horses, severe neuroinvasive disease with even fatal outcomes can occur. Infections in humans predominantly take a subclinical course (80-90% of cases). Some infected individuals (up to 20%) can manifest mild clinical symptoms resembling influenza associated with sudden fever, headache, sour throat and pain in the back, muscles and joints, fatigue, mild transient rash and lymphadenopathy. However, in only a small subset (one per 150 cases) of mostly elderly patients and patients with comorbidities the infection can progress to severe neuroinvasive disease with encephalitis or meningoencephalitis, occasionally with fatal outcomes (Blitvich, 2008; Hrnjaković-Cvjetković et al., 2009). Similarly to humans, WNV infection in horses is usually not accompanied with apparent clinical symptoms. Nevertheless, neurological symptoms can occur in some 10% of cases with a mortality rate that can reach even 50% (Blitvich, 2008; Calistri et al., 2010). The virus has been present in Europe for many years, but dramatic increase of the incidence and severity of epidemic outbreaks with neurological complications in birds, humans and horses has been recorded in central and southern Europe in recent years, which is considered an emerging veterinary and public health problem (Martin-Acebes and Saiz, 2012; Petrović et al., 2013).Apart from some historical data, first reports on the presence of West Nile virus (WNV) in Serbia became available upon testing of horse blood sera for the presence of WNV-specific antibodies in the region of Vojvodina Province. The first study was performed using blood sera collected during 2009 and 2010. The presence of WNV-specific antibodies was detected applying immunoenzyme as-

say (ELISA) and subsequent plaque reduction neutralization test (PRNT) in 12% (46/349) of examined samples (Lupulović et al., 2011). Surprisingly, high percentage of seropositive horses initiated further research, thus, serological testing has continued during following three years. WNV-antibody testing applying ELISA conducted on horse blood sera in 2011 revealed 28.6% (72/252) seropositive animals, whereas 49.2% (64/130) and 46.9% (45/96) seropositive horse blood sera were identified in tests conducted during 2012 and 2013, respectively (Medić et al., 2014; Petrović et al., 2014a; Petrović et al., 2015). Pronounced increase in the prevalence of seropositive horses strongly suggested an intensive circulation of WNV in the environment. In spite of high prevalence, clinical manifestations of the disease have not been reported, i.e., confirmed in the laboratory. Circulation of WNV in the nature has been confirmed by ELISA and PRNT in 5/92 (5.4%) blood sera of wild birds positive to anti-WNV antibodies as well as by virus detection in tissue samples of 8/82 (9.8%) dead wild birds using *real-time RT-PCR*. The analysis included samples of 134 wild birds (belonging to 46 species) and was conducted in the territory of Vojvodina Province of Serbia during the first half of 2012 (Petrović et al., 2013). Besides the aforementioned, the presence of WNV genome was detected by *real-time RT-PCR* in 6% (3/50) and 9.15% (28/306) of the examined mosquito's samples pools collected during 2010 and 2013, respectively (Petrić et al., 2012; Petrović et al., 2015). Moreover, positive reaction to the presence of WNV antibodies in ELISA test was observed in 5.04% (17/337) of examined human samples collected from Vojvodina Province in 2010 (Petrić et al., 2012). Since 2012, when first WNV epidemic associated with apparent clinical picture in humans was reported, the human outbreaks have been recorded every year (ECDC, 2012; Popović et al., 2013). National monitoring programme for WNV funded by the Veterinary Directorate and conducted by scientific and specialized veterinary institutes and field veterinary service in close collaboration with entomologists and ornithologists was launched in 2014. The program proved successful in view of identifying presence and circulation of WNV among sentinel animals (domestic poultry and horses in 2014 and only in horses in other years), and in wild birds and mosquitoes before infection outbreaks in humans in every particular season. The program, with minor modification, has continued during 2015, 2017 and 2018 (Petrović et al., 2014b; Petrović et al., 2018). The main objectives of this program include early detection of WNV in certain region and timely reporting to relevant health service institutions and local authorities responsible for establishing of appropriate mosquito control, sharing the information to the community and taking preventive measures for human and animal health protection. The monitoring programme relies on direct and in-

direct detection (surveillance) of WNV presence in the environment. Surveillance by indirect detection of WNV includes serological testing of sentinel horses and poultry (poultry was included in surveillance only during 2014). The testing is performed periodically during most intense mosquito activity (June-September). The number of tested sentinel animals is defined at the level of each district according to the assessment of the risk of exposure to WNV. Direct monitoring of the presence of WNV involves periodical examination of pooled mosquito samples collected at two-week intervals in the period of their most intense activity (June-September) and testing of wild bird samples (tissues of dead birds and swabs taken from live wild birds of susceptible species) for the presence of WNV. The number of samples is also defined at the level of each district according to estimated risk of WNV infection (Petrović et al., 2018). Some results of the WNV monitoring program conducted during 2014 and 2015 are presented in figures (Figures 1-4).

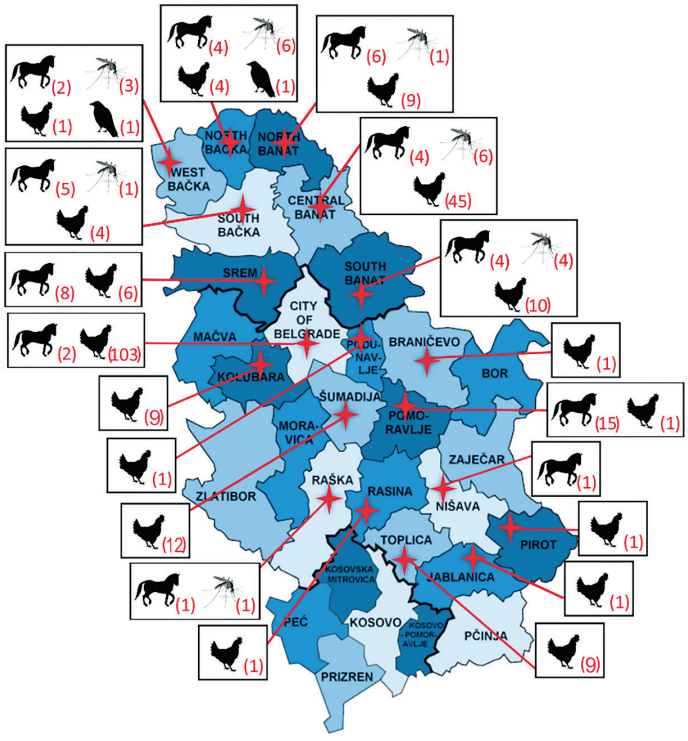

Figure 1. Results of WNV monitoring programme in the Republic of Serbia during 2014 - (n) number of positive findings in mosquitoes and wild birds, WNV specific antibodies in sentinel horses and poultry per Districts level

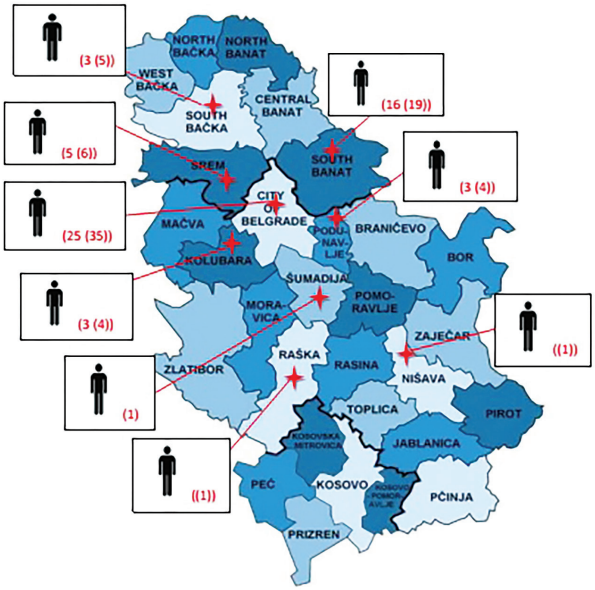

Figure 2. Recorded cases of West Nile Disease in humans in 2014 – (n) reported clinical cases confirmed in the laboratory; ((n)) reported clinical cases not confirmed in the laboratory

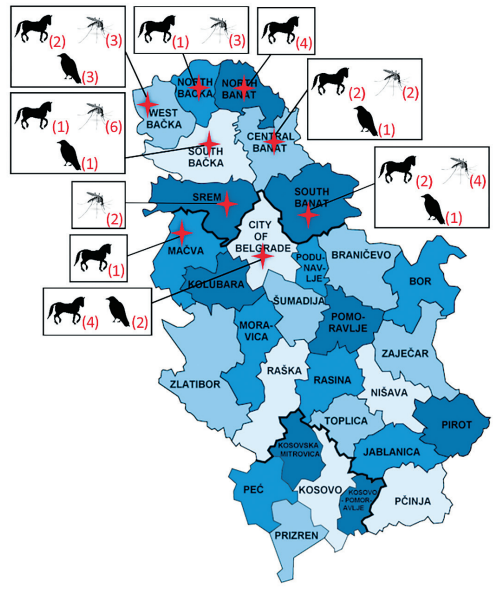

Figure 3. Results of WNV monitoring programme in the Republic of Serbia during 2015 - (n) number of positive findings of WNV and virus specific antibodies in mosquitoes, wild birds and sentinel horses per Districts level

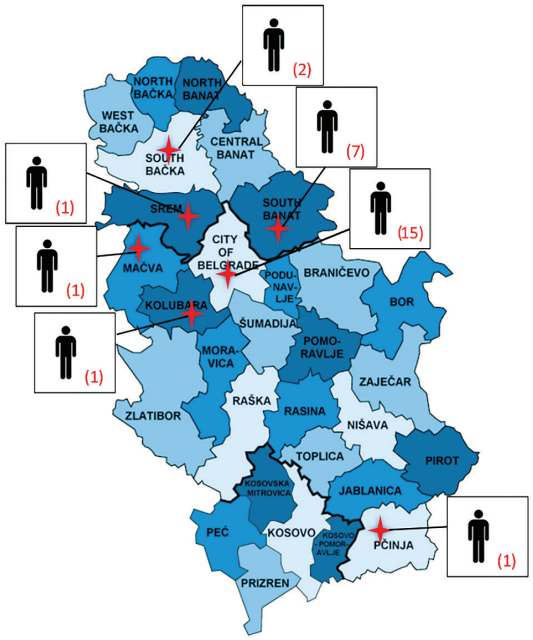

Figure 4. Recorded cases of West Nile Disease in humans in 2015 – (n) reported clinical cases confirmed in the laboratory

PRESENCE AND DISTRIBUTION OF TICK-BORNE ENCEPHALITIS VIRUS (TBEV)

Tick-borne Encephalitis (TBE) is viral infectious disease most commonly distributed in the central and eastern regions of Europe, and Asia. The infection is transmitted predominantly via infected ticks. The disease occurs in endemic areas, naturally active foci in the presence of virus reservoirs (small rodents). There is no direct animal-to-animal or person-to-person virus *transmission. However, animal-to-human transmission is possible via products of animal origin, predominantly by consuming non-pasteurized milk and dairy products, mainly from goats and cattle* (Balogh et al., 2010; Hudopisk et al., 2013). Most often the virus is transmitted to humans by the bite of ticks of the genus *Ixodes*. The ticks can get infected at all stages of their life cycle and remain disease vectors throughout their entire *life*. Virus replication in tick's body and transovarial transmission to following generation enable the maintenance of TBEV among vector population. Majority of clinical infections have been re-

corded in dogs (Pfeffer and Dobler, 2011) and horses (Klaus et al., 2013), while asymptomatic infections are common in domestic ruminants, which represent an important epidemiological problem in view of virus transmission to humans (Balogh et al., 2010; Hudopisk et al., 2013; Caini et al., 2012).

In the territory of former Yugoslavia, the first findings, of virus isolation from the blood of infected person in Slovenia date back to 1953 (Vesenjak-Zmijanac et al., 1955). From this period onwards, the presence of TBEV has been reported in the western part of ex-Yugoslavia, predominantly in Republic of Slovenia as an endemic region. At that time, virus presence was not established in the territory of Serbia (Petrović et al., 2017). First reports on the presence of TBEV in Serbia are based upon serological examinations by hemagglutination-inhibition (HI) assay, which encompassed 1,726 blood serum samples of healthy humans collected in the territory of Republic of Serbia from 1962 to 1969. The presence of TBEV-specific antibodies was established in 1.1% - 52.6% of tested individuals (1.1% from the region of Srem; 2% from the region of Central Serbia; 3.6% from the region of East Serbia; 7.3% from the territory of Belgrade; 8.4% from the region of Banat; 19.4% from the region of West Serbia; 37.8% from the region of Kosovo and 52.6% from the region of Sandžak). The virus was isolated in 1972 from a tick originating from the region of Sandžak (Bordjoški et al., 1972; Petrović et al., 2017). Some more recent serological tests for the presence of TBEV applying ELISA method revealed the presence of antibodies against TBEV in 7.9% (8/101) of healthy individuals in the region of South Bačka, whereas no positive findings (0/80) were reported in Nišava district (Hrnjaković-Cvjetković et al., 2014).

Several decades after the first isolation, the presence of *tick*-borne *encephalitis virus* (TBEV) was confirmed by *real-time RT-PCR,* conventional *RT-PCR* and genome sequencing in ticks of the genus *Ixodes ricinus* collected during 2014 and 2015. Examination of 50 ticks from two localities in Fruška Gora mountain and 15 localities in the region of Belgrade revealed the presence of TBEV in 2% (1/50) and 6.6% (30/450) examined ticks at two out of 17 localities (Andrevlje/Fruška Gora and Manastirska šuma, Rakovica/Belgrade region), respectively. One of the detected TBEV isolates was sequenced and phylogenetically typed as European (Western) TBEV subtype (Figure 5). In addition, low prevalence of TBEV antibodies was established by ELISA in 0.37% (1/267) of the examined blood sera collected in the same period from patients at the Clinic for Infectious Diseases of the Clinical Centre of Vojvodina (Potkonjak et al., 2017).

Some recent data on the presence of TBEV in animals came from serological research that included 200 animal blood sera collected during 2014

and 2015. ELISA analysis of the aforementioned sera revealed the presence of TBEV-specific antibodies in 17.5% (7/40) of dogs, 5% (1/20) horses, 12.5% (5/40) wild boars, 2.5% (1/40) cattle and 2.5% (1/40) of roe deer. The presence of TBEV-specific antibodies was not established in any (0/20) of the examined goat blood sera (Potkonjak et al., 2017). Apart from the abovementioned research, TBEV *was first established in animals in Serbia in 2017. Virus was confirmed by RT-PCR in blood samples of sick 2 year old horse and mare originating from the same household in the region managed by Specialized Veterinary Institute* „Požarevac". Sick animals manifested neurological symptoms accompanied by epileptic seizures with lethal outcome (Živojinović et al., 2017).

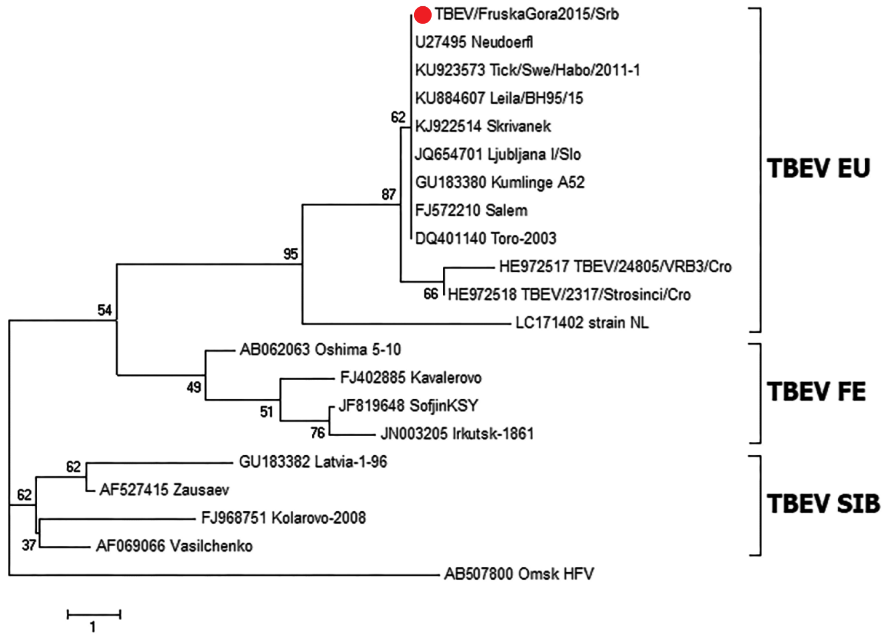

Figure 5. Molecular typing of TBEV isolate detected in tick species *Ixodes ricinus* in the region of Fruška Gora Mountain (marked with red circle) in relation to virus strains available from NCBI GenBank. TBEV EU = strains of European or Western TBEV subtype; TBEV FE = strains of Far eastern TBEV subtype; TBEV SIB = strains of Siberian TBEV subtype (Figure from Potkonjak et al., 2016).

PRESENCE AND DISTRIBUTION OF USUTU VIRUS (USUV)

Usutu virus (USUV) was first isolated in 1959 from *Culex neaevii* mosquitoes in the region along the river Usutu (after which the virus is named)

in Swaziland (since recently, Eswatini – South Africa region) (Williams et al., 1964). First human isolates were detected in 1981 and 2004 in ill persons from the regions of Central African Republic and Burkina Faso, respectively (Nikolay et al., 2011). The presence of USUV in Europe was first established when the first recognized outbreak of USUV occurred among blackbirds *Turdus merula* in Austria in 2001 (Weissenböck et al., 2002), and later also in other European countries. The recently published research confirmed the presence of the virus in archived tissue samples from Italian wild birds found dead in 1996 strongly suggesting that USUV was circulating in the region of Europe even before 2001 (Weissenböck et al., 2013). Natural cycle of USUV is similar to that of the WNV. Diverse species of wild birds are the reservoirs (amplification host) and natural hosts of the virus, while mosquitoes, mainly of the *Culex* genus, serve as major vectors, although USUV was found in other mosquito species (Hrnjaković-Cvjetković et al., 2017; Vilibić-Čavlek et al., 2015; Saiz and Blázquez, 2017). In the infected birds, clinical symptoms of the disease can vary from asymptomatic infection to massive deaths associated with multisystem infection. Lethal USUV infections were reported in Passeriformes birds) with most susceptible blackbird species (*Turdus merula*), house sparrow (*Passer domesticus*) and magpie (*Pica pica*) as well as owls from the order of Strigiformes. In highly susceptible bird species, USUV manifests a broad tissue tropism and tends to replicate in diverse tissues (central nervous system, muscle tissue, fibroblasts, epithelial cells of the intestines and respiratory tract, lymphoid tissues etc.) thus causing dysfunction of multiple organs and lethal outcome. The disease commonly takes acute or per acute course, frequently associated with poor immune response (Vilibić-Čavlek et al., 2015; Saiz and Blázquez, 2017).

Experimental infections proved that gees and chicken can get infected with USUV, but they do not develop clinical symptoms of the disease and shed the virus only intermittently. Once infected with USUV, humans and horses show low level of viraemia, thus becoming accidental final hosts that could not serve as a virus host for further virus spreading ("dead-end" hosts for virus transmission). Clinical manifestations among humans and horses are rare and closely similar to those associated with WNV. The majority of infections are asymptomatic. Neurological manifestations as encephalitis and meningoencephalitis can occur only in limited number of cases (Hrnjaković-Cvjetković et al., 2017; Vilibić-Čavlek et al., 2015; Saiz and Blázquez, 2017). According to data published so far, USUV has been identified in birds and mosquitoes in the territory of Austria, Hungary, Spain, Italy and Belgium. Besides, USUV infection is serologically confirmed in wild birds in England, Czech Republic,

Germany, Italy, Poland, Spain and Switzerland (Hrnjaković-Cvjetković et al., 2017; Vilibić-Čavlek et al., 2015; Saiz and Blázquez, 2017). Seroconversion in horses for the first time was reported in Italy in 2009 (Barbić et al., 2013). First detection of USUV antibodies in wild birds in Serbia dates back from 2012, during the testing for the presence of West Nile virus (Petrović et al., 2013). For the first time, USUV-specific antibodies were detected in horses in Serbia and Croatia in 2009 and 2011, respectively (Lupulović et al., 2011; Barbić et al., 2013). These findings undoubtedly indicated the presence and circulation of the virus in both Serbia and Croatia, which initiated further research predominantly focused on the presence of specific antibodies in humans. During 2015, the testing of blood sera from persons potentially exposed to mosquito-borne arbovirus infections was performed using ELISA test. The examination included 88 blood sera of people from the region of South Bačka, Serbia. The presence of USUV antibodies was confirmed in 5% (4/88) of tested individuals (Hrnjaković-Cvjetković et al., 2014). In Croatia, the presence of USUV-specific antibodies was confirmed in 2012 in one person from Vukovar area, and first clinical cases of human USUV infection were identified in 2013 (3 patients with neuroinvasive disease – meningitis and meningoencephalitis – from the region of Zagreb) (Vilibić-Čavlek et al., 2014; 2015). The first case of human USUV infection in Europe was reported in Italy (Pecorari, 2009).

The presence of USUTU virus for the first time was detected in Serbia using *real-time RT-PCR* and classic *RT-PCR* in 0.9% (2/216) of pooled mosquito samples collected in the territory of South Bačka district during 2015 (42), as well as in 2.75% (3/109) of analysed polled samples of mosquitoes of the species *Culex pipiens* collected in the territory of Vojvodina Province during 2017 (Petrović et al., 2018b). Two of USUTU viruses detected in 2017 were sequenced and identified as lineage 2 of the European - Western USUV subtype (Figure 6). This research has confirmed the previous serological findings in animals and humans from the territory of Serbia.

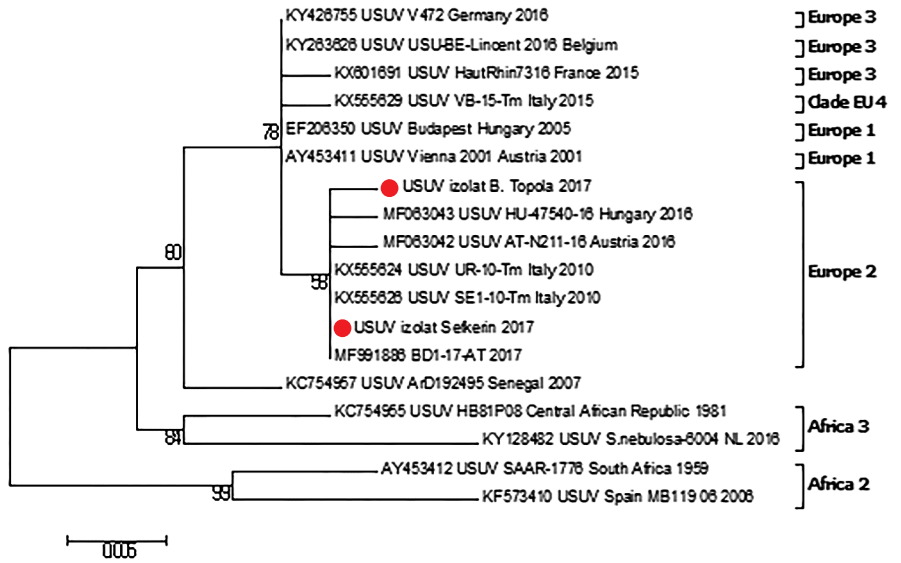

Figure 6. Molecular typing of 2 USUV detected in mosquitoes in the region of Bačka Topola and Sefkerin during 2017 (marked with red circle) in relation to referent virus strains and isolates available from the gene banks (NCBI GenBank). Legend on the right side presents different USUV lineages.

INSTEAD OF A CONCLUSION

The objective of this article was to summarize the research in the field of epidemiology and diagnostics of flavivirus infections conducted in the region of Serbia during past decade and to offer an overview of current epidemiological and epizootic situation as well as of measures of the surveillance of WNV currently in force in Republic of Serbia. Research results obtained through annual surveillance programmes have already offered answers to some questions on the presence and distribution of some flavivirus infections such as WNV. However, they launched other emerging issues related to the incidence and prevalence of TBEV and USUV. Also, there are issues of interaction between these three viral infections in terms of the problem of differential diagnosis due to similar clinical manifestations, as well as the hazards to human and animal health. Understanding of public health importance of the presence and prevalence of different flaviviruses requires further comprehensive sero-epidemiological, clinical and virological research. Apparent and pronounced climatic changes entail intensive spread of specific vectors towards North;

thus, occurrence of certain flavivirus infections so far unknown in the region of Serbia should be expected. In that respect, further research should involve permanent monitoring of vector distribution and their infection status in view of the presence of pathogenic agents of human and animal diseases, as well as the presence of pathogens among the population of natural hosts and reservoirs. Upon that point, establishing of a comprehensive surveillance program is compulsory to obtain all necessary information and to get the answers related to risk analysis and adequate preventive and control measures.

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THE CONTROL PROGRAM OF RED POULTRY MITE (DERMANYSSUS GALLINAE), TODAY

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Abstract

Dermanyssus gallinae control has so far been predominantly based on acaricides (insecticides) and their efficacy has been decreasing in time due to the development of resistance. *D. gallinae* is a species that has developed resistance to all current acaricides. Considering this, we can assume that the new acaricide - fluralaner with its evident efficacy, but also with some downsides, will improve the situation in *D. gallinae* control in the short term. The control of red poultry mite population in intensive poultry production has had an unfavourable tendency for decades. In order for this trend to be stopped and reversed, certain measures must be taken to the control of this disease. It is necessary to eliminate toxicological risks; define the short-term objective (efficient suppression) and the long-term one (eradication); introduce the principles of biosecurity, prevention, and rational control; provide a professional application of formulations and increase the quality of monitoring. The *D. gallinae* program control integrates all the above specified elements into a whole. In our opinion, the currently used program currently used on farms, based on mechanical control and active influence on technological processes, has a bright future. This type of control can be combined with other efficient methods of mite suppression on farms.

Key words: program, control, *Dermanyssus gallinae*

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ПРОГРАМСКА КОНТРОЛА ЦРВЕНЕ КОКОШИЈЕ ГРИЊЕ (*DERMANYSSUS GALLINAE*), ДАНАС

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Kratak sadržaj

Kontrola *D. gallinae* se do sada dominantno zasnivala na akaricidima (insekticidima), čija efikasnost je tokom vremena slabila zbog razvijanja rezistencije. *D. gallinae* je vrsta koja je razvila otpornost na sve do sada korišćene akaricide. Sa tog stanovišta, predpostavka je da će novi akaricid fluralaner, i pored evidentne efikasnosti, ali i nedostataka, dovesti do kratkotrajnog poboljšanja situacije u kontroli *D. gallinae*. Kontrola populacije crvene kokošije grinje u živinarskoj proizvodnji tokom više decenija ima nepovoljnu tendenciju, i da bi taj trend zaustavili i preokrenuli u drugom smeru, moraju se preduzeti određene mere u samom pristupu kontroli ove bolesti. Potrebno je: isključiti toksikološke rizike; odrediti kratkoročan (efikasnog suzbijanja) i dugoročan cilj (eradikacije); uvesti principe biosigurnosti, preventive i racionalne kontrole; obezbediti stručnu primenu formulacija i povećati kvalitet monitoringa. Navedene elemente u celinu objedinjuje program kontrole *D. gallinae*. Aktuelni program koji se koristi na farmama i po našem mišljenju ima perspektivu, bazira se na fizičkoj kontroli i aktivnim uticanjem na tehnološke procese. Takav vid kontrole je moguće kombinovati sa drugim takođe efikasnim metoda suzbijanja grinja na farmama.

Ključne reči: program, kontrola, *Dermanyssus gallinae*

INTRODUCTION

Dermanyssus gallinae is the most significant poultry ectoparasite (Figure 1). The results of several decades of control are the following: high prevalence (Sparagano et al, 2009), health problems disturbance, stress, impact on the general health status of the flock (Emous 2005; Kowalski and Sokol, 2005; Kaoud, 2010), progression of conditional diseases, transferring agents of infec-

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tious diseases (Moro et al, 2005, Huong et al, 2014), decrease in production results, increase of economic loss (Emous 2005, 2017; Flochlay et al, 2017) and evident toxicological risk to human health (Marangi et al, 2012; Giangaspero et al, 2011, 2017), poultry health and the environment. The extent and tendency of all the above mentioned factors prove that current *D. gallinae* control is inadequate and the approach is improper.

"The extent to which the probability of events has changed under the influence of a message can be used as a measurement of the quantity of information contained in that message"- this is an interpretation from the mathematical information theory by Shannon (1948). In addition to the information about the current situation in *D. gallinae* control, we will provide two more examples. In the last few decades of the 20th century, an intensive development of poultry industry started. This is when timely information about the risk and possible spreading of *D. gallinae* should have been provided.

Figure 1. *Dermanyssus gallinae*

Biosecurity measures should have been introduced and mass farm infestations would have been prevented. This information was not provided, and the consequences of this failure are now widely known. The second example is the change of the conventional cage system for housing layers in the EU. That was the moment when the eradication of *D. gallinae* could have easily been affected

in old poultry houses or initial infestation could have been prevented in newly built ones. Despite an extraordinary opportunity to change the situation, necessary information was again not provided. The aim of this paper is to point out the chance to change the widely accepted approach to *D. gallinae* control in order to stop the unfavorable trend, improve practical results, and direct the development towards the permanent solution.

THE BASIC PRINCIPLES OF THE PROGRAM APPROACH FOR CONTROL OF RED POULTRY MITES

The existing *D. gallinae* control needs improvement (Schulz et al, 2014a; Pritchard et al, 2015; Flochlay et al, 2017). Most products for *D. gallinae* control available on the market are not efficient enough. However, even an efficient product is not sufficient for successful control on its own. It also needs to be properly applied. Furthermore, what is necessary is a program - a comprehensive plan that would have a properly defined objective and that would tackle all other factors important for the control. The importance of a program approach to *D. gallinae* control is rarely emphasized, and the efforts to design and implement a program are rarely made. Emous (2005) lists 4 main elements of a program: thorough cleaning of a poultry house between two flocks, prevention of a new infestation, constant monitoring and implementation of efficient control methods. This program proscribed monitoring on a weekly basis (checking the same 20-30 spots) and its aim was suppression. Users are not familiar with its practical value.

The program approach to *D. gallinae* control has been developed in Serbia since 2000 (Pavlićević et al, 2018a).

The objectives of the program are the following:

1. To prevent *D. gallinae* infestation on uninfected farms
2. On infested farms: a) primarily to eliminate safety risks and introduce rational control, and then to raise the level of efficacy and cost-effectiveness of *D. gallinae* control in the short term b) to eradicate *D. gallinae* from production facilities and farms and to introduce biosecurity measures in the long term.

The task of the program is to get a comprehensive overview of all important questions in *D. gallinae* control and to provide the answers. It should cover the following areas: *D. gallinae* biology; environmental characteristics of poultry farms; laboratory tests and clinical trials; detection and monitoring; choice of formulations and methods; preparation of farms and professional application

and introduction of biosecurity measures. This task should be completed in accordance with the principles of preventive veterinary medicine and rational *D. gallinae* control. The program helps to obtain and share important information. Providing quality information to experts and farmers is a way for them to be properly involved in the control program and make an active contribution to the solution.

LIFECYCLE, PHYSIOLOGY OF *D. gallinae* AND IMPACT ON THE EFFICIENCY OF THE CONTROL

D. gallinae biology itself determines the approach and procedure of the control program:

1. Adults are the most problematic stage of *D. gallinae* on which the control measures are focused. They have a great ability to survive. The reasons for this are: ability to hide, mobility, receptors, ability to starve (in some cases for more than a year - Pavlićević et al, 2007), tolerance to environmental conditions (Nordenfors et al, 1999), possibility of alternative nutrition (Nordenfors, 2000).
2. Due to their ability to hide, a prolonged effect of the formulation or method is the key property of an efficient product (Pavlićević et al, 2016). High efficacy on directly exposed mites is obligatory, because without that there is no prolonged effect either.
3. Eggs are resistant to some formulations, but in a properly designed program, this bears no clinical significance. This is due to the fact that egg development is continuous and short (usually from 1 to 3.2 days, depending on the environmental conditions - Nordenfors et al, 1999), and the next developmental stages soon become exposed to the formulation residual effect.
4. Control efficacy must be high due to the great reproductive potential (Pavlovic et al, 2017) and short life cycle of *D. gallinae* (Nordenfors, 2000).
5. *D. gallinae* develops resistance to insecticides (acaricides), but also adapts in other ways (Ebeling, 1971; Zeman and Zelezny, 1985; Zeman, 1987; Marangi et al, 2012; Pavlićević, 2005; Pavlićević et al, 2016; 2018a; Abbas et al, 2014).
6. Molecular diagnostic tests showed that infestation mostly originates from the intensive poultry production, whereas *D. gallinae* originating from the natural environment is secondary (Roy, 2009). This information is important for biosecurity. With the change in the method of poultry housing, infestation from the nature could become more significant.

THE ENVIRONMENT

The environment is one of the key factors in *D. gallinae* control program (Figure 2.). Experts assessed the changes in the method of layer housing in the EU as unfavorable from the aspect of *D. gallinae* control (Flochlay, 2017). In addition to that, the existing practice in *D. gallinae* control does not pay enough attention to the importance of the farm's environment. Not only the method of layer housing, but also the complexity and construction of some models of cages and equipment impose very demanding conditions for *D. gallinae* control, so farmers have long-term indirect losses if they buy those. Poultry experts have not warned farmers about these cases. Regarding *D. gallinae* control, simplicity and functionality are the requirements for every manufacturer of cages and equipment. The model Q – Perch by Vencomatic is designed for active *D. gallinae* control (Dick van de Ven, 2016). The efficacy of this concept is questionable. This approach is not in accordance with the control program that proscribes only security measures for new farms.

In previously used infested poultry houses, the change of cages and equipment provides good conditions for the eradication of *D. gallinae,* and subsequently the introduction of biosecurity measures. The program is especially being developed in the direction of improving the environmental conditions, and in the upcoming period we are expecting results in this area.

Figure 2. An example of improved environmental conditions – the deep gutter on the inner edge of the feed trough is irrelevant for *D. gallinae* control if it is permanently filled with a neutral substance

EFFICACY OF FORMULATIONS

Information on the efficacy of formulations and methods is provided by laboratory tests and clinical trials. Laboratory tests need to determine the effect of a formulation on directly exposed mites, residual effect (on subsequently exposed mites on the treated surface) and resistance (in cases when it occurs). The information we obtain in the lab has a limited, approximate significance. We can form a full profile of a formulation only through clinical trials. The results of clinical trials are influenced by the effect of the formulation, and by other factors depending on the specific conditions as well. Because of this, various factors need to be included and perceived comprehensively by repeating the tests in different conditions. The following factors need to be acknowledged in clinical trials: the results of laboratory tests of the formulation; housing technology; complexity and structure of the environment; hygienic conditions; temperature and humidity (time of year); infestation intensity and extensity; application (the moment of application, concentration and dosage, method of application, controllability); presence of adaptation systems; length of the housing down time; biosecurity measures; monitoring; side effects, flaws, complications and, if necessary, other. The final assessment of a formulation's efficacy should include the whole production period of a layer, a full year (Pavlićević, 2018b).

THE RIGHT CHOICE OF FORMULATION AND METHODS OF *D. gallinae* CONTROL

Laboratory tests and clinical trials provide information which is used in accordance with the criteria of rational control to choose the formulation and method. The choice of the formulation and method should be based on the arguments of safety, efficacy, and cost-effectiveness. In addition to this, it is necessary to define all other features relevant for the successful application in practical conditions.

Prior to the external application of formulations, poultry housing needs to undergo a detailed hygienic preparation, cleaning, washing and disinfection. After the housing has been examined and all necessary conditions fulfilled, a formulation is applied with care. Together with the choice of formulations and methods of *D. gallinae* control, professional application is another precondition for successful control. If necessary, auxiliary measures are taken simultaneously in order for the procedure to be a complete success.

MONITORING THE EFFICACY OF *D. gallinae* CONTROL

Detection and monitoring provide a relevant insight into the presence of *D. gallinae* and infestation intensity and extensity. Detection procedure can be based on methods that provide information from a wider area or from specific spots. An example for the latter are traps, which require multiple repetitions and adequate timing of assessment in order to provide reliable information. Visual examination and early dust detection (in cage systems) are particularly operative methods (Pavlićević et al., 2017). We advocate comprehensive monitoring (staff's observations, anamnestic data, flock's health status, production results) and multiple detection methods which should be implemented throughout the production period at monthly intervals (or more frequently, if necessary). Descriptive presentation of results is preferable to numerical. Complex diagnostic methods such as automated mite counter for *Dermanyssus gallinae* (Mul et al, 2015) are unnecessary expenditures for farmers and we do not think they are justified.

BIOSECURITY MEASURES

Biosecurity measures are supposed to prevent the introduction of *D. gallinae* into the farm or into the poultry house. This measure is essential to the program. It reaches its full purpose in uninfected facilities, and in the examples where eradication has been successfully performed. In infested facilities, biosecurity measures can enhance the effects of suppression. For the biosecurity to be effective, it needs to be based on timely, accurate, and complete information. It is implemented outside and inside the farm alike. The factors that are particularly important for biosecurity in *D. gallinae* control are population of a young flock (the flock itself and transportation cages), removing old flock (transportation cages and vehicles) and purchasing used cages and equipment. When purchasing a young flock, it is important to carry out the forensic assessment correctly. Dermanyssosis is a hidden fault and if not looked for, it usually goes unnoticed. Debris from transportation cages should be shaken off the cages, collected and covered with paper. In the end it should be unloaded and inspected in the presence of the supplier (Pavlićević et al., 2003).

INTEGRATED PEST MANAGEMENT / INTEGRATED HEALTH CARE (IPM/ IHC)

The integrated pest management (IPM) Axtell approach (1998) includes measures that would integrate pest management in the poultry industry: iden-

tification, monitoring and control. The control program is in accordance with the mentioned principles. In large infestations *D. gallinae* can coexist with the housefly - *Musca domestica* (Pavlovic et al, 2016), and very frequently with rodents. P 547/17 has a general acaricide and insecticide effect. However, a more significant coordination with the program is yet to be performed. The development of the program is directed towards integrated health care (IHC), especially by connecting and integrating into the general health care, disease control and toxicology (Pavlićević et al, 2018a,b,c). External machine application of formulations has been successfully performed in order to improve the application of disinfectants. The reasons are excluding manual labor and human error as well as being functional. Disinfection in the true sense of the word cannot be considered successful if there are infestation vectors left in the environment. Therefore, *D. gallinae* control measures need to be synchronized with disease control (Moro et al, 2005; Huong et al, 2014). We must emphasize the significance of salmonellosis that can parasitize in *D. gallinae* for up to 4 months (De Luna, 2008). Housing down time imposed in case of infectious disease can be used to eradicate *D. gallinae* (Pavlićević et al, 2018a).

THE ULTIMATE GOAL OF THE PROGRAM FOR THE CONTROL OF THE POULTRY RED MITE

The possibility of *D. gallinae* eradication from production facilities is the key issue for red poultry mite control, and it is rarely mentioned and considered. The generally accepted expert approach states that eradication is not possible. The control program proves that it is (Pavlićević et al, 2018a). Eradication is the ultimate goal of the program and the final solution for *D. gallinae* control. It eliminates toxicological risk arising from inadequate *D. gallinae* control, the role of *D. gallinae* vectors in intensive poultry production, the adverse effect of *D. gallinae* on the flock's health status. Also, to the greatest possible extent, it protects farmers' economic interest, consumers' health and economic interest and farm staff's interest. Finally, it prevents the development of resistance and further spreading of dermanyssus.

THE CURRENT SELECTION OF ACTIVE SUBSTANCES

The control program (in the narrow sense) is based on mechanical effect, especially on inert substances and during the housing preparation period before the population of the flock. SiO2 formulations are an alternative to acaricides (Kilpinen and Steenberg, 2009; Schulz, 2014a). Their effect focuses on

the absorption by the mites' epicuticular layer and the subsequent dehydration (Ebeling, 1971). In some SiO_2 formulations necessary properties have been recorded in lab conditions (Schulz et al, 2014b) and confirmed in practice (Pavlićević et al, 2018b). There are pros and cons of powdered and liquid forms. What is original in the program of "*D. gallinae*", Cluster, Serbia is the combined application of both liquid and powdered forms of SiO_2. Optimization of their application includes the correct choice of formulations and their combined professional application in both forms during the housing preparation, when there is sufficiently long down time in the temperature conditions when mites are active. Application of powdered and liquid forms of SiO_2 requires special applicators and expertise. Too complex cages and equipment question the rationality of the procedure. In a populated house efficacy is low due to the problem of formulation distribution, low penetrability into the dirt, small biocide capacity per surface unit, the ability to lay eggs even in lethally exposed mites, removing and compromising of the surface layer, influence of moisture (Pavlićević et al, 2018b). Even though successful results of SiO_2 during preparation period have been confirmed, their flaws and demanding application have limited their use to the smaller part of the poultry industry. The flaws have mostly been corrected with the new generation of inert substances P 547/17 (product Pulcap, manufacturer Pulsil LLC" *D. gallinae*" Cluster, Serbia) - Pavlićević et al, 2018a,b,c). The P 547/17 is an emulsion concentrate applied in the form of 20% water emulsion (Figure 3). It is used for the preparation of cages, equipment and environment in general. It is registered as a product for general use. It is not abrasive and does not require special applicators, but the existing spraying applicators for water solutions can be used if they contain the mechanism for mixing while in operation. P 547/17 immobilizes directly exposed mites and prevents *D. gallinae* respiration. Moreover, it probably penetrates the body and disturbs life functions of *D. gallinae*. Comprehensive effect of the formulation on *D. gallinae* is yet to be tested. So far, it is based on the general information about the effect of oils (Agnello, 2002). Liquid form of the emulsion enables better distribution and penetrability. In addition to the high efficacy on directly exposed mites, on unabsorbent surfaces, it forms a layer with long extended effect which is most emphasized in empty poultry houses. Lab tests and clinical trials show that P 547/17 has the necessary properties for highly efficient suppression, but also the potential for eradication (Pavlićević et al, 2018c). The first cases of eradication were recorded in practical conditions (2). Performing eradication in complete systems of intensive poultry production will start in Serbia in 2019 as part of the project funded by The Innovation Fund Serbia (ID = 1115), "Red poultry mite control with a mixture of

inert oils". It will include two production cycles of table eggs with the capacities of 400,000 and 200,000 layers. With the optimization of this concept and the development of the next stages of mechanical control, in the future it will be possible to completely exclude synthetic neurotoxic compounds from egg production.

D. gallinae control program focuses on mechanical methods and housing preparation. In production systems, the program starts on rearing farms. After the rearing finishes, poultry housing is prepared for the production period. A young, uninfested flock is populated in the poultry housing, using uninfested transportation cages. Formulation P 547/17 is proscribed for the treatment of transportation cages, and, if necessary, for used cages and equipment. For the procedure of *D. gallinae* eradication, an important factor is the housing down time (a break after cleaning, washing, disinfection and applying a layer of the substance) in temperature conditions that enable the mite' activity. The recommended length of the down time is 14 days, and optimally 30 days (these figures are approximate, and depend on the temperature conditions).

Figure 3.A cage covered with a layer of Pulcap

Veterinary profession obliges us not only to work on long-term development, but also to constantly look for short-term answers to all the situations that are pressuring the poultry industry. Because of this, in addition to the basic direction of development based on mechanical control, it is our task to adapt the eradication procedure to the current moment and make it less demanding and thus more available for farmers. Especially for those farms where the environmental conditions of alternative methods of layer housing have made *D. gallinae* control more difficult. Isoxazoles are insecticides with specific effect. The assessment of their insecticide potential is announced as "the golden age" (Casida, 2015). Isoxazol Fluralaner is potent inhibitor of parts of arthropod nervous system and acts antagonistically on ligand-gated chloride channels (GABA- receptor and glutamate-receptor). It is registered as a veterinary medicine Exzolt, made by MSD Animal Health. It is administered in drinking water in the dosage of 0.5 mg of fluralaner/kg BW, twice over the period of 7 days. When applied in this way, Exzolt starts to produce its effect in 4 hours, and it reaches its full effect over the period of 14 days, including two development cycles of *D. gallinae* (Technical Manual, Exzolt, MSD Animal Health). However, in our opinion, this residual effect (14 days) is not long enough for a complete *D. gallinae* control. In practical conditions, the development cycle lasts longer than the period stated in the manual for this medicine, which is the minimal length of *D. gallinae* cycle. For example, at the temperature of 25°C the development of a *D. gallinae* generation will end in 16.8 days (Maurer and Baumgartner, 1992). This lasts even longer in winter, at least for the part of the infestation located in the lower levels of cages, floors and walls. In infestations of high intensity and extensity, *D. gallinae* distribution also includes places which are not in the immediate vicinity of hens. Those mites do not have the same feeding dynamics as those close to the hens, which is proven by their colour and dimensions. Toxicological opinion prevents any repeated treatments with the same veterinary medicine within the period shorter than 3 months (instructions for application of Exsolt, MSD Animal Health). Efficacy of fluralaner recorded in laboratory conditions is 90% mortality (LC_{90}) values, the laboratory isolate was susceptible to fluralaner (15.6–62.5 parts per million, ppm). Mite LC_{90} when exposed to fluralaner by blood feeding was < 0.1 ppm (Thomas et al., 2017) and in clinical conditions 99.99% in 4 weeks (Sleeckx et al., 2018), and suppression effect ranges from 56 to 238 days (Thomas et al, 2018). Despite the obvious efficacy manifested by fluralaner, at the same time, it has evident flaws. In cases where conditions for optimal application of inert substances cannot be ensured, it is suggested that inert substances be applied in combination with veterinary medicines based on the

insecticide fluralaner (Pavlićević et al, 2018a). Correct application of insecticides can exclude any further insecticide use, prevent frequent treatments and slow down the development of resistance. The whole procedure needs to be optimized and confirmed in practice. Program application of the chosen inert substances and the new veterinary medicine based on the active substance fluralaner at this moment provides the necessary preconditions for Dermanyssossis to become a controllable health and economic problem. Otherwise, we expect that fluralaner application as currently prescribed, will cause resistance and prevent its further use in the purpose of achieving full efficacy. Therefore, we should immediately switch to its controlled and planned application in the form of a program.

COST BENEFIT ANALYSIS

Despite larger initial expenditures and more effort, eradication is cost-effective for farmers because in this way they can eliminate further spending. We will take a farm of 100,000 hens as an example. Total expenditures incurred by *D. gallinae* are estimated at €0.6 per hen (Emous, 2017; Flochlay et al, 2017). If we take 0.5 € per hen as the annual expenditure caused by red poultry mite, the total amount will be 50,000 €. In case of eradication, this farm would save half a million euros in ten years.

HOW TO CONTRIBUTE TO THE IMPLEMENTATION OF THE PROGRAM?

Reporting *D. gallinae* infestation in poultry houses would be useful for multiple reasons. It would provide information and practical protection for uninfected farms and prevent re-infestation in cases of eradication. It would differentiate the prices of infested and uninfected young flocks and motivate farmers to design a plan of eradication. It would also improve the residue monitoring, provide a continual overview of prevalence, enable the preparation for systematic implementation of *D. gallinae* control program on horizontal and vertical levels, provide assessment of effects of the implemented control measures and monitor the influence on the flock's health status, especially infectious diseases (Pavlićević et al, 2018a). Furthermore, there is a need for an international scientific-expert-business project that would take responsibility for control results on poultry farms, suggest the choice of formulations and methods in accordance with the principles of rational control, and provide adequate application and monitoring in practice. This way it would eliminate

errors that cause health and economic problems, and actively protect the common interest of the poultry industry. At the same time it would contribute to the development of formulations and methods until the final solution of this problem is found (Pavlićević et al, 2018d).

Figure 4. Mites extinct due to a disease of unknown etiology

FINAL CONSIDERATIONS AND EXPECTATIONS

The approach for the control of the poultry red mite has resulted in inadequate control of *D. gallinae* to date. We suggest that existing practice be improved by adopting the *D. gallinae* program control principle. Conditions have been met for Dermanysossis to become a controlled health and economic problem.

In the future it will be possible to completely exclude all synthetic neurotoxic compounds from egg production. We are expecting new improvements of the control program, especially in the field of mechanical control. In addition to this, there is a great potential in studying mite diseases that can cause the extinction of mites in poultry houses (Figure 4.). This phenomenon was recorded in the clinical experience in Germany, Italy and Serbia during the summer period.

The program is open to all new improvements of *D. gallinae* control. Regular tasks are constant testing of new formulations and methods, reviewing, improving and adapting the program to farms' specific conditions and needs.

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SEROPREVALENCE AND SPREADING OF BRUCELLA OVIS IN SOUTH BAČKA AND SREM DISTRICT

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Abstract

Ovine brucellosis can cause epididymitis and reduce fertility in rams and therefore is an important disease in sheep and rams from economic point of view. Sometimes it causes abortions and increased perinatal mortality in sheep as well, but it primarily affects male animals. *Brucella ovis* was first identified in northern region of Serbia, in 2008-2009 during a research study. Specific antibodies against *Brucella ovis* were detected in the sheep that originated from Becej and Titel municipalities in the South Bačka district. The prevalence was low, only 0.89%. For the purpose of this study, a total number of 6,407 serum samples from rams and sheep were used. All of the samples were from the northern part of Serbia, belonging to South Bačka and Srem districts. Serological test was performed in order to determine the presence of specific antibodies against *Brucella ovis* in rams and sheep, by ELISA test (indirect ELISA - iELISA). The prevalence varied from 0% - 19.3% during the years. If we look into the municipalities of South Bačka and Srem district, we can see that the numbers of positive samples are different, and the number of analyzed samples varies. The overall seroprevalence from 2014-2018 in South Bačka and Srem district is 6.15%, but it varies significantly among different municipalities from 0 to 26%. The majority of analyzed sample were from rams and all of the positive findings are in rams. Only a small number of sheep (female) has been examined so far, so the prevalence among females is still unknown.

Key words: *Brucella ovis*, epididimidis, seroprevalence

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SEROPREVALENCA I ŠIRENJE BRUCELLA OVIS U JUŽNOBAČKOM I SREMSKOM OKRUGU

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Kratak sadržaj

Bruceloza ovaca može izazvati epididimitis i smanjenu plodnost kod ovnova zbog toga je ova bolest važna sa ekonomskog gledišta kod gajenja ovaca. Ponekad može da izazove i perinatalna uginuća kod ovaca, ali je primarni efekat oboljenja ipak na ovnovima. U severnom delu Srbije, *Brucella ovis* je po prvi put identifikovana u periodu 2008-2009, tokom naučnog istraživanja. Specifična antitela protiv *Brucella ovis* su utvrđena kod životinja koje potiču iz opština Bečej i Titel, u Južnobačkom okrugu. Prevalenca je bila niska, 0.89%. Za potrebe ovog istraživanja pregledano je ukupno 6.407 seruma ovnova i ovaca. Svi uzorci su poticali iz severnog dela Srbije, odnosno iz Južnobačkog i Sremskog okruga. Serološki testovi su odrađeni sa ciljem da se odredi prisustvo specifičnih antitela protiv *Brucella ovis* u krvnim serumima ovaca i ovnova, ELISA testom (indirektna ELISA). Svi uzorci potiču sa područja Južnobačkog u Sremskog okruga. Tokom perioda od 5 godina, seroprevalenca je varirala od 0% - 19.3%. Ako se pogleda pojedinačno po opštinama u Južnobačkom i Sremskom okrugu, može se videti razlika u broju pozitivnih nalaza, ali i razlika u broju ispitanih uzoraka. Ukupna seroprevalenca za Južnobački i Sremski okrug tokom perioda 2014-2018 godine je 6.15%, ali su značajne razlike u seroprevalenci između različitih opština, od 0 do 26%. Najveći broj uzoraka je poticao od priplodnih ovnova, pa i pozitivni nalazi potiču od ovnova. Samo mali broj uzoraka je poticao od ovaca do sada, tako da je seroprevalenca među ovcama još uvek nepoznata.

Ključne reči: *Brucella ovis*, epididimidis, seroprevalenca

INTRODUCTION

Ovine brucellosis (epididymitis) can cause epididymitis and reduced fertility in rams and therefore is an important disease in sheep and rams from economic point of view. Sometimes it causes abortions and increased perinatal mortality as well, but it primarily affects male animals. The infection causes severe inflamma-

tion of the epididymis in rams, which can later lead to formation of spermatocoeles and fibrinous adhesions. The disease is notifiable to the World Organization for Animal Health (OIE) and it is known to be present worldwide. Unlike most species of *Brucella*, *Brucella ovis* does not seem to infect humans (OIE, 2018)

Etiology

Causative agent of brucellosis in sheep is *Brucella ovis (B. ovis)*. *B. ovis* is a Gram-negative bacteria from the family *Brucellaceae*. The causative agent is a gram-negative coccobacillus or short rod, facultative intracellular pathogen. Rams often become persistently infected with *B. ovis*, and many animals can shed the bacteria in semen for 2-4 years or longer. Shedding can occur with or without clinical signs, and it can be intermittent. Rams can also excrete this organism in urine. Ewes are relatively resistant to infection, and if they become infected, the organism clears the bacteria in a short time. In ewes the organism is shed in vaginal discharges and milk. The infection seems to be transient and rarely spans more than one pregnancy. If mothers are infected, lambs could be infected by nursing, but congenital infection seems to be rare. *B. ovis* can be transmitted from ram to ram via ewes, which in that case act as mechanical vectors. Some of those ewes do not even become infected. Such ewes have been reported to carry this organism in the vagina for at least 2 months. Ram-to-ram transmission is very rare. Some experiments indicate that this organism can enter the body via the prepuce, conjunctiva, and oral, nasal and rectal mucosae, which would mean a possibility of infection by preputial licking or other forms of oral transmission, and transmission when rams mount each other. Despite all the possibilities, the passive genital transmission is a common method of infection.

Contamination of pastures does not seem to be an important method of transmission. Sheep did not become infected when they grazed fields that had recently been occupied by infected animals, or when they were kept in enclosures next to infected animals. Rams are more susceptible than sheep, and the disease affects adult animals (Burgess, 1982).

In the northern region of Serbia, *B. ovis* was identified in 2008-2009 for the first time during a study done on 1,500 sera samples from sheep and rams examined by the method of complement fixation. In that study the findings of specific antibodies against *B. ovis* in dilution 1:5 were considered positive. Specific antibodies against *B. ovis* were detected in the sheep that originated from Becej and Titel municipalities in South Bačka district. The prevalence was low, only 0.89%. In Central Banat district, where only rams were examined, the seroprevalence for *B. ovis* was higher with 4.29% of positive findings, while in Srem district there

were no animals seropositive to *B. ovis* (Grgić et al., 2009), the trade or exchange of rams between herds is common In Serbia. Therefore, this is one of the reasons for the spreading of this contagious diseases to among the herds, especially since a considerable number of breeders does not comply with the recommendations for health check of rams. At approximately the same time, *Brucella ovis* was found in neighboring country of Croatia (Špičić et al., 2009).

Another research was carried out in 2011 in the northern region of Serbia. The total of 88 rams originating from 32 herds in 19 settlements were examined. The average age of animals was 3.1 ±1.43 year. The examined rams were of different breeds: Merinolanscaf, Ile-de-France, Suffolk breed, Tsigai, Charollais and crossbreeds. In this study the diagnostic method performed was ELISA for examining the rams to *Brucella ovis* (ram epididymitis). The percentage of seropositive rams for *B. ovis* was 3.41% (Bugarski et al., 2011). Later on, in another study, it was reported that *B. ovis* was found in rams, in the south part of Serbia, in Pirot municipality (Petrović et al., 2014).

Clinical Signs

Brucellosis caused by *B. ovis* in rams can cause epididymitis, orchitis and reduced fertility. Epididymitis manifests as an enlargement of the epididymis, especially the tail and pain or discomfort in animals on palpation. Palpable lesions can be found in the epididymis and scrotum in 30-50% of animals. The lesions can be unilateral or bilateral, although unilateral lesions are reported to be more common. Nodules or other abnormalities may also be detected, and testicles sometimes shrink in chronic cases. Palpable lesions are permanent in most rams (Arsenault et al., 2004).

In most cases poor quality semen with increased number of white blood cells may be the only sign of infection. Sperm motility and concentration may be decreased, and individual sperm is often abnormal. In ewes *B. ovis* can cause abortions, stillbirths and increased perinatal mortality and birth of weak or small lambs in some ewes, but such reproductive loses are rarely reported in the field. Systemic clinical symptoms are rare in adult ewes and rams. In the 2008-2009 study, in northern region of Serbia, the rams that had symptoms that could be indicating *B. ovis* infection did not have positive results in their blood samples by complement fixation method (Grgić et al., 2009).

Diagnostics

Diagnosis based on clinical symptoms is usually not enough, so the sam-

ples of blood, semen, vaginal swab, milk and the smears of susceptible tissues should be submitted to the laboratory for cultivation of *B. ovis*. According to the OIE Manual of diagnostic tests and vaccines, for detection of infectious agent, staining methods, culturing and PCR can be used (OIE, 2018). The best samples for identification of *B. ovis* are semen, vaginal swabs and milk. On nutritive agar *B. ovis* may be isolated from semen samples in rams, vaginal swabs and milk in ewes, and fetal placenta after the abortion. Serological tests that can be used for diagnostic purposes are several: ELISA, agar gel immunodiffusion (AGID) and complement fixation and are usually used to detect specific antibodies to *B. ovis* in animals (Webb et al., 1980). The most efficient tests and widely used ones are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect Enzyme-Linked Immunosorbent Assay (I-ELISA) (Picard-Hagen et al., 2015). Rams with ambiguous tests should be isolated and retested after 2-4 weeks. Definitive diagnosis requires molecular diagnostic procedures as detection of nucleic acids by PCR (polymerase chain reaction) technique (Costa et al., 2013; OIE, 2018).

The only test that has been prescribed to date by the OIE and the European Union (EU) for international trade was the CFT, but different countries have adopted various standard diagnostic techniques for *B. ovis*. Different independent studies have shown that the ELISA is more sensitive than CFT or AGID test. AGID test and I-ELISA have been reported as more sensitive than the CFT. Also, ELISA was sometimes reported as a less specific method, but this greatly depends on the protocol used (Estein et al., 2002; Nielsen et al., 2004; Praud et al., 2012). There are several commercial ELISA diagnostic kits on the market now, all with same principle of use.

MATERIAL AND METHODS

Samples

A total number of 6,407 serum samples from rams and sheep were used in the study. All of the samples were from the Northern part of Serbia, belonging to South Bačka and Srem districts – the same region where the studies from 2009 and 2011 were performed. The sample collection was done during the annual national surveillance and monitoring program of the country, and it was repeated in the same way every year during the period of 5 years (2014-2018). All the rams with positive to *B. ovis* antibodies were sampled again and the analysis was repeated.

Detection of specific antibodies against B. ovis

Serological test was done in order to detect the presence of specific serum antibodies against *B. ovis* in rams and sheep, by commercial indirect ELISA (iELISA, Ingezim Brucella ovis, Ingenasa, Spain), according to manufacturer's instructions. All the rams that were positive to *B. ovis* antibodies were sampled again and the analysis was repeated. The detection of specific antibodies to *Brucella ovis* in ram and sheep serum samples is based on indirect immunoenzymatic assay technique, which uses a monoclonal antibody (MAb) specific for ruminant's IgG immunoglobulins. The result of each control and sample test was expressed as a percentage (positivity (%)), based on sample optical density (OD) reading and calculated and interpreted according to the instructions of the kit manufacturer.

RESULTS AND DISCUSSION

During the period of 5 years (2014-2018), a total number of 6,407 samples were analyzed for the presence of specific antibodies against *B. ovis*. All the samples were collected in the same region – South Bačka and Srem district, during the obligatory annual surveillance program organized by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water management.

The positive samples were found in percentage from 0% - 19.3%. The annual surveillance program of Serbia started in 2016 and therefore the number of samples has tripled compared to the previous period and continuously has been rising during the following years (Table 1).

Table 1. The number and percentage of examined and positive animals to *Brucella ovis*, during the period from 2014-2018.

Year of examination	Total number of sera	No of sera with positive findings	% of sera with positive findings	No of sera with negative findings	% of sera with negative findings
2014	433	0	0%	433	100%
2015	442	85	19.3%	357	80.7%
2016	1,346	102	7.58%	1242	92.27%
2017	1,784	99	5.55%	1667	93.44%
2018	2,401	107	4.46%	2278	94.87%
Total	6407	394			

In 2014, no specific antibodies against *B. ovis* were found in South Bačka and Srem district. In 2015, positive samples were found and the prevalence was quite high (19.3%) due to the fact that almost all of the samples were collected from that region of Bečej municipality and its surrounding. The aim of the sampling was to find the positive animals and not the monitoring. The majority of the positive samples came from Bečej municipality (Figure 1), the same one where positive animals were found in 2009 (Grgić et al., 2009). Two more positive animals were found in other municipalities – Srbobran, a neighboring municipality to Bečej and Bačka Palanka, further away.

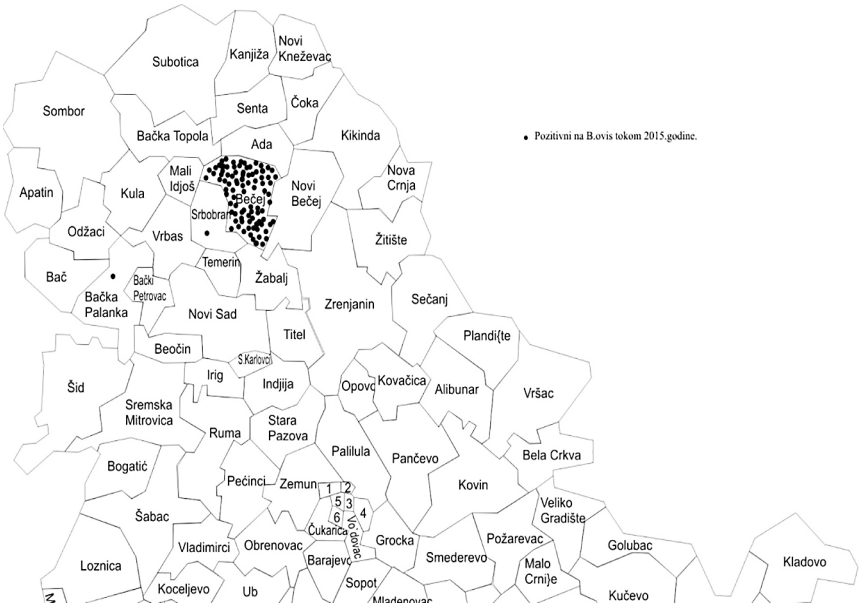

Figure 1. Location of positive animals to *Brucella ovis* found in 2015.

In 2016, there were far more samples examined in total and specific antibodies were found in rams and sheep originating from the following municipalities: Bečej with the highest number of positive samples followed by Žabalj and Titel. Novi Sad and Šid had a few positive findings while there were just one or two positive findings in Ruma, Pećinci, Inđija, Sremska Mitrovica, Srbobran, Bačka Palanka and Plandište (Figure 2). Seroprevalence was lower, due to a large number of samples analyzed, but it was still a higher percentage (7.58%) than in the previous period (2009-2014).

Figure 2. Location of positive animals to *Brucella ovis* found in 2016.

In 2017, there were again more samples analyzed then during the previous year, but the percentage of positive animals for *B. ovis* was similar to the previous year (5.55%). The municipalities where they occurred, however, were different from 2016, meaning there were more municipalities where positive animals were found. The highest number of positive animals were found in the following municipalities: Bečej, Žabalj, Titel, Bačka Palanka, with just a fewer positive findings were Srbobran and Novi Sad followed by Ruma, Pećinci, Inđija, Sremska Mitrovica, Stara Pazova, Temerin and Bač (Figure 3). This indicates that the disease is now found in a broader area, but in total the prevalence is about the same. It is due to the obligational annual government program that the number of examined animals and the number of municipalities has increased.

Figure 3. Location of positive animals to *Brucella ovis* found in 2017.

Until the end of October 2018, the total prevalence was 4.46% and the locations where the samples were collected from were pretty much the same as in 2017. With the higher number of animals examined, the total prevalence seems to be slightly dropping during that last 3 years, but the number of municipalities where the positive animals are found is rising every year. Figure 3 shows all municipalities where animals positive to *B. ovis* were found during the period from 2014-2018.

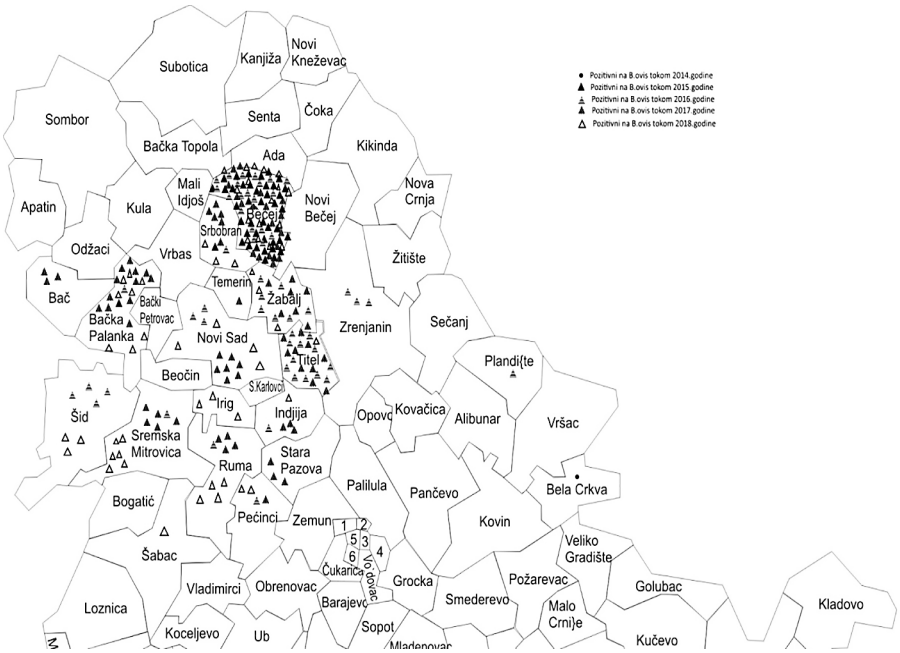

Figure 4. The locations where positive animals to *Brucella ovis* were found during the period 2014-2018.

If we look into the municipalities of South Bačka and Srem district, we can see that the number of positive samples is different and that the number of analyzed samples varies (Table 2). The overall seroprevalence from 2014-2018 in South Bačka and Srem district is 6.15%, but it varies significantly among different municipalities from 0 to 26%. It is also significant to point out that it is not the rule that the more samples were analyzed, the more positive ones were found. There are some municipalities with a low number of positive samples among quite a large number of tested samples that were examined (Table 2).

Table 2. The number and percentage of examined and positive animals to *Brucella ovis*, during the period from 2014-2018 in different municipalities of South Bačka and Srem district.

Municipality	No of analyzed samples	No of positive samples	% of positive samples
Bečej	1224	168	13.73
Bačka Palanka	927	32	3.45
Novi Sad and Sremski Karlovci	401	14	3.49
Srbobran	365	10	2.74
Titel	226	60	26.55
Žabalj	311	27	8.68
Inđija	182	6	3.30
Šid	911	19	2.09
Sremska Mitrovica	603	30	4.98
Pećinci	196	6	3.06
Ruma	306	8	2.61
Stara pazova	131	4	3.05
Bač	275	3	1.09
Bački Petrovac	44	1	2.27
Beočin	71	0	0.00
Irig	120	5	4.17
Temerin	114	1	0.88
TOTAL	6407	394	6.15

In conclusion, *Brucella ovis* in northern part of Serbia exists and is spreading around the municipality where it has been primarily found in 2009 (Grgić et al., 2009). The total prevalence seems to be slightly decreasing during the last 3 years but still not all the animals in the region have been analyzed. Also, mostly rams were analyzed and all of the positive findings are in rams. Only a small number of sheep (female) has been examined so far so the prevalence among females is rather unknown. The national annual surveillance and monitoring program is to be continued, but today it is more familiar which municipalities are with the highest prevalence for *B. ovis*. The sheep breeders must

obey the annual national program in the attempt to stop the spreading of the infection.

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