ISSN 1820-9955
UDK 619

Scientific Veterinary Institute „Novi Sad” Novi Sad
Naučni institut za veterinarstvo „Novi Sad” Novi Sad

# Archives of Veterinary Medicine
# Arhiv veterinarske medicine

| Arch. vet. med. | Vol. 13 | No. 1 | Pg. 1-136 | Novi Sad, 2020. |
|---|---|---|---|---|

ISSN 1820-9955 UDK 619

Scientific Veterinary Institute „Novi Sad" Novi Sad
Naučni institut za veterinarstvo „Novi Sad" Novi Sad

# Archives of Veterinary Medicine
# Arhiv veterinarske medicine



| Arch. vet. med. | Vol. 13 | No. 1 | Pg. 1-136 | Novi Sad, 2020. |
|---|---|---|---|---|

CIP – Каталогизација у публикацији
Библиотека Матице српске, Нови Сад

619

**Archives of Veterinary Medicine = Arhiv veterinarske medicine** / Editor in Chief Sava Lazić. – Vol. 1, br. 1 (2008) –.– Novi Sad : Scientific Veterinary Institute "Novi Sad", 2014 –.– 25 cm

Published twice a year.

ISBN 1820-9955

COBISS.SR-ID 235692807

## EDITORIAL TEAM

**Published twice a year**. Circulation: 100 copies

**Address:**
Scientific Veterinary Institute "Novi Sad",
21000 Novi Sad, Rumenački put 20
Tel. 381 (0)21 4895 321
E-mail: arhiv@niv.ns.ac.rs
Account No: 355-1006444-18 Vojvođanska banka,
ID No.: 08608857,
PIB 100236555

*Review paper*

UDC 636.7:591.69(497.11)
599.742.17: 591.69(497.11)
https://doi.org/10.46784/e-avm.v13i1.238

# ZOONOTIC HELMINTHOSIS OF DOMESTIC AND WILD CARNIVORES IN THE EPIZOOTIOLOGIC TERRITORY OF SERBIA

Tamara Ilić[1], Tamaš Petrović[2], Predrag Stepanović[1]*, Danica Bogunović[1], Bojan Gajić[1], Zoran Kulišić[1], Marko Ristić[4], Milan Hadži Milić[5], Sanda Dimitrijević[1]

[1] Department of Parasitology, University of Belgrade, Faculty of Veterinary Medicine, Republic of Serbia
[2] Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia
[3] Department of equine, small animal, poultry and wild animal diseases, University of Belgrade, Faculty of Veterinary Medicine, Republic of Serbia
[4] Department of Animal Husbandry and Veterinary Medicine, University of Niš, Faculty of Agriculture in Kruševac, Republic of Serbia
[5] Department of Surgery, Orthopaedic and Ophthalmology, University of Belgrade, Faculty of Veterinary Medicine, Republic of Serbia

## Abstract

In the last decade, as the result of climate changes, there have been considerable changes in the parasitofauna of domestic and wild carnivores. The prevalence of the existing parasitic species has varied significantly, showing an increasing tendency, and some parasitic species not present before in this epizootiologic territory have been diagnosed as well. It is thought that the reason for such an epizootiological situation is increased presence of owners with their pet animals in the regions endemic for particular zoonotic helminthoses during summer holidays and touristic visits. This tendency has become especially conspicuous in the last several years characterized by warm winters and very hot summers due to global warming effects, with abundant atmospheric precipitation. Oral vaccination of foxes against rabies, regulated in Serbia by appropriate laws since 2010, has led to an increased number of foxes and rise of prevalence of the parasitic diseases

[1*] Corresponding author: pedja@vet.bg.ac.rs; prstepanovic@gmail.com

for which foxes represent the infection source/reservoir. Continued urbanization of Serbian cities, with the extension of urban belts into the suburbia and recreational ("weekend") settlements, lead to a closer contact of street dogs and owned dogs with foxes, which results in a significant change in the parasitic fauna of dogs. It is an additional factor, which in the chain fox - street dog - owned dog - human increases the risk and tendency for the occurrence of human infections with zoonotic endoparasites of wild and domestic carnivores. In order to reliably predict the degree of spread of particular zoonotic helminthoses in particular regions in Serbia, for which wild carnivores represent the infection source, it is necessary to institute continued monitoring of the parasitic fauna in this type of wild animals.

**Key words:** carnivores, parasitological screening, zoonoses, endemic parasitoses, Serbia

# ZOONOZNE HELMINTOZE DOMAĆIH I DIVLJIH MESOJEDA NA EPIZOOTIOLOŠKOM PODRUČJU SRBIJE

Tamara Ilić[1], Tamaš Petrović[2], Predrag Stepanović[3*],
Danica Bogunović[1], Bojan Gajić[1], Zoran Kulišić[1],
Marko Ristić[4], Milan Hadži Milić[5], Sanda Dimitrijević[1]

[1] Katedra za parazitologiju, Univerzitet u Beogradu, Fakultet Veterinarske Medicine, Republika Srbija
[2] Naučni Institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija
[3] Katedra za bolesti kopitara, mesojeda, živine i divljači, Univerzitet u Beogradu, Fakultet Veterinarske Medicine, Republika Srbija
[4] Katedra za stočarstvo i veterinu, Univerzitet u Nišu, Poljoprivredni Fakultet, Kruševac, Republika Srbija
[5] Katedra za hirurgiju, ortopediju i oftalmologiju, Univerzitet u Beogradu, Fakultet Veterinarske Medicine, Republika Srbija

## Kratak sadržaj

U toku poslednje decenije u Srbiji je usled klimatskih promena došlo do značajnijih promena parazitofaune domaćih i divljih mesojeda. Prevalencija postojećih parazitskih vrsta je značajno varirala, i pokazala tendenciju povećanja, a dijagnostikovane su i neke vrste parazita koje ranije nisu

bile prisutne na ovom epizootiološkom području. Smatra se da je razlog za ovakvu epizootiološku situaciju, povećana učestalost kretanja vlasnika sa svojim ljubimcima, u endemska područja pojedinih zoonoznih helmintoza, tokom letovanja i turističkih putovanja. Ovaj trend je naročito došao do izražaja poslednjih nekoliko godina, koje su usled globalnog zagrevanja praćene pojavom blagih zima i veoma toplih leta, sa velikom količinom atmosferskih padavina. Oralna vakcinacija lisica protiv besnila, zakonski regulisana u Srbiji od 2010. godine, uslovila je povećanje broja lisica i rast prevalencije parazitoza za koje lisice predstavljaju izvore/rezervoare infekcije. Urbanizacija gradova u Srbiji sa širenjem gradskog pojasa na periferiju i područja vikend naselja, dovodi do bliskih kontakata pasa lutalica i vlasničkih pasa sa lisicama, što je rezultiralo značajnom promenom parazitofaune pasa. To je samo dodatni faktor koji u lancu: lisica - pas lutalica - vlasnički pas - čovek, značajno povećava rizik i tendenciju za nastanak infekcije ljudi zoonoznim endoparazitima divljih i domaćih mesojeda. Da bi se predvidele razmere širenja izvesnih zoonoznih helmintoza u pojedinim regionima Srbije, za koje divlji mesojedi predstavljaju izvore infekcije, neophodno je sprovoditi kontinuiran monitoring parazitske faune ove vrste divljači.

**Ključne reči:** mesojedi, parazitološki skrining, zoonoze, endemične parazitoze, Srbija

## INTRODUCTION

There is a great number of zoonotic endoparasites infesting carnivorous animals which represent a risk for the health of pets or human public health, and the most important among them are the species from the genera *Toxocara*, *Echinococcus*, *Taenia*, *Dirofilaria*, *Dipylidium caninum*, *Capillaria aerophila* and *Thelazia callipaeda* (Overgaauw and van Knapen, 2013; Marino et al., 2018; ESCCAP, 2018).

Special risk factors associated with the infection of carnivores with endoparasites are free roaming, contacts with stray dogs or cats, feeding on carcasses of paratenic hosts, feeding on intermediate host animals, age-related susceptibility, animal hormonal status (gravidity/lactation), contacts with children or immunodeficient individuals and travelling to the regions endemic for particular endoparasitoses (McNamara et al., 2018).

As the consequence of global warming and active migrations of owners with their pet animals into the countries of this region and Europe, there has been an increase in the prevalence of helminths with zoonotic potential in domestic and wild carnivores in the territory of Serbia in the last ten years. Many of the European countries have already been registered as endemic regions for certain zoonotic nematodoses (Wall and Morgan, 2009; ESCCAP, 2018). Such an epizootiologic situation, associated with the action of the above predilection factors, has caused increased occurrence of particular cardiorespiratory, subcutaneous, ocular and intestinal parasitoses, some of which have developed endemic characteristics in the territory of Serbia as well.

Routine treatment and prevention of endoparasitoses affecting carnivores depend on legislation in individual countries and information available to doctors of veterinary medicine, the most important of which are parasite epidemiology, education of pet owners and individual risk estimations. In accordance with the ESCCAP guidelines (2018), it is recommended that every protocol of planned dehelminthization should be implemented after the following: 1) completed clinical examination; 2) coprological examination after the request by the owner; and 3) coprological diagnosis within the preparation for vaccinal immunization, in accordance with the advice given by the doctor of veterinary medicine.

## CARDIOPULMONARY AND SUBCUTANEOUS DIROFILARIOSIS

The first research of dirofilariosis in dogs in the territory of former Yugoslavia took place at the end of the last century. Up to then, there were sporadic reports in the literature about the findings of *Dirofilaria immitis* species in the heart of dogs (Dimitrijević, 1999), mostly as incidental autopsy observations (Milosavljević and Kulišić, 1989; Blitva-Mihajlović et al., 1995). After that, some autochthonous cases of cardiopulmonary dirofilariosis in dogs in Serbia were diagnosed (Kulišić and Milosavljević, 1994; Dimitrijević et al., 2007). The study by Tasić et al. (2008) in the territory of Vojvodina confirmed that this region was the northernmost border for cardiopulmonary dirofilariosis in dogs in the Balkans and one of European regions with the highest prevalence of subcutaneous dirofilariosis in dogs. The authors diagnosed *Dirofilaria repens* in 49.2% of dogs, *D. immitis* in 7.2%, and *Acanthocheilonema reconditum* in 2.1% of dogs. Tasić et al. (2012) reported about the occurrence of filariosis in dogs in the territory of Pančevo and Veliko Gradište municipalities, where cardiopulmonary dirofilariosis was found in 12.3%, and subcutaneous dirofilariosis in 42.6% of dogs. The first cases of *D. immitis* in dogs in the territory

of Novi Sad were detected by Savić-Jevđenić et al. (2004). The prevalence of dirofilariosis in dogs in Vojvodina was examined by Savić et al. (2012) and they established the seroprevalence of *D. immitis* of 18% in police dogs, and in the same region in the period 2009-2013, the presence of microfilaria of *D. immitis* was detected in 27.6% of dogs (Savić et al., 2014). Stepanović et al. (2015) reported about their finding of *D. immitis* in 68% of police dogs in the Belgrade municipality, which had been declared endemic for this nematodosis several years before (Jovanović, 2012). *D. immitis* infection was established in 24.2% of non-owned dogs in the Belgrade municipality (Gajić, 2016). Krstić et al. (2016) established the presence of *D. immitis* in 12.7% of tested animals without clinical symptoms. Out of the total number of positive animals, 44% were dogs from dog shelters and 60% were pets.

The prevalence of adult forms of *D. immitis* in wild carnivores in Serbia was monitored by Penezić et al. (2014). In the period 2009-2013 they diagnosed this filaria in 7.32% of golden jackals, in 1.55% of foxes, in 1.43% of wolves and 7.69% of wild cats. Cardiopulmonary dirofilariosis was demonstrated on autopsy in 13.33% of foxes (Gavrilović et al., 2014) and in one wolf (Gavrilović et al., 2015) from the South Banat District.

Microfilaremia established in the above host animals indicated that they represented an infection source for mosquitoes. High prevalence percentages of *D. immitis* of 18.52% in jackals from Romania (Ionică et al., 2016), 9.6% in those from Bulgaria (Kirkova et al., 2011) and 7.4% in animals from Hungary (Tolnai et al., 2014) suggest that this species of wild carnivores has an important role in the maintenance of infection. According to some authors (Otranto and Deplazes, 2019) it is necessary to elucidate the epidemiological role of foxes as well, especially bearing in mind the report on 32.3% of foxes positive to *D. immitis* in some of the irrigated regions in Spain (Gortázar et al., 1998).

The first study of *Dirofilaria spp*. in disease vectors in Serbia was performed by Kurucz et al. (2016). In the period May-August, by way of molecular analysis of grouped samples from 13 municipalities in Vojvodina, 11 species of mosquitoes were identified, and in 60% of samples the presence of *Dirofilaria spp*. was found. *Dirofilaria immitis* was diagnosed in 80% of positive grouped samples of *Culex pipiens, Coquillettidia richiardii and Ochlerotatus caspius* species, while *D. repens* was diagnosed in 20% of positive samples of *Aedes vexans, Cx. pipiens* and *Oc. sticticus* species, without any *D. immitis* and *D. repens* coinfections.

Several factors have influenced the accelerated spread of *Dirofilaria spp*. into European countries in which these nematodes have not been reported before. Increased duration of warm periods due to climatic change is one of

the main factors of impact on the development, activity and seasonal survival of mosquitoes, as well as the development of larval forms of dirofilarios in disease vectors (Farkas et al., 2020). The introduction of the „Pet Travel Scheme" in 2000 has contributed to the spread of dirofilariosis, facilitating the movement of infected, microfilaremic dogs from endemic regions to other parts of Europe (Genchi et al., 2011).

Since the prevalence of dirofilariosis in dogs in hyperendemic regions (northern parts of Serbia) exceeds 60% (Tasić et al., 2008) human infections with the parasite should be expected as well. In the territory of Serbia and Montenegro, individual cases of human infection with *D. repens* have been reported (Kulišić et al., 1989; Kranjčić-Zec et al., 2001; Džamić et al., 2009). In people from different parts of Serbia (Pančevo, Novi Sad, Zaječar, Leskovac, Vranje, Niš, Pirot), Tasić-Otašević et al. (2014) demonstrated seropositivity of 15.4% to dirofilaria antigens. Specific antibodies against *D. repens* were found in 9.7%, against *D. immitis* in 8.1%, while antibodies against both dirofilaria species were found in 2.3% of the examined individuals. Momčilović et al. (2019) diagnosed *D. repens* in buccal mucosa in a man aged 45 years from central Serbia. A striking buccal mucosa edema along the lateral edge of the right maxilla was observed in this patient, followed by leukocytosis. Surgical extirpation was performed, and subsequent histopathological, parasitological, and molecular analysis of the specimen revealed the presence of the *D. repens* nematode. Of the 13 such cases reported so far worldwide, this is the first case analyzed by molecular methods.

## RESPIRATORY CAPILLARIOSIS

Considering the available epizootiological data on the distribution of *Capillaria aerophila* species in domestic and wild carnivores in Europe, it is certain that global warming has an important impact on the distribution of this parasite (Traversa and Di Cesare, 2014; Ilić et al., 2015; Otranto and Deplazes, 2019).

Climatic change, animal migrations, destruction of animal habitats by humans, maritime transport of goods, as well as travelling of pets with their owners, all have had a significant role in the spread of this parasitosis and its global distribution (Traversa et al., 2010; Otranto et al., 2013). Changed habitats of wild animals, as the consequence of human activity, have led to a closer contact between wild and domestic animals, which caused this nematode to appear in domestic animals as well (Di Cesare et al., 2014).

The parasite has been diagnosed by way of regular coprological examinations in dogs and/or cats in Spain (Miro et al., 2004), Germany (Epe et al.,

2004), Portugal (Madeira de Carvalho et al., 2009), Romania (Mircean et al., 2010) and Italy (Traversa et al., 2009; Di Cesare et al., 2011; Traversa and Di Cesare, 2016), with evident clinical symptoms of the disease. In the period February-March 2019, in a parasitological examination of dog feces samples taken from three public city parks in Niš, *C. aerophila* nematode was diagnosed with the prevalence of 8-14% (Ristić et al., 2020a).

Di Cesare et al. (2012) have described 15 genetic haplotypes of *C. aerophila*, out of which five were identified in pet animals in Italy and in wild animals in Serbia, and three genetic subtypes were isolated in both domestic cats and foxes in Serbia, Romania and Portugal. It should be emphasized that the genetic haplotypes described in foxes in Serbia were also found in cats and dogs in Italy and Romania. A couple of years later, it was demonstrated that different genetic haplotypes of *C. aerophila* could be simultaneously identified in foxes, beech martens, cats and dogs in European countries, confirming a common pattern of parasite transmission between wild animals and pets (Di Cesare et al., 2014).

The prevalence of respiratory capillariosis in red foxes in Europe is rather high, reaching 97% in Lithuania (Bružinskaitė-Schmidhalter et al., 2012), 88% in Norway (Davidson et al., 2006), 74.1% in Denmark (Saeed et al., 2006), 76.2% in Poland (Karamon et al., 2018), 67-75% in Germany (Schug et al., 2018), 66% in Hungary (Sréter et al., 2003), 46.8% in the Netherlands (Borgsteede, 1984) and 41.8% in Italy (Magi et al., 2015). Otranto et al. (2015) have reported about the finding of *C. aerophila*in European wild cats (33.3%), jackals (5%) and raccoon dogs (32%).

Ilić et al. (2016a) have reported about the prevalence of respiratory capillariosis in foxes in particular areas in Northern, Eastern and Western Serbia, which was 49.02% in the period 2008-2012. The highest prevalence was found in foxes from the hunting grounds in the Districts of Zaječar (74.04%) and Raška (52.63%). Examining the endoparasitic fauna of foxes and jackals in 8 epizootiological regions in Serbia between 2010 and 2014, Ilić et al. (2016b) diagnosed *C. aerophila* in 23.56% of foxes aged above one year. In foxes below one year of age and in jackals, this nematode could not be identified.

There is a close association between the population of foxes and populations of dogs and cats. It is assumed that the process of urbanization and extension of urban belts into the natural habitats of foxes can lead to the opportunities for closer contacts between street dogs and cats, and foxes (Ilić et al., 2017a). The population of foxes thus represents a permanent source of infection for dogs and cats in suburbia, which is especially important for the epidemiology of respiratory capillariosis.

It is supposed that one of the reservoirs of this zoonosis in Serbia are foxes from the territory of Vojvodina, which is additionally confirmed by a case of pulmonary capillariosis diagnosed in an individual from Bačka Palanka (Lalošević et al., 2008). Supporting to this are the results of some authors, who examined the respiratory tract of foxes from different regions of Vojvodina and diagnosed *C. aerophila* nematode in the trachea in 84% (Lalošević et al., 2013) and 30-37.50% of foxes (Ilić et al., 2016a). These epidemiologic data additionally corroborate the hypothesis that wild carnivores represent the principal definitive hosts responsible for the transmission of *C. aerophila* (Otranto and Deplazes, 2019).

## OCULAR THELAZIOSIS

After the original report about the finding of this nematode in dogs in Italy (Rossi and Bertaglia, 1989), *T. callipaeda* has swiftly spread across Europe, involving France (Dorchies et al., 2007), Switzerland (Malacrida et al., 2008), Germany (Magnis et al., 2010), Spain (Miró et al., 2011), Portugal (Vieira et al., 2012), Slovakia (Čabanová et al., 2017) and Greece (Papadopoulos et al., 2018).

The first autochthonous cases of ocular thelaziosis in dogs and cats in Serbia have been diagnosed in central, western, and southern parts of the country (Gajić et al., 2014; Tasić-Otašević et al., 2016). The isolated parasites were morphologically identified as *T. callipaeda*, while the molecular analyses of cytochrome oxidase-1 (cox1) gene established the presence of h1 haplotype, so far the only established haplotype of this parasite in Europe (Gajić et al., 2014). Hadži Milić et al. (2016) reported their finding of a relatively high overall prevalence of *T. callipaeda* (35.52%) in dogs in different regions of Serbia. Infected dogs in this study were from the regions in Northern (43.58% from North Banat District and 28.94% from South Bačka District), Central (41.83% from Belgrade suburbia and 21.68% from Braničevo District), Eastern (47.50% from Bor District and 30.92% from Zaječar District) and Southern Serbia (25.00% from Pčinja District). Infection of wolves with *T. callipaeda* has recently been documented, with an overall prevalence of 38.1% (Gajić et al., 2019). This finding suggests the significance of the epizootiological role of wolves as reservoirs of infection for thelazia, although other wild carnivores' role should also be addressed in the studies.

The presence of *T. callipaeda* in this epizootiological area is not surprising for the geographical coordinates of Serbia, positioned between lat 41°53'Nand 46°11'N and long 18°49'Eand 23°00'E, in Southeastern Europe in the central

part of the Balkan Peninsula. Endemic cases of ocular thelaziosis in dogs have been reported in many countries worldwide with similar geographical coordinates (between 39° and 46°) (Otranto et al., 2013). For the most part, Serbia is characterized by moderate continental climate. In the South-Western part the climate varies between subtropical and continental, with the average yearly precipitation of 896 mm, resembling those climatic conditions in the countries with reported thelaziosis cases (Hadži Milić et al., 2016).

According to the literature data, a high prevalence rate of ocular thelaziosis is present in foxes, ranging from 27.71% in Bosnia and Herzegovina (Hodžić et al., 2014), 29.38% in Romania (Ionică et al., 2018) and 49.3% in Southern Italy (Otranto et al., 2009), and cases of the disease have been reported in Portugal as well (Sargo et al., 2014). Furthermore, Pan-European distribution of the vector *Phortica variegata* (*Drosophilidae, Steganinae*) best explains the abilities and spreading potential of *T. callipaeda* (Máca and Otranto, 2014).

Circulation of the nematode across different animal species and its recent appearance in Europe is in accordance with the common genetic haplotype of *T. callipaeda* present in all examined wild and domestic animals (Otranto and Deplazes, 2019). Considering the fact that wild carnivores have a very broad movement area, 10 to 30 km for foxes (Doncaster and Macdonald, 1991) and even up to 800 km for wolves (Mech, 1970), they contribute considerably to the maintenance and spread of the infection. Moreover, they represent a significant infection reservoir for *T. callipaeda*, in both endemic and non-endemic regions (Mihalca et al., 2016), such as Great Britain, where only imported cases of thelaziosis in dogs have been reported (Graham-Brown et al., 2017) despite an endemic vector (Palfreyman et al., 2018).

In the last six years, in the countries nearby Serbia (Romania, Croatia, Bosnia and Herzegovina, Bulgaria, Austria) there have been several reported autochthonous cases of ocular thelaziosis in dogs and cats (Hodžić et al., 2014; Mihalca et al., 2015; Hodžić et al., 2019), and in Bosnia and Herzegovina in foxes as well (Hodžić et al., 2014). In 2016, the zoonotic potential of the parasite in these regions was additionally confirmed by the report of two cases of thelaziosis in humans: one in a 36-year-old man from Serbia (Tasić-Otašević et al., 2016) and one in 82-year-old man from Croatia (Paradžik et al., 2016).

Since *T. callipaeda* has got zoonotic potential and presents a risk for human health, it is necessary that veterinarians, doctors and ophthalmologists should include this nematodosis with ocular manifestations into their differential diagnostic considerations. Such a clinical approach is especially important in regions where ocular thelaziosis has assumed endemic character, such as in Serbia.

## INTESTINAL ZOONOTIC HELMINTHOSES

Regarding geographical distribution and clinical relevance, *Toxocara canis*, *Ancylostomatidae* and *Trichuris vulpis* are the most prevalent intestinal helminths affecting dogs, the importance of which is often unacknowledged by doctors of veterinary medicine, medical doctors and general public (Traversa et al., 2014). Depending on the intensity of dog infection, we should not overlook some cestodes (*D. caninum* and *Taenia* spp.), as well as trematodes (*Alaria alata*) and protozoans (*Giardia intestinalis*, *Amoeba* spp., and *Cryptosporidium* spp.) (Möhl et al., 2009; Traversa, 2012; ESCCAP, 2018). From the medical, veterinary, economic and environmental point of view, enzootic infections are the most important, the sources of which are linked to natural sites.

In the last three decades, a large number of authors have studied parasitic fauna of the digestive tract of domestic and wild carnivores from the territory of Serbia (Kulišić et al., 1992; Pavlović and Kulišić, 1994; Antanasijević et al., 1997; Dimitrijević et al., 2005; Nikolić et al., 2008; Pavlović et al., 2010; Ilić et al., 2016b; Ilić et al., 2017b; Ristić et al., 2020a).

In owned dogs, stray dogs and military working dogs in the territory of Belgrade, Nikolić et al. (2008) have found a high prevalence rate of intestinal zoonotic parasites (75.50%). In most of these animals, the infection with *T. vulpis* nematode was diagnosed (47.00%). In dogs and cats aged 1-8 years in the territory of Belgrade, Ilić et al. (2017b) have diagnosed toxocarosis (15.88-16.62%), ancylostomatidosis (1.87-3.80%) and trichuriosis (0.93-4.03%), with the finding of highest prevalence of *Dipylidium caninum* infection, ranging from 21.49% in cats to 24.70% in dogs.

Kostić (2016) has reported on the finding of intestinal parasites in street dogs in the territory of the city of Kruševac. Parasitological studies revealed the predominance of *G. duodenalis* (52.11%) and *D. caninum* cestode (36.61%). *Trichuris vulpis* nematode was found in one third of examined dogs (32.39%), and *T. canis* (22.53%), *Taenia* spp. (5.63%), and *E. granulosus* (2.82%) were diagnosed as well. In the period from September 2017 to June 2018, in a study of endoparasitoses in street dogs aged one year and over from six dog shelters in the Republic of Serbia, the highest prevalence of endoparasitic infections was found in the Shelter for street dogs and cats in Požarevac (69.54%). In dogs below one year of age, most prevalent were toxocarosis (42.85%) and ancylostomatidosis (26.53%) in the Shelter for abandoned dogs in Zemun, while toxocarosis was most prevalent (35.14%) in the Shelter for abandoned dogs in Subotica (Nišavić, 2019).

Monitoring endoparasitic fauna of foxes and jackals from eight different localities in Serbia in the period 2010-2014, Ilić et al. (2016b) have diagnosed as

the most prevalent helminths *A. alata* (49.41% in foxes and 30.00% in jackals), *T. canis* (49.41% in foxes and 23.33% in jackals), ancylostomatids (40.69% in foxes and 33.33% in jackals) and *T. vulpis* (55.23% in foxes and 11.66% in jackals).

Ristić et al. (2020a) have studied the prevalence of zoonotic intestinal parasites in dogs from the public parks in the city of Niš and assessed the health risks they presented for people in public places and children's playgrounds. Endoparasitoses were diagnosed with overall prevalence of 58-70%. Depending on the season of the study, four most common endoparasites were *T. canis* (36.66-38%), ancylostomatids (24.66-32%), *T. vulpis* (20-28%) and *A. alata* (28%). Certainly worth mentioning was the fact that these helminths were identified in soil/sand samples taken from these public parks in Niš. In soil samples, a high and medium degree of contamination with *T. canis* ascaridid was found (14-22%), as well as a low and medium degreeof contamination with ancylostomatids (4-12%), medium degree of contamination with *T. vulpis* species (4-6%), and medium and high degree of contamination with *A. alata* trematode (2%). In sand samples, different degrees of contamination with helminths *T. canis* (26%), ancylostomatids (8%), *T. vulpis* (4%) and *A. alata* (16%) (Ristić et al., 2020b) were established. Based on the results of this parasitological screening, the authors concluded that a large number of street dogs circulated in public parks in Niš, which presented the reservoir of numerous parasitic zoonoses for owned dogs and people (particularly for preschool and school children).

The literature data suggest that *T. canis* is overall the most prevalent helminth in dogs, the prevalence of which varies between European countries from 7.5% (Riggio et al., 2013) to 22.1% (Habluetzel et al., 2003) in Italy; 17.72% in Spain (Martínez-Moreno et al., 2007), from 11.9% (Papajová et al., 2014) to 45.1% (Rudohradská et al., 2011) in Slovakia; and from 0.5% (Ferreira et al., 2017) to 15.8% (Otero et al., 2014) in Portugal. The finding of this ascaridid always involves a high degree of risk for human populations, since after the infection its larvae migrate to individual internal organs, causing the syndrome of visceral and ocular *larva migrans* (Overgaauw and van Knapen, 2013).

In recent years, there has been a growing interest for the infection with *A. alata* trematode, which is very prevalent in Europe, has a potential zoonotic importance since it causes larval alariosis, and is diagnosed in a number of countries nearby Serbia, such as Croatia, Romania and Bulgaria (Lalošević et al., 2014).

A high intensity infection with this trematode has been reported to occur in intermediate hosts (wild pigs) living in the areas with high prevalence of alariosis in definitive hosts (domestic and wild carnivores). In addition to rodents, reptiles and amphibians (Esite et al., 2012), humans as well can be incidental paratenic hosts if they consume insufficiently thermally processed

frog legs, pork (Wójcik et al., 2002; Möhl et al., 2009) or wild goose meat (Kramer et al., 1996) infected with *A. alata* mesocercarias.

In recent years, larval alariosis has been reported in other European countries as well. The first finding in the meat of wild pigs in Bulgaria was reported by Portier et al. (2014), when the overall prevalence of 0.6% was established. The study of the vitality of mesocercarias in products made from infected wild pig meat, prepared in a traditional fashion in Germany, has shown than only fresh products contain living mesocercarias and could be the source of infection for humans (González-Fuentes et al., 2014).

In addition to trichinellosis, wild pig meat can be the source of infection with trematode mesocercarias, so that any preparation of thermally unprocessed products (e.g., sausages) cannot be recommended. Safe for human nutrition is only well cooked wild pig meat. Bearing in mind this information, it is necessary to adjust accordingly the legislation on food safety for human consumption in Serbia (Lalošević et al., 2014).

In view of human population, zoonotic parasites from the feces of carnivores can threaten mostly dog owners or dog breeders who disregard the necessity to perform dehelminthization of their litters, children who do not wash their hands after their contacts with animals, or those with geophagic practices, agricultural workers and greengrocers (especially in semirural and rural areas where dogs freely defecate in the vicinity of vegetables).

In people in Serbia, the cases of a familial epidemic of cryptosporidiosis have been coprologically diagnosed in three immunocompetent patients (Gvozdenović et al., 2012) and there is also the case of 4.5-year-old girl in whom toxocarosis has been detected serologically (Mijatović et al., 2015). A retrospective analysis of the results of examination of feces samples from healthy people in Southern Serbia to detect possible presence of *G. intestinalis* revealed the highest prevalence in 2005 (4.9%), and the lowest in 2014 (0.57%) (Miladinović Tasić et al., 2017). Perić et al. (2017) have reported on their two cases for whom there were no anamnestic data concerning their previous travelling abroad, and in whom cutaneous *larva migrans* was diagnosed in 2016. The first described patient was a 72-year- old man from Western Serbia with changes localized in the chest region, and the other was a 31-year-old man from Central Serbia with changes localized in his right arm.

## CONCLUSION

In order to prevent and control zoonotic helminthoses of domestic and wild carnivores it is essential that their occurrence, infection spread and maintenance should be continually monitored in foxes, dogs and humans; that the

problem of abandoned dogs should be effectively resolved in most urban environments; that the measures to control vector populations should be undertaken; that all goods, commodities, services and traffic in international trade should be monitored; that all persons who travel with their pets to the regions endemic for these diseases or who return from these to Serbia should be monitored; that continued education of pet owners is organized; and that synchronized collaboration of appropriate services of veterinary medicine and human medicine is in place.

## ACKNOWLEDGEMENTS

This paper was realized in keeping with the projects No. TR31084, No. 173001 and No. TR30188 financed by the Ministry of Education and Science of the Republic of Serbia.

### Authors' contributions

IT, PT, SP, BD, GB, KZ, RM, HM and DS were participated in the design of the study, conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

### Competing interests

The author(s) declare that they have no competing interests.

## REFERENCES

1. Antanasijević S., Petrović M., Ignjatović R., Kulišić Z., Pavlović I. 1997. Echinococcosis and hydatigenosis od domestic animals in Niš area-socio-economic aspects. In Archivos internationales de la hydatidosis. XVIII International Congress of Hydatolgy, Vol XXXII, Lisboa, 286.
2. Blitva-Mihajlović G., Ralić M., Miletić B. 1995. Bolest srčane gliste. U Simpozijum Male životinje - život i zdravlje, Beograd, 34.
3. Borgsteede F.H. 1984. Helminth parasites of wild foxes (*Vulpes vulpes L.*) in The Netherlands. Zeitschrift für Parasitenkunde, 70, 281-285.
4. Bružinskaitė-Schmidhalter R., Šarkūnas M., Malakauskas A., Mathis A., Torgerson P.R., Deplazes P. 2012. Helminths of red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) in Lithuania. Parasitology, 139, 120-127. doi:10.1017/S0031182011001715

5. Čabanová V., Kocák P., Víchová B., Miterpáková M. 2017. Firstautochthonous cases of canine thelaziosis in Slovakia: a new affected area in Central Europe. Parasites &Vectors, 10, Article No 179. doi:10.1186/s13071-017-2128-2.
6. Davidson R.K., Gjerde B., Vikøren T., Lillehaug A., Handeland K. 2006. Prevalence of *Trichinella* larvae and extra-intestinal nematodes in Norwegian red foxes (*Vulpes vulpes*). Veterinary Parasitology, 136, 307-316. doi:10.1016/j.vetpar.2005.11.015.
7. Di Cesare A., Castagna G., Otranto D., Meloni S., Milillo P., Latrofa SM., Paoletti B., Bartoliniet R., Otranto D. 2012. Molecular detection of *Capillaria aerophila*, an agent of canine and feline pulmonary capillariosis. Journal of Clinical Microbiology, 50, 1958-1963. doi:10.1128/JCM.00103-12.
8. Di Cesare A., Castagna G., Meloni S., Milillo P., Latrofa S., Otranto D., Traversa D. *2011.* Canine and feline infections by cardiopulmonary nematodes in central and southern Italy. *Parasitology Research,* 109, 1, 87-96. doi:10.1007/s00436-011-2405-5.
9. Di Cesare A., Otranto D., Latrofa SM., Veronesi F., Perrucci S., Lalosević D., Gherman MC., Traversa D. 2014. Genetic variability of *Eucoleus aerophilus* from domestic and wild hosts. Research in *Veterinary Science*, 96, 512-515. doi:10.1016/j.rvsc.2014.03.018.
10. Dimitrijević S., Ilić T., Nikolić A, Bečkei Ž. 2005. Zastupljenost parazitskih infekcija mesojeda na teritoriji grada Beograda.U Zbornik radova VII Simpozijuma „Clinica Veterinaria", Ohrid, 326.
11. Dimitrijević S.: Dirofilarioza *ante portas*. U Zbornik radova Prvog savetovanja iz kliničke patologije i terapije životinja "Clinica veterinaria 99", Septembar 13-17, Budva, 58.
12. Dimitrijević S., Tasić A., Tasić S., Adamović V., Ilić T., Miladinović-Tasić N. 2007. Filariosis in dogs in Serbia. In Proceedings of the first European Dirofilaria days, edited by Genchi C., Rinaldi L., Cringoli G., Zagreb, Croatia, 201.
13. Doncaster C.P. and Macdonald D.W. 1991. Ecology and ranging behaviour of red foxes (*Vulpes vulpes*) in the city of Oxford. Hystrix 3, 11-20.
14. Dorchies P., Chaudieu G., Siméon L.A., Cazalot G., Cantacessi C., Otranto D. 2007. First reports of autochthonous eyeworm infection by *Thelazia callipaeda* (Spirurida, Thelaziidae) in dogs and cat from France. Veterinary Parasitology, 149, 294-297. doi:10.1016/j.vetpar.2007.08.005.
15. Džamić A.M., Colović I.V., Arsić-Arsenijević, V.S., Stepanović S., Boricić I., Džamić Z., Mitrović S.M., Rasić D.M., Stefanović I., Latković Z., Kranjcić-Zec I.F. 2009. Human Dirofilaria repens infection in Serbia. Journal of helminthology, 83, 2, 129–137. doi:10.1017/S0022149X09341346.
16. Epe C., Coati N., Schnieder T. *(2004).* Results of parasitological examinations of faecal samples from horses, ruminants, pigs, dogs, cats, hedge-

hogs and rabbits between 1998 and 2002. *Deutsche Tierarztliche Wochenschrift,111,* 6, 243-247.
17. ESCCAP. 2018. Worm control in dogs and cats. ESCCAP Guideliene 01 Third edition July 2017 - Accessed 8 Jan 2018, 40.
18. Esite Z., Deksne G., Bagrade G. 2012. Overview of *Alaria alata* distribution in different host animals in Latvia. Animals, health, food hygiene. In Proceedings of Conference on "Current events in veterinary research and practice", 22-23 November, Jelgava, Latvia, 36-39.
19. Farkas R., Mag V., Gyurkovszky M., Takács N., Vörös K., Solymosi N. 2020. The current situation of canine dirofilariosis in Hungary. *Parasitology Research*, 119, 129-135. https://doi.org/10.1007/s00436-019-06478-5.
20. Ferreira A., Alho A.M., Otero D., Gomes L., Nijsse R., Overgaauw PA., Madeira de Carvalho L. 2017. Urban dog parks as sources of canine parasites: contamination rates and pet owner behaviours in Lisbon, Portugal. *Journal of Environmental and Public Health*, Article ID 5984086, 1-7. doi:10.1155/2017/5984086.
21. Gajić B. 2016. Prevalencija vektorski prenosivih bolesti kod nevlasničkih pasa na području Beograda. Akademski specijalistički rad, Fakultet veterinarske medicine,Univerzitet u Beogradu, 51.
22. Gajić B., Bogunović D., Stevanović J., Kulišić Z., Simeunović P., Stanimirović Z. 2014. Canine and feline thelaziosis caused by *Thelazia callipaeda* in Serbia. Acta Veterinaria-Beograd, 64, 4, 447-455. doi:10.2478/acve-2014-0042.
23. Gajić B., Bugarski-Stanojević V., Penezić A., Kuručki M., Bogdanović N., Ćirović D. 2019. First report of eyeworm infection by *Thelazia callipaeda* in gray wolf (*Canis lupus*) from Serbia. Parasitology Research, 118, 3549-3553. doi:10.1007/s00436-019-06519-z.
24. Gavrilović P., Blitva-Robertson G., Özvegy J., Kiskároly F., Becskei Zs. 2015. Case Report of dirofilariasis in grey wolf in Serbia. Acta Parasitologica, 60, 175-178. doi:10.1007/BF03406010.
25. Gavrilović P., Vasković N., Živulj A., Jovanović M. 2014. Examination of *D. immitis* presence in foxes in Serbia (in Serbian with English summary). *Veterinarski Glasnik*, 68, 5-6, 387-393. doi:10.2298/VETGL1406387G.
26. Genchi C., Mortarino M., Rinaldi L., Cringoli G., Traldi G., Genchi M. 2011. Changing climate and changing vector-borne disease distribution: the example of *Dirofilaria* in Europe. Veterinary Parasitology, 176, 295-299. doi:10.1016/j.vetpar.2011.01.012.
27. González-Fuentes H., Hamedy A., Borell E., Luecker E., Riehn K. 2014. Tenacity of *Alaria alata* mesocercariae in homemade German meat products. International Journal of Food Microbiology, 176, 9-14. doi:10.1016/j.ijfoodmicro.2014.01.020.

28. Gortázar C., Villafuerte R., Lucientres J., Fernández-de-Luco D. 1998. Habitat related differences in helminth parasites of red foxes in the Ebro valley. Veterinary Parasitology, 80, 75-81. doi:10.1016/s0304-4017(98)00192-7.
29. Graham-Brown J., Gilmore P., Colella V., Moss L., Dixon C., Andrews M., Arbeid P., Barber J., Timofte D., Mc Garry J., Otranto D., Williams D. 2017. Three cases of imported eyeworm infection in dogs: a new threat for the United Kingdom. Veterinary Record, 181, 346. doi:10.1136/vr.104378.
30. Gvozdenović E., Mitrović M., Dakić Z., Stojković-Švirtlih N., Dulović O. 2012. Family outbreak of cryptosporidiosis in Serbia: Case report. Srpski arhiv za celokupno lekarstvo, 140, 9-10, 653-657. doi:10.2298/SARH1210653G.
31. Habluetzel A., Traldi G., Riggieri S., Attili AR., Scuppa P., Marchetti R., Menghini G., Esposito F. 2003. An estimation of *Toxocara canis* prevalence in dogs, environmental egg contamination and risk of human infection in the Marche region of Italy. Veterinary Parasitology, 113, 243-252. doi:10.1016/S0304-4017(03)00082-7.
32. Hadži-Milić M., Ilić T., Stepanović P., Đorđević J., Dimitrijević S. 2016. Serbia: Another endemic region for canine ocular thelaziosis. Medycyna Weterynaryjna, 72, 9, 558-563. doi:10.21521/mw.5561.
33. Hodžić A., Latrofa MS., Annoscia G., Alić A., Beck R., Lia RP., Dantas-Torres F., Otranto D. 2014. The spread of zoonotic *Thelazia callipaeda* in the Balkan area. Parasites & Vectors, 7, 352. doi:10.1186/1756-3305-7-352.
34. Hodžić A., Payer A., Duscher G.G. 2019. The first autochthonous case of feline ocular thelaziosis in Austria. Parasitology Research, 118, 1321-1324. doi:10.1007/s00436-019-06275-0.
35. Ilić T., Mandić M., Stepanović P., Dimitrijević S. 2015. Dog and cat respiratory capilariasis - clinical, parasitological and epidemiological significance. Veterinarski Glasnik, 69, 5-6, 417-428. doi:10.2298/VETGL1506417I.
36. Ilić T., Becskei Zs., Tasić A., Stepanović P., Radisavljević K., Đurić B., Dimitrijević S. 2016a. Red foxes (*Vulpes vulpes*) as reservoirs of respiratory capillariosis in Serbia. Journal of Veterinary Research, 60, 153-157. doi:10.1515/jvetres-2016-0022.
37. Ilić T., Becskei Zs., Petrović T., Polaček V., Ristić B., Milić S., Stepanović P., Radisavljević K., Dimitrijević S. 2016b. Endoparasitic fauna of red foxes (*Vulpes vulpes*) and golden jackals (*Canis aureus*) in Serbia. Acta Parasitologica, 61, 2, 389-396. doi: 10.1515/ap-2016-0051.
38. Ilić T., Mandić M., Stepanović P., Dimitrijević S. 2017a. The cardiopulmonary metastrongylidosis of dogs and cats contribution to diagnose. Veterinarski Glasnik, 71, 2, 69-86. doi:10.2298/VETGL170310010I.

39. Ilić T., Kulišić Z., Antić N., Radisavljević K., Dimitrijević S. 2017b. Prevalence of zoonotic intestinal helminths in pet dogs and cats in the Belgrade area. Journal of Applied Animal Research, 45, 1, 204-208. doi:10.1080/09712119.2016.1141779.
40. Ionică A.M., Matei I.A., D'Amico G., Daskalaki A.A., Juránková J., Ionescu DT., Mihalca AD., Modrý D., Gherman C.M. 2016. Role of golden jackals (*Canis aureus*) as natural reservoirs of *Dirofilaria* spp. in Romania. Parasites & Vectors, 9, 240. doi:10.1186/s13071-016-1524-3.
41. Ionică A.M., Deak G., Matei I.A., D'Amico G., Cotuțiu V.D., Gherman C.M., Mihalca A.D. 2018. *Thelazia callipaeda*, an endemic parasite of red foxes (*Vulpes vulpes*) in Western Romania. Journal of Willife Diseases, 54, 829-833. doi:10.7589/2017-10-251.
42. Jovanović M. 2012. Prilog poznavanju dijagnostike, terapije i profilakse dirofilarioze pasa na teritoriji Grada Beograda. Academic specialisation (on post graduate course for pathology and therapy of small animals), Faculty of Veterinary Medicine, University of Belgrade, Serbia, 76.
43. Karamon J., Dąbrowska J., Kochanowski M., Samorek-Pieróg M., Sroka J., Różycki M., Cencek T. 2018. Prevalence of intestinal helminths of red foxes (*Vulpes vulpes*) in central Europe (Poland): a significant zoonotic threat. Parasites & Vectors, 11, 436. doi:10.1186/s13071-018-3021-3.
44. Kirkova Z., Raychev E., Georgieva D. 2011. Studies on feeding habits and parasitological status of red fox, golden jackal, wild cat and stone marten in Sredna Gora, Bulgaria. Life Science Journal, 5, 264-270.
45. Kostić B.2016. Ekološki i društveni značaj rešavanja problema napuštenih pasa na teritoriji grada Kruševca. Doktorska disertacija, Fakultet za ekonomiju i inžinjerski menadžment, Univerzitet Privredna akademija, Novi Sad, 187.
46. Kramer M.H., Eberhard M.L., Blankenberg T.A. 1996. Respiratory symptoms and subcutaneous granuloma caused by mesocercariae: a case report. American Journal of Tropical Medicine and Hygiene, 55, 447-448. doi:10.4269/ajtmh.1996.55.447.
47. Kranjčić-Zec I., Džamić A., Arsić V., Mitrović S. 2001. Human infections by the nematodes of the *Dirofilaria sp*. In Proceedings of Scientific Meeting: Tissue helminthoses of man and animals (in Serbian), 11-20.
48. Krstić M., Gabrielli S., Ignjatović M., Savić S., Cancrini G., Ranđelović G., Momčilović S., Stojnev S., Otašević S. 2016. An appraisal of canine and human cases reveals an endemic status of dirofilariosis in parts of Serbia, *Molecular and Cellular Probes*, 31, 37-41. doi:10.1016/j.mcp.2016.08.005.
49. Kulišić Z., Kranjčić-Zec I., Mitrović S., Radojičić B. 1989. Novi slučaj dirofilarioze ljudi u Jugoslaviji. U 6. Kongres mikrobiologov Jugoslavije, Maribor, 124.

50. Kulišić Z., Pavlović I., Nikolić A., Veljović T., Kokoškov N., Sikimić P., Bojkovski J. 1992. Paraziti digestivnog trakta pasa na području Beograda. U Zbornik kratkih sadržaja V Savetovanja veterinara Srbije, Kopaonik, 90.
51. Kulišić, Z. and Milosavljević, P. 1994. Savremene metode dijagnostike dirofilarioze pasa. Veterinarski Glasnik, 48, 9, 745-749.
52. Kurucz K., Kepner A., Krtinic B., Zana B., Földes F., Banyai K., Oldal M, Jakab F., Kemenesi G. 2016. First molecular identification of *Dirofilaria* spp. (Onchocercidae) in mosquitoes from Serbia. International Journal of Infectious Diseases, 53, 156. doi:10.1016/j.ijid.2016.11.381.
53. Lalošević D., Lalošević V., Klem I., Stanojev-Jovanović D., Pozio E. 2008. Pulmonary capillariasis miming bronchial carcinoma. American Journal of Tropical Medicine and Hygiene, 78, 14-16.
54. Lalošević V., Lalošević D., Čapo I., Simin V., Galfi A., Traversa D. 2013. High infection rate of zoonotic *Eucoleus aerophilus* infection in foxes from Serbia. Parasite, 20, Article No3, 1-5. doi:10.1051/parasite/2012003.
55. Lalošević D., Lalošević V., Putić S., Simin S., Kuruca Lj. 2014. *Alaria alata* mezocerkarije u mesu divlje svinje, novoregistrovani patogen u Srbiji. MD - Medical Data, 6, 4, 363-365.
56. Máca J. and Otranto D. 2014. Drosophilidae feeding on animals and the inherent mystery of their parasitism. Parasites & Vectors, 7, 516. doi:10.1186/s13071-014-0516-4.
57. Madeira de Carvalho LM. Pereira da Fonseca LM. Gomes L., Meireles JM. *2009.* Lungworms in domestic and wild carnivores in Portugal: rare parasites or rarely diagnosed? *In* Proceedings of the Bayer Angiostrongylosis Forum, 19th Annual Congress of the European College of Veterinary Internal Medicine - Companion Animals, Porto, Portugal, 28.
58. Magi M., Guardone L., Prati M.C., Mignone W., Macchioni F. 2015. Extraintestinal nematodes of the red fox *Vulpes vulpes* in north-west Italy. Journal of Helminthology, 89, 4, 506-511. doi:10.1017/S0022149X1400025X.
59. Magnis J., Naucke T.J., Mathis A., Deplazes P., Schnyder M. 2010. Local transmission of the eye worm *Thelazia callipaeda* in southern Germany. Parasitology Research, 106, 715-717. doi:10.1007/s00436-009-1678-4.
60. Malacrida F., Hegglin D., Bacciarini L., Otranto D., Nägeli F., Nägeli C., Bernasconi C., Scheu U., Balli A., Marenco M., Togni L., Deplazes P., Schnyder M. 2008. Emergence of canine ocular thelaziosis caused by *Thelazia callipaeda* in southern Switzerland. Veterinary Parasitology, 157, 321-327. doi:10.1016/j.vetpar.2008.07.029.
61. Marino V., Gálvez R., Colella V., Sarquis J., Checa R., Montoya A., Barrera PJ., Domínguez S., Lia PR., Otranto D., Miró G. 2018. Detection of *Thelazia*

*callipaeda* in *Phortica variegata* and spread of canine thelaziosis to new areas in Spain. Parasites & Vectors, 11, Article No195, 1-10. doi:10.1186/s13071-018-2773-0.

62. Martínez-Moreno F.J., Hernández S., López-Cobos E., Becerra C., Acosta I., Martínez-Moreno A. 2007. Estimation of canine intestinal parasites in Córdoba (Spain) and their risk to public health. Veterinary Parasitology, 143, 1, 7-13. doi:10.1016/j.vetpar.2006.08.004.
63. McNamara J., Drake J., Wiseman S., Wright J. 2018. Survey of European pet owners quantifying endoparasitic infection risk and implications for deworming recommendations. Parasites & Vectors, 11, 571. doi:10.1186/s13071-018-3149-1.
64. Mech L.D. 1970. The wolf: the ecology and behavior of an endangered species. The Natural History Press, Garden City, New York.
65. Mihalca AD., D'Amico G., Scurtu I., Chirilă R., Matei IA., Ionică AM. 2015. Further spreading of canine oriental eyeworm in Europe: first report of *Thelazia callipaeda* in Romania. Parasites & Vectors, 8, 48. doi:10.1186/s13071-015-0663-2.
66. Mihalca A.D., Ionică A.M., D'Amico G., Daskalaki A.A., Deak G., Matei I.A., Șimonca V., Iordache D., Modrý D., Gherman C.M. 2016. *Thelazia callipaeda* in wild carnivores from Romania: new host and geographical records. Parasites & Vectors, 9, 350. doi:10.1186/s13071-016-1628-9.
67. Mijatović D., Ćalasan N., Simin V., Lalošević D. 2015. Nadzor pacijenta sa toksokarijazom - Prikaz slučaja (In Serbian). MD - Medical Data 7, 4, 327-329.
68. Miladinović Tasić N., Đorđević N., Zdravković D., Tasić A. 2017. Prevalencija đardioze kod asimptomatskih osoba na području grada Niša (In Serbian). Acta Medica Medianae, 56, 3, 48-54. doi:10.5633/amm.2017.0308.
69. Milosavljević P. and Kulišić Z. 1989. Prvi slučajevi dirofilarioze pasa u Jugoslaviji. Veterinarski Glasnik, 43, 1, 71-76.
70. Mircean V., Titilincu A., Vasile C. *2010.* Prevalence of endoparasites in household cat (*Felis catus*) populations from Transylvania (Romania) and association with risk factors. Veterinary Parasitology, 171, 163-166. doi:10.1016/j.vetpar.2010.03.005.
71. Miro G., Montoyaa A., Jimenez S., Frisuelosa C., Mateoa M., Fuentes I. *2004.* Prevalence of antibodies to *Toxoplasma gondii* and intestinal parasites in stray, farm and household cats in Spain. Veterinary Parasitology, 3, 249-255. doi:10.1016/j.vetpar.2004.08.015.
72. Miró G., Montoya A., Hernández L., Dado D., Vázquez M.V., Benito M., Villagrasa M., Brianti E., Otranto D. 2011. *Thelazia callipaeda* infection in dogs: a new parasite for Spain. Parasites & Vectors, 4, 148. doi:10.1186/1756-3305-4-148.

73. Momčilović S., Gabrielli S., Golubović M., Smilić T., Krstić M., Đenić S., Ranđelović M., Tasić-Otašević S. 2019. Human dirofilariosis of buccal mucosa - first molecularly confirmed case and literature review. Parasitology International, 73, Article ID 101960, 1-7. doi:10.1016/j.parint.2019.101960.
74. Möhl K., Grosse K., Hamedy A., Wüste T., Kabelitz P., Lücker E. 2009. Biology of *Alaria alata* and human exposition risk to *Alaria* mesocercaria - a review. Parasitology Research, 105, Article No 1. doi:10.1007/s00436-009-1444-7.
75. Nikolić A., Dimitrijević S., Katić-Radivojević S., Klun I., Bobić B., Djurković-Djaković O. 2008. High prevalence of intestinal zoonotic parasites in dogs from Belgrade, Serbia - Short communication. Acta Veterinaria Hungarica, 56, 3, 335-340. doi:10.1556/avet.56.2008.3.7.
76. Nišavić U. 2019. Endoparazitoze pasa u odabranim prihvatilištima na području Srbije. Akademski specijalistički rad, Fakultet veterinarske medicine, Univerzitet u Beogradu, 62.
77. Otero D., Nijsse R., Gomes L., Alho A., Overgaauw P., Hoek D., Madeira de Carvalho LM. 2014. Prevalência de ovos de *Toxocara* spp., no solo de parquespublicos da area da Grande Lisboa, Portgal - resultadospreliminares. (In Portuguese). Acta Parasitologica Portuguesa, 20, 1-2, 47-50.
78. Otranto D. and Deplazes P. 2019. Zoonotic nematodes of wild carnivores. International Journal for Parasitology: Parasites and Wildlife, 9, 370-383. doi:10.1016/j.ijppaw.2018.12.011.
79. Otranto D., Cantacessi C., Pfeffer M., Dantas-Torres F., Brianti E., Deplazes P., Genchi C., Guberti V., Capelli G. 2015. The role of wild canids and felids in spreading parasites to dogs and cats in Europe. Part I: Protozoa and tick-borne agents. Veterinary Parasitology, 213, 12-23. doi:10.1016/j.vetpar.2015.04.022.
80. Otranto D., Dantas-Torres F., Brianti E., Traversa D., Petrić D., Genchi C., Capelli G. 2013. Vector-borne helminths of dogs and humans in Europe. Parasites & Vectors, 6, 16. doi:10.1186/1756-3305-6-16.
81. Otranto D., Dantas-Torres F., Mallia E., Di Geronimo P.M., Brianti E., Testini G., Traversa D., Lia R.P. 2009. *Thelazia callipaeda* (Spirurida, Thelaziidae) in wild animals: report of new host species and ecological implications. Veterinary Parasitology, 166, 262-267. doi:10.1016/j.vetpar.2009.08.027.
82. Overgaauw P.A. and van Knapen F. 2013. Veterinary and public health aspects of *Toxocara* spp. Veterinary Parasitology, 193, 398-403. doi:10.1016/j.vetpar.2012.12.035.
83. Palfreyman J., Graham-Brown J., Caminade C., Gilmore P., Otranto D., Williams D.J.L. 2018. Predicting the distribution of *Phortica variegata* and

potential for *Thelazia callipaeda* transmission in Europe and the United Kingdom. Parasites & Vectors, 11, Article No 72. doi:10.1186/s13071-018-2842-4.

84. Papadopoulos E., Komnenou A., Thomas A., Ioannidou E., Colella V., Otranto D. 2018. Spreading of *Thelazia callipaeda* in Greece. Transboundary and Emerging Diseases, 65, 248-252. doi:10.1111/tbed.12626.
85. Papajová I., Pipiková J., Papaj J., Ĉiţmár A. 2014. Parasitic contamination of urban and rural environments in the Slovak Republic: dog's excrements as a source. Helminthologia, 51, 4, 273-280. doi:10.2478/s11687-014-0241-8.
86. Paradžik M.T., Samardžić K., Živičnjak T., Martinković F., Janjetović Ž., Miletić-Medved M. 2016. *Thelazia callipaeda*: first human case of thelaziosis in Croatia. *Wiener Klinische Wochenschrift*, 128, 221-223. doi:10.1007/s00508-015-0889-1.
87. Pavlović I., Kulišić Z., Ljubić B., Radivojević S., Terzin V., Stokić-Nikolić S., Rajković M., Anđelić-Buzadžić G. 2010. Raširenost parazitskih infekcija pasa i kontaminiranost javnih površina-rizik nastanka humanih infekcija. U Zbornik VII Kongresa mikrobiologa Srbije, Beograd, 1-2.
88. Pavlović I. and Kulišić Z. 1994. Prevalencija askarida kod lisica i pasa na području Beograda. U Zbornik kratkih sadržaja VII Savetovanja veterinara Srbije, Zlatibor, 28.
89. Penezić A., Selaković S., Pavlović I., Ćirović D. 2014. First findings and prevalence of adult heartworms (*Dirofilaria immitis*) in wild carnivores from Serbia. Parasitology Research, 113, 3281-3285. doi:10.1007/s00436-014-3991-9.
90. Perić J., Lekić B., Reljić V., Ćirković L., Škiljević D. 2017. Cutaneous larva migrans - report of 2 new cases locally acquired in Serbia. Serbian Journal of Dermatology and Venerology, 9, 4, 149-153. doi:10.1515/sjdv-2017-0016.
91. Portier J., Vallée I., Lacour S.A., Martin-Schaller R., Ferté H., Durand B. 2014. Increasing circulation of *Alaria alata* mesocercaria in wild boar populations of the Rhine valley, France, 2007-2011. Veterinary Parasitology, 199, 3–4, 153-159. doi:10.1016/j.vetpar.2013.09.029.
92. Riggio F., Mannella R., Ariti G., Perrucci S. 2013. Intestinal and lung parasites in owned dogs and cats from central Italy. Veterinary Parasitology, 193, 1-3, 78-84. doi:10.1016/j.vetpar.2012.11.026.
93. Ristić M., Dimitrijević S., Višnjić A., Bogunović D., Gajić B., Stojanović M., Ilić T. 2020a. Dogs from public city parks as a potential source of environmental pollution by zoonotic parasites. Indian Journal of Animal Sciences, April 2020 issue 90 (4): 35-42, 2020a. In press.

94. Ristić M., Miladinović-Tasić N., Dimitrijević S., Nenadović K., Bogunović D., Stepanović P., Ilić T. 2020b. Soil and sand contamination with canine intestinal parasite eggs as a risk factor for human health in public parks in Niš (Serbia). Helminthologia, 57, 2, 109-119. doi:10.2478/helm-2020-0018.
95. Rossi L. and Bertaglia P.P. 1989. Presence of *Thelazia callipaeda* Railliet & Henry, 1910, in Piedmont, Italy. Parassitologia, 31, 167-172.
96. Rudohradská P., Papajová I., Juriš P. 2011. Pets as a source of parasitic soil contamination in the settlements of marginal ised groups of inhabitants. Folia Veterinaria, 55, 1, 33-35.
97. Saeed I., Maddox-Hyttel C., Monrad J., Kapel C.M.O. 2006. Helminths of red foxes (*Vulpes vulpes*) in Denmark. Veterinary Parasitology, 139, 1-3, 168-179. doi:10.1016/j.vetpar.2006.02.015.
98. Savić S., Vidić B., Grgić Z., Potkonjak A., Spasojević Lj. 2014. Emerging Vector-Borne Diseases - Incidence through Vectors. Frontiers in Public Health, 2, 267. doi:10.3389/fpubh.2014.00267.
99. Savić S., Vidić B., Grgić Ž., Jurišić A., Ćurčić V., Ružić M., Lolić Z. 2012. Canine vector borne zoonozes in Vojvodina. Arhiv veterinarske medicine, 5,1, 77-87. doi:10.46784/e-avm.v5i1.164.
100. Savić-Jevđenić S., Vidić B., Grgić Ž., Milovanović A. 2004. Brza dijagnostika dirofilarioze pasa u regionu Novog Sada. Veterinarski Glasnik, 58, 5-6, 693-698.
101. Schug K., Krämer F., Schaper R., Hirzmann J., Failing K., Hermosilla C., Taubert A. 2018. Prevalence survey on lungworm (*Angiostrongylus vasorum, Crenosoma vulpis, Eucoleus aerophilus*) infections of wild red foxes (*Vulpes vulpes*) in central Germany. Parasites & Vectors, 11, 85. doi:10.1186/s13071-018-2672-4.
102. Sréter T., Széll Z., Marucci G., Pozio E., Varga I. 2003. Extraintestinal nematode infections of red foxes (*Vulpes vulpes*) in Hungary. Veterinary Parasitology, 115, 329-334. doi:10.1016/S0304-4017(03)00217-6.
103. Stepanović P., Ilić T., Krstić N., Dimitrijević S. 2015. Efficiency of modified therapeutic protocol in the treatment of some varieties of canine cardiovascular dirofilariasis. *Bulletin of Veterinary Institute in Pulawy*, 59, 505-509, 2015. doi:10.1515/bvip-2015-0075.
104. Tasić A., Rossi L., Tasić S., Miladinović-Tasić N., Ilić T., Dimitrijević S. 2008. Survey of canine dirofilariasis in Vojvodina. Serbia. Parasitology Research, 103, 1297-1302. doi:10.1007/s00436-008-1132-z.
105. Tasić A., Tasić-Otašević S., Gabrielli S., Miladinović-Tasić N., Ignjatović A., Dornević J., Dimitrijević S., Cancrini G. 2012. Canine dirofilariosis in two non-investigated areas of Serbia: epidemiological and genetical

aspects. Vector-Borne and Zoonotic Diseases, 12, 1031-1035. doi:10.1089/vbz.2011.0949.

106. Tasić-Otašević S., Gabrielli S., Trenkić-Božinović M., Petrović A., Gajić B., Colella V., Momčilović S., Cancrini G. Otranto D. 2016. Eyeworm infections in dogs and in a human patient in Serbia: a one health approach is needed. Comparative Immunology, Microbiology & Infectious Diseases, 45, 20-22. doi:10.1016/j.cimid.2016.01.003.
107. Tasić-Otašević S.A., Gabrielli S.V., Tasić A.V., Miladinović-Tasić N.L., Kostić J.T., Ignjatović A.M., Popović-Dragonjić L.D., Milošević Z.G., Arsić-Arsenijević S., Cancrini G.A. 2014. Seroreactivity to *Dirofilaria* antigens in people from different areas of Serbia. BMC Infectious Diseases, 14, 68. doi:10.1186/1471-2334-14-68.
108. Tolnai Z., Széll Z., Sproch Á., Szeredi L., Sréter T. 2014. *Dirofilaria immitis*: an emerging parasite in dogs, red foxes and golden jackals in Hungary. Veterinary Parasitology, 203, 3-4, 339-342. doi:10.1016/j.vetpar.2014.04.004.
109. Traversa D., Di Cesare A., Milillo P., Iorio R., Otranto D. 2009. Infection by *Eucoleus aerophilus* in dogs and cats: is another extra-intestinal parasitic nematode of pets emerging in Italy? Research in Veterinary Science, 87, 270-272. doi:10.1016/j.rvsc.2009.02.006.
110. Traversa D. and Di Cesare A. 2014. Cardio-pulmonary parasitic nematodes affecting cats in Europe: unraveling the past, depicting the present, and predicting the future. Frontiers in Veterinary Science, 1, 11, 1-9. doi:10.3389/fvets.2014.00011.
111. Traversa D. and Di Cesare A. 2016. Diagnosis and management of lungworm infections in cats: Cornerstones, dilemmas and new avenues. Journal of Feline Medicine and Surgery, 18, 7-20. doi:10.1177/1098612X15623113.
112. Traversa D., Di Cezare A., Conboy G. 2010. Canine and feline cardiopulmonary parasitic nematodes in Europe: emerging and understimated. Parasites & Vectors, 3, 62. doi:10.1186/1756-3305-3-62.
113. Traversa D., Frangipane di Regalbono A., Di Cesare A., La Torre F., Drake J., Pietrobelli M. 2014. Environmental contamination by canine geohelminths. Parasites & Vectors, 7, 67. doi:10.1186/1756-3305-7-67.
114. Traversa D. 2012. Pet roundworms and hookworms: A continuing need for global worming. Parasites & Vectors, 5, 91. doi:10.1186/1756-3305-5-91.
115. Vieira L., Rodrigues F.T., Costa Á., Diz-Lopes D., Machado J., Coutinho T., Tuna J., Latrofa M., Cardoso L., Otranto D. 2012. First report of canine ocular thelaziosis by *Thelazia callipaeda* in Portugal. Parasites & Vectors, 5, 124. doi:10.1186/1756-3305-5-124.

116. Wall R. and Morgan E. 2009. Veterinary parasitology and climate change. Veterinary Parasitology, 163, 263. doi:10.1016/j.vetpar.2009.03.023.
117. Wójcik A.R., Franckiewicz-Grygon B., Żbikowska E. 2002. Badania nad przywrą *Alaria alata* (Goeze, 1782) [Article in Polish]. Medycyna Weterynaryjna, 58, 517-519.

Submitted: 10.05.2020.
Accepted: 19.06.2020.

*Original research article*

UDC 636.32/.39.09:616.98(497.113)
https://doi.org/10.46784/e-avm.v13i1.53

# PREVALENCE OF SMALL RUMINANT LENTIVIRUS INFECTIONS IN SHEEP AND GOATS IN SOME REGIONS OF VOJVODINA PROVINCE

Sara Savić[*1], Marina Žekić Stošić[1], Dejan Bugarski[1], Doroteja Marčić[1], Aleksandar Milovanović[1], Aleksandar Potkonjak[2]

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

## Abstract

Small Ruminant Lentivirus causes a chronic lifelong, multisystemic diseases in sheep and goats with or without clinical manifestation. Maedivisna virus (MVV) and Caprine arthritis encephalitis virus (CAEV) are often considered together as small ruminants lentivirus (SRLV) because of their phylogenetic correlation and the interspecies transmission between sheep and goats.

During a five-year period, from 2014 to 2018, a monitoring on male animals (rams and bucks) used for breeding was conducted with the aim to determine antibodies against SRLV using ELISA serological method. In total, serum samples from 5,039 animals were analysed and the overall seroprevalence of 5.59% was found. The seroprevalence for the whole period was 5.15% in male animals while in female animals it was 6.15%. During the whole 2014-2018 period, seroprevalence was 4.52% in rams, 17.61% in bucks, 1.14% in sheep and 12.57% in goats. Compared to earlier studies and within the study period, the seroprevalence for SRLV actually decreased in rams and bucks. The study results show that annual monitoring program is very important and has to be conducted in the future in order to keep the disease under control. The program helps in raising awareness and familiarizing owners with SRLV and the importance of breeding seronegative animals.

**Key words:** SRLV, Meadi Visna virus, sheep, goats

[1*] Corresponding author: sara@niv.ns.ac.rs

# PREVALENCA INFEKCIJE LENTIVIRUSOM MALIH PREŽIVARA KOD OVACA I KOZA U NEKIM DELOVIMA VOJVODINE

Sara Savić[1], Marina Žekić Stošić[1], Dejan Bugarski[1],
Doroteja Marčić[1], Aleksandar Milovanović[1], Aleksandar Potkonjak[2]

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

## Kratak sadržaj

Lentivirus malih preživara izaziva dugotrajno, multisistemsko hronično oboljenje kod ovaca i koza koje može biti sa ili bez kliničkih simptoma. Medi-visna virus (MVV) i virus artritisa i encefalitisa koza se smatraju lentivirusima malih preživara (SRLV) zbog njihove filogenetske sličnosti i prenošenja viruse među vrstama.

Tokom perioda od 5 godina, od 2014-2018, sprovođen je godišnji monitoring program za muške priplodne životinje, radi utvrđivanja antitela protiv SRLV virusa pomoću ELISA serološke metode. Ukupno je ispitano 5039 uzoraka krvnih seruma životinja i utvrđena ukupna seroprevalenca od 5.59%. Kod muških životinja seroprevalenca za ceo period je 5,15%, a kod ženskih životinja je 6,15%. Tokom posmatranog perioda utvrđena je seroprevalenca kod ovnova 4.52%, kod jarčeva 17.61% , kod ovaca 1.14% i kod koza 12.57%. U poređenju sa rezultatima prethodnih studija, a i tokom perioda ovog istraživanja, utvrđeno je da je seroprevalenca za virus SRLV zapravo opala kod ovnova i jarčeva. Iz ovakvih rezultata se može zaključiti da je godišnji monitoring program veoma važan i mora da se održi i u budućnosti, da bi se ovo oboljenje držalo pod kontrolom. Program takođe doprinosi i povećanju svesti kod vlasnika životinja / farmera o SRLV i značaju gajenja seronegativnih životinja.

**Ključne reči**: SRLV, Medi Visna virus, ovce, koze

## INTRODUCTION

Maedi-visna virus (MVV) and Caprine arthritis encephalitis virus (CAEV) are often considered together as small ruminants lentivirus (SRLV) because of their phylogenetic correlation and the interspecies transmission between sheep and goats. SRLV belongs to genus *Lentivirus*, subfamily *Orthoretrovirinae* and family Retroviridae. SRLV can cause a chronic lifelong, multisystemic diseases in sheep and goats with clinical manifestation in adults. Due to a slow progress of the disease, the possibility of infection within a flock is high. Most of infected animals never develop clinical symptoms. In sheep, name "visna" stands for the disease that affects central nervous system, a neurological form of the disease and predominantly causes meningoencephalitis in sheep. The term "maedi" stands for infection of the lungs. Besides this, the virus can also affect other organs such as udder.

Small ruminant lentiviruses include viruses with diverse genotypes that frequently cross the species barrier between sheep and goats that display a great genetic variability. There are five genotypes (A-E) of SRLV (Minguijón et al., 2015). Genetic variability is a key feature of small ruminant lentivirus genome and is essential for accurate diagnosis. There is a slight variation of the strains of SRLV and specific symptoms can be more dominant depending on the differences in genetic susceptibility patterns. Depending on the cell type, visna/maedi virus infection may lead to a cytopathic effect, or remain unnoticed.

Caprine arthritis and encephalitis virus (CAEV) can affect joints, central nervous system and udder in goats (Marcom et al., 1991). Arthritis can be found in infected animals which are 1 to 6 years old, with swollen carpal joints, causing limp and weight loss in sick animals (Blacklaws, 2004).

Serological diagnostic methods are considered the most convenient for detection of SRLV infections (de Andres et al. 2005). Different serology methods can be used for diagnostics of the disease, like agar gel immunodiffusion test, indirect immunofluorescence test and ELISA test (Larruskain and Jugo, 2013). After infection, seroconversion takes several weeks, mostly between 2 and 8 (Carrozza et al., 2009; Reina et al., 2011).

Domesticated host animals of the virus are sheep (*Ovis aries*) and goats (*Capra hircus*). European mouflon (*Ovis aries musimon*) is also recognized as a host in the wilderness (Straub, 2004). The earliest reports on the disease come from South Africa and the USA (Straub, 2004, Randall et al. 1988), but nowadays the disease can be found in all the countries where sheep are bred. This virus is distributed all over Europe and in many other countries worldwide. According to International Animal Health organization (OIE), back in 2005, the disease was either reported as present or known to be present in most of

European countries (Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Hungary, Iceland, Ireland, Luxembourg, Netherlands, Norway, Poland, Portugal, Sweden, Switzerland and UK) (OIE, Handistatus, 2005). One of the most important factors that affected the spreading of this disease in different countries (such as Denmark, Norway, Finland and the United Kingdom) is the trade of animals without control (Gjerset et al 2007). In some other countries there was serological evidence and/or isolation of the agent (Latvia, Slovakia) and in some countries the virus was not found at that time. Later on, the evidence of the disease appeared in those countries too, like in Serbia and Romania (Vidić et al., 2008; Mihai et al., 2018). So far, SRLV-free countries are New Zealand and Australia. The first survey in Serbia was conducted in 2008, when a prevalence of 16.24% was found in the whole country by ELISA test (Vidić et al., 2008). The attention was brought to this disease at that time and then in 2012 a seroprevalence of 21% was found in sheep (varying from 14-30% in different regions) to 6.81% in rams (Savić et al., 2012). It is very important to identify the presence of Maedi-visna infection because of the long incubation period, and control of this disease is complicated since there is no treatment, no vaccine and the options for prevention are limited. This is why a monitoring program for breeding animals was introduced in 2016. The purpose of this study was to analyse the seroprevalence in sheep, rams, goats and bucks after the 3 years of active monitoring program in breeding animals for SRLV and to show if and how seroprevalence has changed over the study period.

Different studies have shown that all productivity parameter measures appeared to be reduced in the seropositive groups for both goats and sheep, even though the differences were not statistically significant (Leginagoikoa et al., 2010; Barquero et al., 2013; Junkuszew et al., 2016).

## MATERIAL AND METHODS

### *Material/sampling and sample distribution*

During a five-year period, from 2014-2018, an annual monitoring on male animals (rams and bucks) used for breeding was conducted. The purpose of this program was determination of antibodies against SRLV with ELISA serological method. This program was proposed and coordinated by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management. The monitoring program is planned and announced every year and it applies to the whole country, including all regions. The analyses in this study were done according to that mandatory annual program introduced by the

government of Serbia and so they included all male animals (rams and bucks) used for breeding. Besides male animals, female animals were also analysed when there was a suspicion that the disease is present in a flock. The region in which the sampling was performed was South Backa and Srem District – a region which is controlled by the Scientific Veterinary Institute "Novi Sad". This means that during the whole study period, each year the animals from the same regions were analysed. In total, serum samples from 5,039 animals were included in the study. Out of the total, 1,316 samples were from sheep, 2,587 samples were from rams, 994 samples were from goats and 142 samples were from bucks. There were 2,729 samples from male and 2,310 samples from female animals analysed for the presence of antibodies against SRLV.

### *The ELISA methods*

The kits used were ELISA diagnostic kits of two types. First, the screening diagnostic kit was used in order to identify samples positive for antibodies against SRLV. Positive samples identified with screening ELISA kit (ID Screen MVV-CAEV Indirect Screening test, IDvet, France) were then analysed with a conformation ELISA kit (INgezim MAEDI Confirmation, Ingenaza, Spain) in order to obtain the conformation of positive samples. Screening kit is designed to detect antibodies against SRLV in sheep and goat serum samples, based on an indirect ELISA technique using a purified pool of SRLV antigenic peptides. Conformation assay was designed to confirm the positive samples obtained in the screening test by detection of specific antibodies against SRLV through the use of specific peptides of genotypes A and B. The sensitivity and specificity of the kits were 95%.

## RESULTS

During the five-year period, the results were categorized by year and by animal species and categories into tables and pictures. From the total number of 5,039 analysed animal samples, 282 were positive for SRLV, which is 5.59% of anlysed animals. The seroprevalence for the whole period was 5.15% only in male animals, while and in female animals it was 6.15%.

Seroprevalence for SRLV for the whole 2014-2018 period was 4.52% in rams, and 17.61% in bucks. In sheep, seroprevalence for the whole period was 1.14% and in goats it was 12.57%. The number of positive samples for antibodies against SRLV and seroprevalence in sheep, rams, goats and bucks is shown in Table 1 and Figures 1 and 2.

Table 1. Number of analysed and positive samples of rams, sheep, goats and bucks for antibodies against SRLV during the 2014-2018 period

| Year | Total sample No | Sheep | | | Rams | | | Goats | | | Bucks | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No of analysed samples | No of positive samples | Seroprevalence % | No of analysed samples | No of positive samples | Seroprevalence % | No of analysed samples | No of positive samples | Seroprevalence % | No of analysed samples | No of positive samples | Seroprevalence % |
| **2014** | 310 | 124 | 0 | - | 19 | 0 | - | 162 | 0 | - | 5 | 0 | - |
| **2015** | 168 | 137 | 2 | 1.46 | 31 | 0 | - | 0 | 0 | - | 0 | 0 | - |
| **2016** | 920 | 351 | 3 | 0.85 | 532 | 53 | 9.96 | 2 | 0 | - | 35 | 8 | 22.86 |
| **2017** | 781 | 258 | 5 | 1.93 | 502 | 18 | 3.58 | 0 | 0 | - | 21 | 10 | 47.62 |
| **2018** | 2860 | 446 | 5 | 1.12 | 1,503 | 46 | 3.06 | 830 | 125 | 15.06 | 81 | 7 | 8.64 |
| **Total** | 5039 | 1316 | 15 | 1.14 | 2,587 | 117 | 4.52 | 994 | 125 | 12.57 | 142 | 25 | 17.61 |

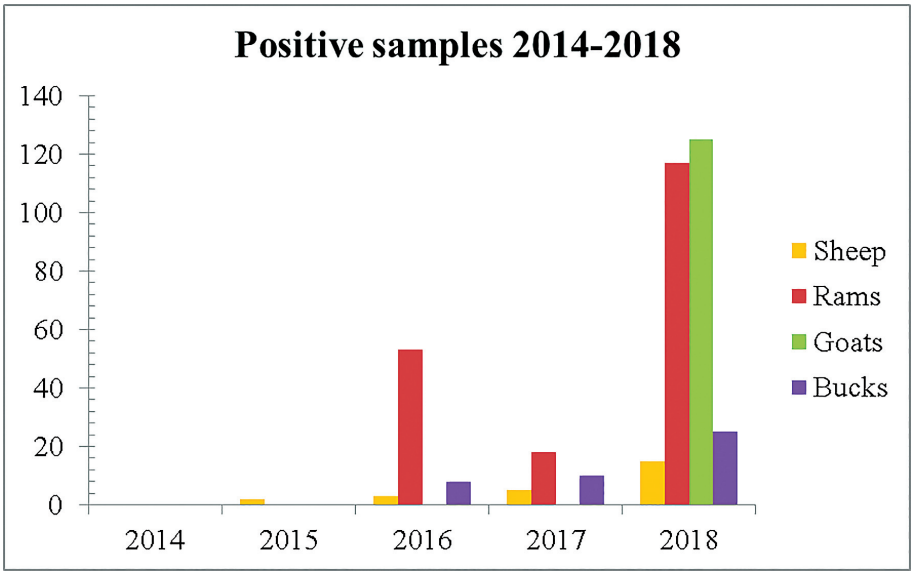


Figure 1. Number of positive samples for antibodies against SRLV virus in sheep, rams, goats and bucks for the 2014-2018 period

The number of positive animals in the years 2014 and 2015 was 0 and 2 respectively, leading to no seroprevalence and a very low seroprevalence, respectively. This was most probably due to a very low number of total samples taken during those two years, and also due to a low number of samples taken from goats/bucks. The monitoring program was in preparation and the actual numbers were gained during the next three years, over the period from 2016 to 2018. During this period, the seroprevalence did not change much in sheep (0.85 – 1.93%), but in rams it significantly decreased from 9.96 to 3.06%. The seroprevalence in goats was 15.05% in 2018, which is rather high compared to other categories, but in the previous years it was not looked into. The seroprevalence in bucks was mostly inconsistent, but it was decreasing significantly from 47.62% to 8.64%. Seroprevalence in sheep and rams (3.38%) is significantly lower than in goats and bucks (13.20%).

The overall seroprevalence for the whole study period (2014-2018) in sheep, rams, goats and bucks is shown in Figure 2.

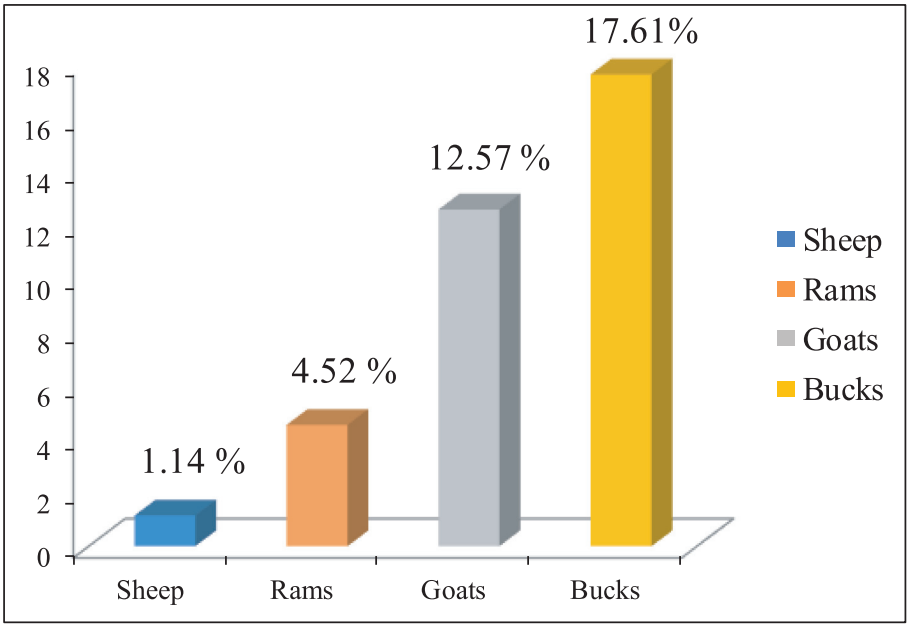


Figure 2. The overall seroprevalence for the whole study period (2014-2018) in sheep, rams, goats and bucks

## DISCUSSION

During the 2014-2018 period, seroprevalence for SRLV decreased from 9.96% to 3.06% in rams and from 47.62% to 8.64% in bucks. In sheep, the seroprevalence did not change much over the years (0.85-1.93%), but in goats it appeared to be very high during the last year of the study (15.06%). Compared to previous studies, it can be observed that the overall seroprevalence decreased to 5.59%, and it is very interesting regarding the sex and species categories.

The first survey in Serbia was conducted in 2008 and seroprevalence of 16.24% was found in the whole country (Vidić et al., 2008). A study from 2012 showed a seroprevalence of 21% in sheep in Vojvodina region, while in rams it was 6.81% (Savić et al., 2012). Then, in the period from 2013 to 2015 seroprevalence seemed to be very low, but it was only because a very small number of samples (or even none) were analysed. When the monitoring program coordinated by the Veterinary Directorate started in 2016, a significantly higher number of samples were analysed, but still not from all the categories. Only in 2018, a representative number of samples were gathered from sheep, rams, goats and bucks. What is also important is that seroprevalence in goats and bucks together (13.20%) was significantly higher than seroprevalence in sheep and rams (3.38%), even though the number of analysed samples was significantly lower in goats and bucks.

The characteristic of SRLV antibodies is that they are slow in appearance after the infection and this has to be taken into consideration when findings are discussed. Passively obtained antibodies can last for 6 months and when serological examination of sheep is performed before this period, this fact needs to be taken into account as well. Positive serology findings do not mean that symptoms will appear. It means that the animals were in contact with the virus, but the animals will not necessarily be sick. Prevalence can be high in a flock, especially in older animals meaning that there was a contact between the flock and the virus (Gomez-Lucia et al., 2018).

The prevalence of SRLV seems to be much higher in farms where lambs and kids are fed with a pool of colostrum or milk from the tank, a practice that favours transmission to the management system (Leginagoikoa et al., 2010; Barquero et al., 2013). Besides a type of management system (higher SRLV seroprevalence in intensively reared sheep than in semi-intensively and extensively reared sheep), flock size also can be a factor. According to Junkuszew et al. (2016) 70 dams are the limit above which there is an increased risk for lentiviral infection. The route of transmission is related to body fluids, mainly respiratory exudates and milk or colostrum and also airborne, favoured by overcrowding (Barquero et al., 2015).

The region in the study area is very developed in agriculture with plant production and soil exploitation. Thus, sheep and goats are mostly kept in stables with a lot of animals in a small area. Traditionally, the size of the flock is from one hundred to a few hundred animals. Only in the places where the soil is not suitable enough for plant production, animals can be kept on pastures where they can easily get in contact with other flocks. Also, traditionally, male animals are traded between different flocks. Sheep are most commonly used only for meat production and lambs are not separated from mothers that nurse them, which carries a great risk of getting infected with SRLV through milk or colostrum. There is also a great risk of other ways of infection due to the direct contact with older animals in flock which are all kept together. Goat breeding is oriented exclusively towards milk production and lambs and kids are separated from dams at early stages of life, so it is more appropriate to take measures in order to diminish the risk of infection with SRLV.

The main economic losses resulting from SRLV infection come from the effect of subclinical infection on the productivity of infected flock. The economic impact of this disease is a direct damage from increased mortality in a flock. But besides that, there is a decreased production of animals, a higher level of illness incidence, etc. Seropositive animals have lower level of milk production, shorter lactation period and reduced reproduction abilities. The presence of the disease in a flock significantly affects the value of the animals on the market.

## CONCLUSION

The number of positive animals is a significant finding especially with regards to a longer screening period. It is important to keep the disease under control, because the losses resulting from this disease are significant, which means that the annual monitoring program is crucial. What has not been accomplished so far is including a larger number of goats and bucks in the program in order to gain much more precise results, and to identify the infected flocks.

In the observed region there are seropositive animals within the flock which need to be under control. There is uneven distribution of seropositive animals within the region. However, all the municipalities are affected by SRLV virus.

Annual monitoring program organized by the Veterinary Directorate is an ongoing and continuing program. The obtained results show that this program is very important and needs to be maintained in the future in order to keep the disease under control. The program helps in raising the awareness and

familiarizing the owners with SRLV and the importance of breeding seronegative animals. A monitoring program that includes more bucks and goats is required in the future as well as a program for seropositive animals. Also, a plan on what to do with them while the seroprevalence in the country is still relatively low is required.

## ACKNOWLEDGEMENT

This paper is part of the research done in the project TR31071 granted by the Serbian Ministry of Education and Science.

## Author's contributions:

Individual contributions of authors to the manuscript, specified by initials in this section are the following: SS made contributions to the concept and design of the study, organisation of work, writing the manuscript and data analysis and prepared the final draft of the manuscript. MŽS completed laboratory analysis of all the samples, drew the maps and worked on technical preparation of the manuscript. DB participated in writing of the manuscript and contributed mostly to data analysis process. DM collected all the epizootical data needed for map drawings and participated in the writing of the manuscript. AM participated in writing of the manuscript and preparation of the final draft of the manuscript, mostly contributing to data analysis. AP participated in writing of the manuscript and preparation of the final draft of the manuscript.

## Competing Interests

All the authors declare that they have no competing interests for the work presented in the Manuscript. The manuscript is not influenced by any personal or financial relationship with people or organizations.

## REFERENCES

1. Barquero N., Domenech A., Gomez-Lucia E. 2015. Infections by Small Ruminant Lentiviruses, LAP Lambert Academic Publishing, Saarbrücken, Germany, ISBN-13: 978-3659744051.
2. Barquero N., Gomez-Lucia E., Arjona A., Toural C., Las Heras A., Fernández-Garayzábal J.F., Ruiz-Santa Quiteria J.A., Doménech A. 2013. Investigation of risk factors associated with infections caused by small ruminant

lentiviruses. Bulletin of the Veterinary Institute in Pulawy, 57, 4, 473–478. doi: 10.2478/bvip-2013-0082.
3. Blacklaws B.A., Berriatua E. Torsteinsdottir S., Watt N.J., de Andres D., Klein D., Harkiss G.D. 2004. Transmission of small ruminant lentiviruses. Veterinary Microbiology, 101, 3, 199-208. doi:10.1016/j.vetmic.2004.04.006.
4. Carrozza M.L., Mazzei M., Lacerenza D., Del Chiaro L., Giammarioli M., Marini C., Rutili D., Rosati S., Tolari F. 2009. Seroconversion against SU5 derived synthetic peptides in sheep experimentally infected with different SRLV genotypes. Veterinary Microbiology, 369-374, 137, 3–4. doi:10.1016/j.vetmic.2009.01.032.
5. Cutlip R.C., Lehmkuhl H.D., Schmerr M.J., Brogden K.A. 1988. Ovine progressive pneumonia (maedi-visna) in sheep. Veterinary Microbiology, 17, 3, 237-250. doi:10.1016/0378-1135(88)90068-5.
6. de Andres D., Klein D., Watt N.J., Berriatua E., Torsteinsdottir S., Blacklaws B.A., Harkiss G.D. 2005. Diagnostic tests for small ruminant lentiviruses. Veterinary microbiology, 107, 49-62. doi:10.1016/j.vetmic.2005.01.012.
7. Gjerset B., Jonassen C.M., Rimstad E. 2007. Natural transmission and comparative analysis of small ruminant lentiviruses in the Norwegian sheep and goat populations. Virus Research, 125, 2, 153-161. doi:10.1016/j.virusres.2006.12.014.
8. Gomez-Lucia E., Barquero N., Domenech A. 2018. Maedi-Visna virus: current perspectives, Veterinary Medicine (Auckland, N.Z.), 9, 11–21. doi: 10.2147/VMRR.S136705.
9. Junkuszew A., Dudko P., Bojar W., Olech M., Osiński Z., Gruszecki T.M., Kania M.G., Kuźmak J., Czerski G. 2016. Risk factors associated with small ruminant lentivirus infection in eastern Poland sheep flocks. Preventive Veterinary Medicine, 127, 44–49. doi:10.1016/j.prevetmed.2016.03.011.
10. Larruskain A. and Jugo B.M. 2013. Retroviral Infections in Sheep and Goats: Small Ruminant Lentiviruses and Host Interaction. Viruses. 5, 8, 2043-2061. doi:10.3390/v5082043.
11. Leginagoikoa I., Minguijón E., Juste R.A., Barandika J., Amorena B., de Andrés D., Badiola J.J., Luján L., .Berriatua E. 2010. Effects of housing on the incidence of Visna/Maedi virus infection in sheep flocks. Research in Veterinary Science, 88, 3, 415–421. doi:10.1016/j.rvsc.2009.11.006.
12. Marcom K.A., Pearson L.D., Chung C.S., Poulson J.M., Demartini J.C. 1991. Epitope analysis of capsid and matrix proteins of North American ovine lentivirus field isolates. Journal of clinical microbiology, 29, 7, 1472-1479. doi: 10.1128/JCM.

13. Mihai I., Crivei I.C., Horhogea C., Savuta G., Velescud E. 2018. Preliminary Serological Investigation on Caprine Arthritis and Encephalitis Virus Infection in a Goat Farm from North-Eastern Romanian Region. Bulletin UASVM Veterinary Medicine, 75, 2. doi:10.15835/buasvmcn-vm:2017.0061.
14. Minguijón E., Reina R., Pérez M., Polledo L., Villoria M., Ramírez H., Leginagoikoa I., Badiola J.J., García-Marín J.F., de Andrés D., Luján L., Amorena B., Juste R.A. 2015. Small ruminant lentivirus infections and diseases. Veterinary Microbiology, 14, 181, 1-2, 75-89. doi: 10.1016/j.vetmic.2015.08.007.
15. OIE: Handistatus. 2005. Available at: https://www.cabi.org/isc/datasheet/59452#59945D22-0E77-4B2E-8B26-03F4A7569A1B.
16. Reina R., Juganaru M.M., Profiti M., Cascio P., Cerutti F., Bertolotti L., De Meneghi D., Amorena B., Rosati S. 2011. Immunological parameters in goats experimentally infected with SRLV genotype E, strain Roccaverano. Veterinary Immunology and Immunopathology, 139, 2–4, 237-244. doi:10.1016/j.vetimm.2010.11.001.
17. Savić S., Vidić B., Bugarski D., Grgić Ž. 2012. Findings of specific antibodies against Maedi-Visna virus in sheep population in the region of Vojvodina. In *Conference proceedings,* International Conference on BioScience: Biotechnology and Biodiversity – Step in the Future – The Forth Joint UNS – PSU, Institute of Field and Vegetable Crops, Novi Sad, 109-114. ISBN 978-86-80417-40-0.
18. Straub O.C. 2004. Maedi–Visna virus infection in sheep. History and present knowledge. Comparative Immunology, Microbiology and Infectious Diseases, 27, 1, 1-5. doi:10.1016/S0147-9571(02)00078-4.
19. Vidić B., Grgić Ž., Savić-Jevđenić S., Maljković M., Milanov D. 2008. Prevalence of maedi-visna in sheep herds. Arhiv veterinarske medicine, 1, 1, 30-40. ISSN 1820-9955. doi:10.46784/e-avm.v1i1.221.

Received: 13.01.2020.
Accepted: 09.05.2020.

Original research article

UDC 636.1/.4.087.7:[615.918:582.28
https://doi.org/10.46784/e-avm.v13i1.65

# IN VITRO ASSESSMENT OF BINDING CAPACITY OF COMBINED ADSORBENT (BENTONITE WITH YEAST CELL WALL EXTRACTS) AND AFLATOXIN B1

Ksenija Nešić [1*], Sandra Jakšić[2], Nenad Popov[2],
Milica Živkov-Baloš[2], Marko Pajić[2], Bojan Zloh[3], Vladimir Polaček[2]

[1] Institute of Veterinary Medicine of Serbia, Belgrade, Republic of Serbia
[2] Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia
[3] INBERG d.o.o., Boljevci, Republic of Serbia

## Abstract

The contamination of animal feed with mycotoxins is a worldwide problem in the animal husbandry, but it also represents a serious threat for the whole food chain. The health of both animals and humans is potentially endangered. From this point of view aflatoxins are a class of mycotoxins especially well known. Therefore, new strategies to combat these natural contaminants are constantly being developed. The most applied method to protect animals against aflatoxicosis is the utilization of feed additives aimed to adsorb aflatoxins. In order to estimate adsorbing potential of feed additive "MycoStop DUPLO", designed for the prevention and/or alleviation of adverse effects of aflatoxin $B_1$ in animal nutrition, *in vitro* trial was conducted. As a result of the experiment, conducted at pH 5 during 120 minutes of incubation at 37°C, the optimal formulation of the adsorbent was revealed. This product, in low concentration and in the presence of high amounts of toxin, met the stringent European regulation requirements for minimum 90% aflatoxin binding efficiency (90.1% achieved with 0.02% adsorbent and 4 mg/L toxin concentration). In higher adsorbent (0.2%), and lower toxin (0.2 mg/L) conditions, adsorption was 99.6%. Such outcome indicated the validity of *in vitro* experimental approach which can serve as a reliable fast tool for triage of adsorbents and preselect them for *in vivo* tests.

**Key words**: feed, feed additives, mycotoxins

[1*] Corresponding author: ksenija.nesic@gmail.com

# IN VITRO PROCENA KAPACITETA VEZIVANJA KOMBINOVANOG ADSORBENTA (BENTONIT I EKSTRAKT ĆELIJSKOG ZIDA KVASCA) I AFLATOKSINA $B_1$

Ksenija Nešić[1], Sandra Jakšić[2], Nenad Popov[2], Milica Živkov-Baloš[2], Marko Pajić[2], Bojan Zloh[3], Vladimir Polaček[2]

[1] Naučni institut za veterinarstvo Srbije, Beograd, Republika Srbija
[2] Naučni institut za veterinarstvo "Novi Sad", Novi Sad, Republika Srbija
[3] INBERG d.o.o., Boljevci, Republika Srbija

## Kratak sadržaj

Kontaminacija hrane za životinje mikotoksinima predstavlja svetski problem u stočarstvu, ali i ozbiljnu pretnju u čitavom lancu hrane. Potencijalno je ugroženo zdravlje i životinja i ljudi, a sa ovog aspekta aflatoksini su naročito značajna klasa mikotoksina. Stoga se neprestano razvijaju nove strategije za borbu protiv ovih prirodnih hazarda. Najčešći vid zaštite životinja od aflatoksikoze jeste korišćenje dodataka u hrani za životinje koji adsorbuju aflatoksine. U cilju procene kapaciteta adsorpcije aditiva „MycoStop DUPLO", namenjenog prevenciji i/ili ublažavanju štetnih efekata aflatoksina $B_1$ u ishrani životinja, sprovedeno je *in vitro* ispitivanje. Kao rezultat eksperimenta izvedenog na pH 5 tokom 120 minuta inkubacije na 37°C, otkrivena je optimalna formulacija adsorbenta, koji je u niskoj koncentraciji i u prisustvu velike količine toksina ispunio stroge zahteve evropskih propisa za minimalnom efikasnošću vezivanja aflatoksina od 90% (90,1% ostvareno sa 0,02% adsorbenta i 4 mg/L toksina). U uslovima veće količine adsorbenta (0,2%) i manje toksina (0,2 mg/L) vezalo se 99,6%. Takav ishod ukazuje na validnost *in vitro* eksperimentalnog pristupa koji može da posluži kao pouzdan brzi alat za trijažu adsorbenasa i njihovu predselekciju za *in vivo* testove.

**Ključne reči:** hrana za životinje, dodaci hrani za životinje, mikotoksini

## INTRODUCTION

Mycotoxins contaminate food chain through food and feed crops, mainly cereals, which become infested prior to and during harvest, or during (improper) storage. They are produced as secondary metabolites of different types of fungus under the favourable environmental conditions, when temperature and moisture are appropriate. Climate changes during the last decade in particular contributed to the escalation of this problem (Nešić et al., 2014; Nešić, 2018; Jakšić et al., 2017, 2018, 2019). Aflatoxins are strong (Class I, IARC, 2002) carcinogens in mammalian species, difuranocoumarin derivatives produced by different species of *Aspergillus* (*Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius* and *Aspergillus pseudotamarii*). Several types of aflatoxin (14 or more) are found in nature, and $B_1$, $B_2$, $G_1$, G2 and $M_1$ are of major importance. Aflatoxins $B_1$, $B_2$, $G_1$ and G2 are direct secondary metabolites of fungi, whereas aflatoxin M1 is produced by metabolizing aflatoxin $B_1$ ($AFB_1$), which is usually a major product of toxigenic strains (WHO, 2018). The presence of mycotoxin in feed results in huge economic losses for animal breeders caused by decreased performance and production, increased susceptibility to diseases and other adverse effects (Rawal et al., 2010).

Since the mycotoxins have an important impact, there is a continuous effort to develop various ways to alleviate and/or prevent their harmfulness. The approach by using different feed additives, which either adsorb mycotoxins on their surface or foment enzyme degradation of mycotoxins proved to be particularly effective (EFSA, 2009; Nedeljkovic-Trailovic et al., 2015). The output depends mostly on the chemical structure of the adsorbent, as well as on the type of present mycotoxin. Mineral adsorbents (e.g. hydrated sodium calcium aluminosilicate, sodium bentonit, dietary clay and zeolites) and active charcol are among the most used for this purpose. These are the substances that are not resorbable from the gut and that physically bind target chemicals and consequently block their resorption (Nešić et al., 2014). The feasibility of utilizing organic adsorbents has also been examined, particularly those isolated from the yeast cell wall that possess significant adsorption capacity (Devegowda et al., 2004; Nešić et al., 2008). Recently, some new types of additives which contain microorganisms have been developed. They have the ability to enzymatically modify the mycotoxin structure (Fuchs et al. 2002; Nešić et al. 2011, 2012).

The ability of different adsorbents to ameliorate aflatoxin $B_1$ toxicity was tested in *in vitro* and *in vivo* conditions and the findings mostly correlated (Vekiru et al., 2015). Supplementation of diets with selected adsorbents, especially of the bentonite type, seems to almost fully protect animals against

aflatoxicosis, so the EFSA Scientific Report gives an actual and comprehensive overview on this topic (EFSA, 2009). Bentonites are composed predominantly of smectite. However, a wide variety of other minerals may occur as impurities. The dioctahedral smectite mineral montmorillonite is present in most bentonites. Depending on the dominant exchangeable cations, bentonite may be referred to as calcium or sodium bentonite. Sodium bentonite swells and expands to a greater degree than its calcium equivalent. Calcium bentonite may be converted to sodium bentonite, then termed sodium activated bentonite. The type of the cation on the surface of the aluminium sheet (Ca or Na) may affect the binding capacity of the montmorillonite (EFSA, 2011).

Besides its excellent nutritional value, yeasts and yeast cell wall can also be used as adsorbents for mycotoxins. The adsorption of mycotoxins can be enhanced by using yeast cell walls instead of whole cells. The cell walls harbouring polysaccharides (glucan, mannan), proteins and lipids exhibit numerous different and easily accessible adsorption centers including different adsorption mechanisms, e.g. hydrogen bonding, ionic, or hydrophobic interaction (Huwig et al., 2001). Regarding polysaccharides, including β-D-glucan and α-mannan, it has been proposed that their antigenotoxic action mechanism is related to their action as antioxidant agents (Pereyra et al., 2012). The ability of β-D-glucan to partially prevent DNA damage induced by $AFB_1$ in mouse hepatocytes was determined in a trial (Madrigal-Bujaidar, 2015). The data suggested the formation of a supramolecular complex between $AFB_1$ and β-D-glucan. Mannan oligosaccharide is a potent immunomodulator which alleviates the damages of $AFB_1$ (Sun et al., 2019).

The aim of the presented *in vitro* trial was to estimate adsorbing potential of "MycoStop DUPLO". It is a feed additive which combines bentonite and yeast components and is intended for prevention and/or alleviation of adverse effects of aflatoxin $B_1$ in animal nutrition.

## MATERIAL AND METHODS

### *Chemicals and mycotoxin adsorbents*

$AFB_1$ standard was purchased from Sigma-Aldrich, Cat No A6636 ((St. Louis, MO, USA). Stock solution of $AFB_1$ was prepared by dissolving the toxin in acetonitrile (10 μg/mL). Acetonitrile HPLC gradient grade was purchased from Fisher Scientific (Leicestershire, UK). To perform the adsorption experiments, appropriate volume of stock standard solution was evaporated to dryness and dissolved in buffer solution. Acetate buffer (pH5), 0.1 mol/L was prepared by dissolving 0.82 g of sodium acetate ($C_2H_3NaO_2$; Lach-Ner, Nera-

tovice, Czech Republic) in approximately 90 mL of distilled water. Then, the solution was adjusted to pH5 with acetic acid analytical grade ($C_2H_4O_2$), and filled up to 100 mL with distilled water.

Five samples of different adsorbents (1 - 5) were provided by INBERG d.o.o. (Belgrade, Republic of Serbia). Four of them (2 - 5) consisted of bentonite (smectite - dioctahedral montmorillonite) and yeast cell wall extracts, in different combination, while one (labelled 1) contained zeolite as inorganic component.

### *Physico-chemical characterization tests*

The physico-chemical properties of adsorbents were examined as moisture content, acidity and swell index. Moisture content in adsorbents was determined by drying in oven (Memmert UNB 500, Germany) at 105°C to constant mass. The acidity of the adsorbent samples was measured in 1:10 adsorbent: water suspension (De Mil et al., 2015). The suspensions were shaken for 2 h and were left to sediment for next 2h under closed lid. The pH of the supernatant was measured using pH meter (Consort, Turnhout, Belgium). For determination of the swell index, the ASTMD5890, 2011 method was used.

### *In vitro* experiment design

The assessment of aflatoxin $B_1$ adsorption capacity was accomplished in accordance with the Regulation (EU) No 1060/2013 (European Commission, 2013), an approved method for the evaluation of bentonites authorized as feed additives against $AFB_1$. The test was carried out in a buffer solution at pH 5.0, at 37°C, for 120 minutes, with a concentration of 4 mg/L for $AFB_1$ and 0.02% (*w/v*) for the adsorbent (phase I). The best performing adsorbents from the phase I were examined in the second phase of the experiment. In this phase, II binding capacity was investigated using a standard solution of 0.2 mg/L $AFB_1$ and the adsorbent in the concentration of 0.2% (*w/v*; Prapapanpong et al., 2019). All the tests were done in triplicate.

After incubation (shaking for 2 h at 37°C), samples were filtered (syringe filters 0.22 µm; LLG-Labware, Meckenheim, Germany) and the solution was analyzed by an HPLC Dionex UltiMate 3000 Series system equipped with a FLD 3100 detector (Thermo Scientific, Germering, Germany) at 30°C, and $\lambda_{ex}$ 365 nm, $\lambda_{em}$ 435 nm. The HPLC column was Supelcosil™ LC-18-DB, 250 x 4.6 mm (particle size 5 µm; Merck, Darmstadt, Germany), fitted with a guard column. The mobile phase was acetonitrile: water (50:50, *v/v*) filtered through 0.22 µm membrane filter, at a flow rate of 1.2 mL/min. The system was controlled by Chromeleon® 7 software (Thermo Scientific, Germering,

Germany). The peak areas at aflatoxin $B_1$ retention times were compared to the corresponding calibration curves. Calculation of $AFB_1$ adsorption rates (%) was performed according to the following equation:

$BC_{AFB1} = (1-C_I/C_0) \times 100\%$

$BC_{AFB1}$ = binding capacity; $C_I$ = concentration of free $AFB_1$ after the incubation period; $C_0$ = initial fortified concentration of the $AFB_1$.

## RESULTS

The results of physico-chemical characterization tests showed different properties of adsorbents regarding moisture content, swell index and acidity (Table 1).

Table 1. Results of physico-chemical characterization tests

| Sample label | Moisture content (%) | Swell index (mL/2g) | pH |
|---|---|---|---|
| **1** | 4.80 ± 0.18 | 2 | 6.4 ± 0.1 |
| **2** | 10.46 ± 0.18 | 8 | 6.5 ± 0.4 |
| **3** | 9.80 ± 0.15 | 13 | 7.6 ± 0.1 |
| **4** | 7.73 ± 0.03 | 5 | 6.5 ± 0.1 |
| **5** | 7.22 ± 0.11 | 4 | 7.4 ± 0.2 |

Test results for the adsorption of aflatoxin $B_1$ (4 mg/l) by different adsorbents 1 - 5 (0.02% w/v) at pH 5 after 120 minutes (phase I) were from 9.1 ± 1.9 % to 90.1 ± 0.2 %. In the second phase (phase II) of the trial, which was performed with high adsorbent (0.2% w/v) and low toxin concentration (0.2 mg/L), the best performance was confirmed for the sample number 4 as binding capacity was 99.6 ± 0.03 % (Table 2).

Table 2. Binding results for different adsorbents (1 – 5) and aflatoxin $B_1$ in the phase I and II (pH5, 37°C, 120 min) [%]

| Sample label | Phase I: $AFB_1$ 4 mg/l + adsorbent 0.02% w/v | Phase II: $AFB_1$ 0.2 mg/L + adsorbent 0.2% w/v |
|---|---|---|
| **1** | 9.1 ± 1.9 | / |
| **2** | 88.3 ± 1.3 | 97.2 ± 0.3 |
| **3** | 85.7 ± 0.8 | 93.8 ± 2.4 |
| **4** | 90.1 ± 0.2 | 99.6 ± 0.03 |
| **5** | 27.0 ± 0.5 | / |

The characterization study showed that all samples differed in physico-chemical properties, such as moisture content and swelling index. Besides that, their ability to adsorb aflatoxin $B_1$ also varied greatly.

## DISCUSSION

The variable properties of adsorbents are the result of their different composition in terms of the ratio of organic and inorganic components. Sample number 1 mostly differed, as it contained zeolite for mineral component instead of montmorillonite. However, more samples need to be investigated for correlation assessment between physico-chemical properties of adsorbents and the amounts of adsorbed toxin.

According to Regulation (EU) No 1060/2013 (European Commission, 2013), $AFB_1$ good binding capacity ($BC_{AFB1}$) should be above 90%, which was achieved with the product number 4. The adsorbent was tested under „intensified conditions" (low binder concentration and high toxin concentration), as described by Vekiru et al. (2015) and such concept was carried out to get closer to the limit of the product's adsorption capacities. In the Phase II, an extremely high percentage of binding for the same sample confirmed the previous good outcome.

Based on the obtained results, the material labelled with number 4 was categorized as good and its composition proved to be the best of all the examined samples. Bentonite, which is the main component of this product, is well known for its ability to bind aflatoxins (EFSA, 2009, 2011), while natural yeast extracts, a cell wall derivatives of *Saccharomyces cerevisiae*, show considerable binding ability with several commonly occurring mycotoxins (Devegowda and Murthy, 2005) and are beneficial in minimizing their adverse effects in animals (Nešić et al., 2008). Such multilevel mechanism of action, achieved through the complex composition of this adsorbent, indicate various usage potentials and also enable further efficiency testing of this feed additive.

The poorest performing of the zeolite sample was sample (No 1), which is in accordance with the results of Thieu and Pettersson (2008) who reported that bentonite has a better ability to adsorb AFB1 than zeolite. According to Marroquín-Cardona et al. (2009), this could be due to the smaller size of the zeolite pores (4 - 7 Å in case of natural clinoptilolite) in comparison with AFB1 size (10.4 - 12.8 Å), which limits the adsorption to the external surface only.

As reported in the case of charcoal, *in vitro* success is not always a sufficient criterion to choose an adsorbent for practical use, so *in vivo* trials should verify its usefulness (Diaz et al., 2002; 2004). Even among good binders, there were

differences in *in vivo* efficacy, indicating that *in vitro* testing alone is not always adequate for complete evaluation of the additive (Vekiru et al., 2015). Nevertheless, the advantage of *in vitro* test is the possibility of rapid screening efficiency of a large number of adsorbents. In this way, the reduction of mycotoxin toxicity is also indirectly confirmed. Thus, *in vitro* experimental approach can serve as a reliable fast tool for triage of adsorbents. Vekiru et al. (2015) showed in their trial that it is a helpful to preselect an $AFB_1$ adsorbent and to predict the *in vivo* $AFB_1$ detoxifying performance.

## CONCLUSION

As a result of the presented experiment, the optimal formulation of the adsorbent No 4 "MycoStop DUPLO" was found, which at low concentration and in the presence of high amounts of toxin met the stringent European regulation requirements for the minimum 90% aflatoxin binding efficiency. Based on good *in vitro* aflatoxin $B_1$ adsorption results, it seems pertinent to extend *in vivo* studies of the selected adsorbent. As, according to literature data, it combines bentonite and yeast polysaccharides, it is reasonable to perform assays with other mycotoxins in the future and expect promising results.

Although there is a regulation on the *in vitro* testing of bentonite in the EU, many national regulations worldwide do not cover estimation of binding capacity of adsorbents used as additives in animal feed. Also, there is no unique methodology for analyzing adsorbents and variously designed experiments could be found in the literature. Therefore, the information on the adsorption capacity is obtained in different ways and is not always comparable. It would be necessary to standardize this procedure and establish regulations to cover this significant area.

## ACKNOWLEDGEMENT

This paper is published as part of the project financed by the Inovation Fund of the Republic of Serbia. The authors cordially thank INBERG d.o.o., Belgrade, Serbia for providing tested adsorbents.

## Author's contributions:

KN-conducted the experiment and paper concept and writing, SJ- concept and design of the study, NP- experiment performance, MZB-experiment concept and revising the manuscript critically, MP- providing material and data

collection, BZ-initial idea and providing material, VP-experiment organization and material provision.

## Competing Interests

The authors declare that they have no competing interests.

## REFERENCES

1. ASTM D5890 Bentonite swell index, Technical note, Global Syntetics.
2. De Mil T., Devreese M., De Baere S., Van Ranst E., Eeckhout M., De Bacjer P., Croubels S. 2015. Characterization of 27 Mycotoxin Binders and the Relation with in Vitro Zearalenone Adsorption at a Single concentration. Toxins, 7, 21-33. doi: 10.3390/toxins7010021.
3. Devegowda G., Reddy N.B., Shashidhara R.G. 2004. Ability of modified glucomannan to sequestrate T-2 toxin in the gastrointestinal tract of chicken. Asian Australian Journal of Animal Science, 17, 2, 259. doi: 10.5713/ajas.2004.259.
4. Devegowda G. and Murthy T.N.K. 2005. Mycotoxins: Their adverse effects in poultry and some practical solutions. In: *The Mycotoxin Blue Book*. Ed. D. E. Diaz, Nottingham University Press, Nottingham, UK, 25-56.
5. Diaz D.E., Hagler W.M., Hopkins B.A., Whitlow, L.W. 2002. Aflatoxin binders I: in vitro binding assay for aflatoxin B1 by several potential sequestering agents. Mycopathologia, 156, 223-226. doi: 10.1023/a:1023388321713.
6. Diaz D.E., Hagler W.M., Blackwelder J.T., Eve J.A., Hopkins B.A., Anderson K.L., Jones F.T., Whitlow, L.W. 2004. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathologia, 157, 233-241. doi:10.1023/B:MYCO.0000020587.93872.59.
7. European Commission (EC). 2013. Commission Implementing Regulation (EU) No 1060/2013 of 29 October 2013 concerning the authorisation of bentonite as a feed additive for all animal species, Official Journal of the European Union, L 289: 33-37.
8. European Food Safety Authority (EFSA). 2009. Review of mycotoxin-detoxifying agents used as feed additives: mode of action, efficacy and feed/food safety. EFSA-Q-2009-00839. http://www.efsa.europa.eu/en/scdocs/doc/22e.pdf
9. European Food Safety Authority (EFSA). 2011. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Scientific Opinion

on the safety and efficacy of bentonite (dioctahedral montmorillonite) as feed additive for all species. EFSA Journal, 9, 2, 24.
10. Fuchs E., Binder E.M., Heidler D., Krska R. 2002. Structural characterization of metabolites after the microbial degradation of type A trichothecens by the bacterial strain BBSH 797. Food Additives and Contaminants, 19, 4, 379–386. doi: 10.1080/02652030110091154.
11. Huwig A., Freimund S., Kappeli O., Dutler H. 2001. Mycotoxin detoxication of animal feed by different adsorbents. Toxicology Letters 122, 179 – 188. doi: 10.1016/s0378-4274(01)00360-5.
12. IARC - International Agency for Research on Cancer. 2002. IARC Monograph on the Evaluation of Carcinogenic Risk to Humans. 82, 171.
13. Jakšić S., Živkov-Baloš M., Prodanov-Radulović J., Jajić I., Krstović S., Stojanov I., Mašić Z. 2017. Aflatoxin M1 in milk and assessing the possibility of its occurrence in milk products. Archives of Veterinary Medicine, 10, 1, 37- 50. doi:10.46784/e-avm.v10i1.80.
14. Jakšić S., Popov N., Živkov Baloš M., Prodanov-Radulović J., Abramović B. 2018. Fumonisins in pig feed. Arhiv veterinarske medicine, 11, 2, 43 - 51. doi:10.46784/e-avm.v11i2.25.
15. Jakšić S., Zivkov Balošs M., Jajic I., Abramović B. 2019. Fumonisins in Serbian Corn: Long-time Assessment under Actual Climate Change Conditions. Cereal Research Communications, 47, 4, 714-723. doi: 10.1556/0806.47.2019.45.
16. Madrigal-Bujaidar E., Morales-González J.A., Sánchez-Gutiérrez M., Izquierdo-Vega J.A., Reyes-Arellano A., Álvarez-González I., Pérez-Pasten R., Madrigal-Santillán E. 2015. Prevention of Aflatoxin B1 Induced DNA Breaks by β-D-Glucan. Toxins, 7, 2145-2158. doi: 10.3390/toxins7062145.
17. Marroquín-Cardona A., Deng Y., Taylor J.F., Hallmark C.T., Johnson N.M., Phillips T.D. 2009. In vitro and in vivo characterization of mycotoxin-binding additives used for animal feeds in Mexico. Food Additives and Contaminants, 26, Part A, 733-743. doi: 10.1080/02652030802641872.
18. Nedeljkovic-Trailovic J., Trailovic S., Resanovic R., Milicevic D., Jovanovic M., Vasiljevic M. 2015. Comparative Investigation of the Efficacy of Three Different Adsorbents against OTA-Induced Toxicity in Broiler Chickens. Toxins, 7, 1174-1191. doi: 10.3390/toxins7041174.
19. Nešić K., Resanovic R., Nešić V., Sinovec Z. 2008. Efficacy of mineral and organic adsorbent in alleviating harmful effects of zearalenone on pigs performance and health. Acta Veterinaria Beograd, 58, 2–3, 211–219. doi: 10.2298/AVB0803211N.

20. Nešić K., Resanovic R., Jakic-Dimic D., Nešić V. 2011. Efficiency of various feed additives on the performance of broilers treated with T-2 toxin. Biotechnology in Animal Husbandry 2-3, 2, 705–711. doi: 10.2298/BAH1103705N.
21. Nešić V., Resanovic R., Marinkovic D., Nešić K., Lazarevic M., Nešić S., Aleksic-Kovacevic S. 2012. Efficacy of T-2 toxin detoxifying agent in broiler chickens. Acta Veterinaria Beograd, 62, 2–3, 171–182. doi: 10.2298/AVB1203171N.
22. Nešić K., Ivanovic S., Nešić V. 2014. Fusarial Toxins - Secondary Metabolites of Fusarium Fungi. Reviews of Environmental Contamination and Toxicology, 228, 101-120. doi: 10.1007/978-3-319-01619-1_5.
23. Nešić K. 2018. Mycotoxins – climate impact and steps to prevention based on prediction. Acta Veterinaria-Beograd, 68, 1, 1-15. doi: 10.2478/acve-2018-0001.
24. Pereyra C.M., Cavaglieri L.R., Chiacciera S.M., Dalcero A. 2012. The corn influence on the adsorption levels of aflatoxin B1, and zearalenone by yeast cell wall. Journal of Applied Microbiology, 114, 655–662. doi: 10.1111/jam.12082.
25. Prapapanpong J. , Udomkusonsri P. , Mahavorasirikul W. , Choochuay S., Tansakul N. 2019. In vitro studies on gastrointestinal monogastric and avian models to evaluate the binding efficacy of mycotoxin adsorbents by liquid chromatography-tandem mass spectrometry. Journal of Advanced Veterinary and Animal Research, 6, 1, 125-132. doi: 10.5455/javar.2019.f322.
26. Rawal S., Kim J.E., Coulombe Jr., R. 2010. Aflatoxin B1 in poultry: toxicology, metabolism and prevention. Research in Veterinary Science, 89, 325-331. doi: 10.1016/j.rvsc.2010.04.011.
27. Sun Y., Su J., Yang S., Liu Z., Liu D, Gan F., Chen X., Huang K. 2019. Mannan Oligosaccharide Protects against the Aflatoxin-B1-Promoted Influenza Replication and Tissue Damages in a Toll-Like-Receptor-4-Dependent Manner. Journal of Agricultural and Food Chemistry. 16, 67, 2, 735-745. doi: 10.1021/acs.jafc.8b05829.
28. Thieu N.Q. and Pettersson H. 2008. In vitro evaluation of the capacity of zeolite and bentonite to adsorb aflatoxin B1 in simulated gastrointestinal fluids. Mycotoxin Research, 24, 124-129. doi: 10.1007/BF03032338.
29. Vekiru E., Fruhauf S., Rodrigues I., Ottner F., Krska R., Schatzmayr G., Ledoux D.R., Rottinghaus G.E., Bermudez A.J. 2015. In vitro binding assessment and in vivo efficacy of several adsorbents against aflatoxin B1. World Mycotoxin Journal, 8, 4, 477-488. doi:10.3920/WMJ2014.1800.

30. WHO 2018. Available at: https://www.who.int/foodsafety/FSDigest_Aflatoxins_EN.pdf. Accessed 6.2.2020.

Received: 03.03.2020.
Accepted: 17.05.2020.

Original research article
UDC 597.552.512:639.2.09(497.1)
https://doi.org/10.46784/e-avm.v13i1.78

# THE FIRST OUTBREAK OF LACTOCOCCOSIS CAUSED BY LACTOCOCCUS GARVIEAE IN SERBIA

Vladimir Radosavljevic[1*], Oliver Radanovic[1], Nemanja Zdravkovic[1], Bozidar Savic[1], Marko Stankovic[2], Jelena Maksimovic-Zoric[1], Ljubiša Veljović[1], Ksenija Nesic[1]

[1] Institute of Veterinary Medicine of Serbia, Belgrade, Republic of Serbia
[2] Faculty of Agriculture, University of Belgrade, Zemun, Republic of Serbia

## Abstract

The outbreak of lactococcosis affecting rainbow trout, weighing 70 - 120 g, occurred at a trout aquaculture facility in Central-West Serbia during July, 2018. This outbreak lasted for three weeks, and cumulative mortality attributed to the disease was around 40%. The diseased fish showed erratic swimming, dark discoloration and exophthalmia, with the cumulative mortality of around 40%. A pure Gram-positive cocci isolates were obtained from the eye and kidney samples. Based on colony morphology, phenotypic and biochemical characteristics, the isolated bacterium was presumably identified as *Lactococcus garvieae*. Using the BBL CRYSTAL GP ID system, the isolate was identified as *L. garvieae* and the identity of the isolate was confirmed with MALDI-TOF Mass Spectrometry. Blast analysis of 16S rRNA sequence of our isolate had a 99.4 to 99.6% similarity to the *L. garvieae* strains previously isolated from diseased fish. The antibacterial activity of 15 antimicrobials against *L. garvieae* was evaluated using disc diffusion. In this paper, we report the first case of lactococcosis in rainbow trout in Serbia, isolation and characterization of causative agent, *Lactococcus garvieae* from diseased rainbow trout.

**Key words:** Lactococcosis, *Lactococcus garvieae*, rainbow trout

[1*] Corresponding author: vladimiradosavljevic@yahoo.co.uk

# PRVI SLUČAJ LAKTOKOKOZE IZAZVANE SA *LACTOCOCCUS GARVIEAE* U SRBIJI

Vladimir Radosavljević[1]*, Oliver Radanović[1],
Nemanja Zdravković[1], Bozidar Savic[1], Marko Stanković[2],
Jelena Maksimović-Zorić[1], Ljubiša Veljović[1], Ksenija Nešić[1]

[1] Naučni institut za veterinarstvo Srbije, Beograd, Republika Srbija
[2] Poljoprivredni fakultet, Univerzitet u Beogradu, Zemun, Republika Srbija

## Kratak sadržaj

Pojava laktokokoze u zapatu kalifornijske pastrmke, težine 70 - 120 g, je utvrđena na pastrmskom ribnjaku u zapadnoj Srbiji u julu 2018. godine. Bolest je trajala tri nedelje, a kumulativni mortalitet nastao kao posledica oboljenja je iznosio oko 40%. Klinička slika se karakterisala promenama u plivanju, tamnom pigmentacijom kože i izraženim egzoftalmusom. Na osnovu morfoloških i biohemijskih karakteristika, izolovana bakterija je identifikovana kao *Lactococcus garvieae*. Korišćenjem BBL CRYSTAL GP ID sistema, izolat je identifikovan kao *L. garvieae*, a identitet izolata je potvrđen pomoću MALDI-TOF masene spektrometrije. Analiza 16Sr RNK sekvence našeg izolata pokazala je sličnost od 99,4 do 99,6% sa sekvencama sojeva *L. garvieae* prethodno izolovanih iz obolelih riba. Antibakterijska aktivnost 15 antimikrobnih lekova protiv *L. garvieae* procenjena je metodom disk difuzije. U radu je opisan prvi slučaj pojave laktokokoze kalifornijske pastrmke u Srbiji i izolacija i karakterizacija uzročnika bolesti.

**Ključne reči:** laktokokoza, *Lactococcus garvieae*, kalifornijska pastrmka

## INTRODUCTION

Lactococcosis is a significant fish disease caused by the *Lactococcus garvieae* bacteria. It is a systemic hyper-acute infection with the occurrence of widespread haemorrhaging (Austin & Austin 2016), described for the first time at the end of the 1950s in Japan, where the first cases were diagnosed in rainbow trout (Vendrell et al. 2006). Now, the disease is present in many parts of the world, affecting sea fish and freshwater fish in aquaculture (Meyburgh et al. 2017). Rainbow trout is an important species for aquaculture in Serbia,

with more than 100 farms in business. Trout farms are mainly concentrated in South-East part of the country. In Europe, the first outbreak of this disease in rainbow trout was reported in Spain in 1989 (Palacios et al., 1993). After that, *L. garvieae* was isolated in Italy (Reimundo et al. 2011), the UK (Algöet et al. 2009), France (Eyngor et al. 2004), Portugal (Pereira et al. 2004), Greece (Savvidis et al. 2007), Spain (Aguado-Urda et al. 2011), Turkey (Diler et al. 2002) and Bulgaria (Eyngor et al. 2004). Lactococcosis is the single most important risk factor in the European trout industry, with losses approximating 50% of the total annual production (Eyngor et al., 2004). The impact of lactococcosis is particularly significant as losses often occur when fish reach market size (Ceschia et al. 1998). The disease causes significant losses at temperatures above 15°C. The oral route is the main route of *L. garvieae* transmission (Nakai et al. 1999), but the results of a study conducted by Avci et al. (2010) suggest that the gills and eyes are major spots of attachment and the proliferation of *L. garvieae* during infection period. Infected fish exhibit a variety of clinical signs, such as anorexia, exophthalmia, melanosis, conjunctivitis, erect swimming, severe internal haemorrhage and congestion of blood vessel, peritonitis, abscess of spleen and liver, meningoencephalitis, and bacterial septicemia (Eldaret al.1999, Evans et al.2009, Pereira et al. 2004).

The causative agent, *L. garvieae* is one of the most important bacterial fish pathogens indifferent freshwater and marine fish species in many countries (Vendrell et al. 2006; Evans et al. 2009, Meyburgh et al. 2017), with the highest economic impact in rainbow trout aquaculture. *L. garvieae* is a non-motile, non-sporulating, facultative anaerobic, catalase and cytochrome oxidase negative, Gram-positive coccus. It is a lactic acid bacterium, first isolated from a case of bovine mastitis it the UK (Collins et al. 1983), and later from other animal hosts, such as cows, buffalos, pigs, dolphins, water buffalos, cats and dogs (Aguado-Urda et al. 2013, Tejedor et al. 2011). The bacterium was isolated from rivers and sewage waters, vegetables, meat and dairy products (Aguado-Urda et al. 2013). *L. garvieae* strains of dairy origin have been found to be free from virulence determinants, such as haemolysins and gelatinase (Fortina et al., 2007), suggesting that *L. garvieae* dairy strains are unrelated to the pathogenic ones (Foschino et al., 2008). Also, *L. garvieae* was involved in an increasing number of human clinical cases including infective endocarditis, septicemia, urinary and skin infections (Aguado-Urda et al. 2011), giving rise to the status of an emerging zoonotic pathogen. Although *L. garvieae* is a well-known fish pathogen, human infections are usually related to a contact with raw fish. It has gained recognition as an emerging zoonotic opportunistic pathogen, with the ingestion of contaminated foodstuffs being a likely route

of infection. Handling and of raw fish is reported as a source or risk factor in the majority of clinical cases (Gibello et al. 2016). High levels of antibiotic resistance and resistance genes in *L. garvieae* strains should be considered as a potential danger for trout culture as well as for public health (Raissy and Moumeni, 2016).

In this paper, we report the first case of lactococcosis in rainbow trout in Serbia, isolation and characterization of causative agent, *L. garvieae* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum).

## MATERIAL AND METHODS

A total of 20 rainbow trout (weighing 70-120g) that showed the clinical signs of the disease were collected from a trout aquaculture facility in Central-West Serbia in July, 2018. The samples for bacterial isolation were obtained from the kidney, liver, spleen and eye of the diseased fish. They were streaked on blood agar plates containing 10% defibrinated sheep blood (BA), Mueller-Hinton (MH) agar and trypticase-soy agar (TSA) plates, and incubated at 20°C for 48h. Single colonies were restreaked on the same media to obtain pure isolates. Pure colonies were subjected to Gram staining, followed by catalase and oxidase tests. The routine tests for the determination of biochemical characteristics were carried out as previously described (Austin & Austin 2016). Additionally, the BBL CRYSTAL™ Gram-Positive (GP) Identification (ID) system was used for the biochemical identification of isolated bacterium.

The isolate was tested for antimicrobial susceptibility by the disc diffusion method on MH agar. After 24h of the incubation, incubation zone diameters were measured and evaluated. The isolates were classified as sensitive (S), intermediary sensitive (I), or resistant (R), on the basis of the size of the zone of bacterial growth inhibition, according to the National Committee for Clinical Laboratory Standards (CLSI, 2019).

### *MALDI-TOF*

Protein mass spectroscopy analysis was carried out by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as previously described (Heras Cañas et al. 2015) using a VITEK MS Mass spectrometry microbial identification system (VITEK MS, bioMerieux, France).

### *16S rRNA sequence analysis*

Total bacterial DNA of the isolate was extracted using the Cador Pathogen Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The extracted DNA from the isolate was used as a template to amplify a 1419bp segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique using the universal prokaryotic primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' GGTTACCTTGTTACGACTT-3'). The composition of PCR mixture was as recommended by manufacturer (HotStarTaq Master Mix Kit, Qiagen, Germany). The PCR cycling regime was the following: one cycle of 15 min denaturation and Taq activation at 95°C, 35 cycles of 40 s at 95°C, 40 s at 55°C, and 90 s at 72°C, and final extension cycle of 10 min at 72°C. A small quantity of PCR products was verified by standard agarose gel electrophoresis. The amplicons were purified using QIAquick PCR Purification kit (Qiagen, Germany) and sequenced using the primers 27F and 1492R. The sequencing of PCR product was performed by Macrogen. The isolate sequence was compared to sequences from the NCBI database, using the BLASTn algorithm. The limit fixed for identification of a bacterial species was 98% nucleotide identity for the 16S rRNA gene. The phylogenetic relationships of the isolates were determined by comparative 16S rRNA gene sequence analysis. Genetic distances matrix was obtained using Kimura's two-parameter model, and an evolutionary tree was created using the Neighbour-Joining method with Mega X (Kumar et al.2018).

## RESULTS

The disease outbreak affecting rainbow trout weighing 70 - 120 g occurred at a trout aquaculture facility in Zaovine Lake (43°52'46.3"N 19°24'08.4"E) during July, 2018. This outbreak lasted for three weeks, and cumulative mortality attributed to this pathogen was around 40%. The water temperature during the outbreak was consistently higher than 14°C (with highest temperature of 20°C). The infected fish exhibited lethargy, anorexia, dark skin coloration, marked unilateral and bilateral exophthalmos with the presence of generalized hemorrhaging or blood spots in the eye, eyeball disruption and loss of eye or eyes (Figure 1).

Figure 1. The hemorrhaging in the eye and exophthalmos in diseased rainbow trout caused by lactococcosis

Macroscopic examination revealed the accumulation of fluid in the body cavity. Skeletal muscle and liver were anemic with congestion. Liver and spleen were enlarged. Hemorrhages were present in liver, adipose tissue, pyloric caeca and muscle. Enlargement of the spleen and liver, and hemorrhagic enteritis with yellow, gelatinous fluid in the lumen of intestine were observed (Figure 2).

Figure 2. Lactococcosis in rainbow trout - hemorrhages in internal organs, enlargement of the spleen and liver, and yellow, gelatinous fluid in the lumen of intestine

After 48h incubation, pure cultures of cream-colored, opaque, round and convex colonies were recovered from eye and kidney samples (Figure 3).

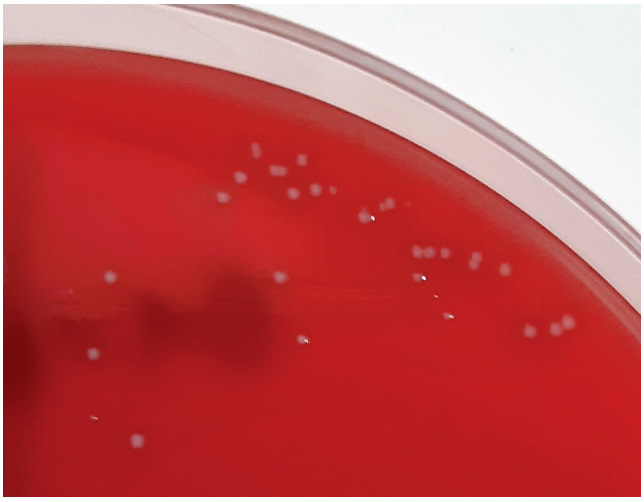

Figure 3. Growth of colonies of *L. garvieae* SRB NIVS-1 strain on a blood agar plate after 24 hours of incubation in aerobic conditions.

The bacterial cells were α-hemolytic, non-motile, oxidase-negative, catalase-negative, Gram-positive cocci occurring in pairs and short chains (Figure 4).

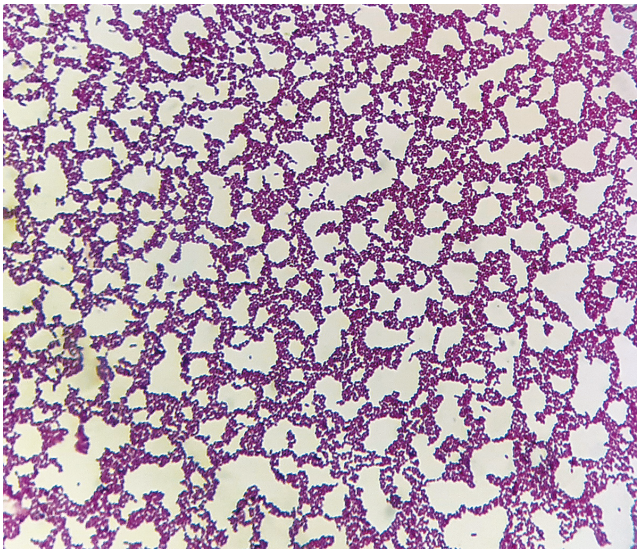

Figure 4. Gram stain of *L. garvieae* SRB NIVS-1 strain with clusters and short chains of gram-positive cocci.

Based on colony morphology, phenotypic and biochemical characteristics, the isolated bacterium was presumably identified as *L. garvieae* (Table 1).

Table 1. Phenotypic characteristic of *L. garvieae* SRB NIVS-1 strain isolated from diseased rainbow trout.

| **Phenotypic characteristics** | ***L. garvieae* SRB NIVS-1** | ***L. garvieae* ATCC 43921** |
|---|---|---|
| Colony morphology | smooth | smooth |
| Cell morphology | ovoid-cocci | ovoid-cocci |
| Gram staining | + | + |
| Motility | - | - |
| Production of oxidase | - | - |
| Production of catalase | - | - |
| Production of indole | - | - |
| Production of $H_2S$ | - | - |
| Citrate utilization | - | - |
| Methyl red | + | + |
| Voges Proskauer | + | + |
| Nitrate reduction | - | - |
| Starch hydrolysis | - | - |
| Growth on nutrient agar | + | + |
| Growth on trypticase soy agar | + | + |
| Growth on brain heart infusion agar | + | + |
| Growth on Mueller-Hinton agar | + | + |
| Growth on MacConkey agar | - | - |
| Hemolysis in blood agar | α | α |

Using the BBL CRYSTAL GP ID system, the isolate was identified as *L. garvieae* ID: 3440571723 (Table 2).

Table 2. The BBL Crystal profile for *L. garvieae* isolated from diseased rainbow trout

| | | | |
|---|---|---|---|
| L-phenylalanine-AMC | + | p-nitrophenyl-β-D-cellobioside | + |
| L-tryptophan-AMC | + | p-nitrophenyl-α-D-maltoside | + |
| Trehalose | + | Esculin | + |
| Sucrose | + | L-valine-AMC | +- |
| Arabinose | - | pyroglutamic acid-AMC | +- |

| | | | |
|---|---|---|---|
| p-nitrophenyl-β-D-glucoside | + | 4MU-N-acetyl-β-D-glucosaminide | +- |
| p-nitrophenyl-phosphate | - | L-isoleucine-AMC | + |
| Urea | - | Methyl-α & β-glucoside | + |
| 4MU-β-D-glucoside | + | Maltotriose | + |
| 4MU-α-D-glucoside | + | Fructose | + |
| L-arginine-AMC | +- | Proline & Leucine-p-nitroanilide | + |
| Lactose | - | o-nitrophenyl-β-D-galactoside (ONPG) & p-nitrophenyl-α-D-galactoside | - |
| Mannitol | + | ID: 3440571723 *Lactococcus garvieae* | |
| Glycerol | - | | |

The *L. garvieae* isolate was tested in the present study with 15 different antimicrobials in terms of antimicrobial sensitivity and results are shown in Table 3.

Table 3. Antibiotic sensitivity of *L. garvieae* isolate SRB NIVS-1 isolated from diseased rainbow trout

| Antibiotic | Sensitivity |
|---|---|
| Flumequine (15 μg) | R |
| Oxytetracycline (30 μg) | S |
| Sulfamethoxazole/trimethoprim (25 μg) | R |
| Enrofloxacin (5 μg) | R |
| Florfenicol (30 μg) | S |
| Penicillin G (10 IU) | S |
| Ampicillin (10 μg) | S |
| Amoxycillin (25 μg) | S |
| Tetracycline (30 μg) | S |
| Gentamycin(10 μg) | R |
| Neomycin (10 μg ) | R |
| Streptomycin (10 μg ) | R |
| Trimethoprim (5 μg) | R |
| Erythromycin (15 μg) | S |
| Oxacillin (1 μg) | R |

To further confirm our result, the *L. garvieae* strain SRB NIVS-1 was identified with MALDI-TOF, which also confirmed the identity of our isolate. To

determine the genotype identity, we have extracted and sequenced the 16S rRNA (1419 bp, NCBI Genbank accession number MT000099) of strain SRB NIVS-1 and blasted using BLAST search program in the GenBank of NCBI, which revealed 99.6% sequence identity with *L. garvieae*. The sequence was compared with the sequences of reference species in Genebank data library by the BLAST program. Blast analysis of 16S rRNA sequence of our isolate presented a 99.4 to 99.6% similarity in sequences to the L. garvieae strains previously isolated from diseased fish. The isolate showed high percentage sequence similarity to other strains of *L. garvieae*: 99.6 % sequence similarity to MG016446 from China, 99.5 % sequence similarity to KT428591 from Turkey and 99.4 % sequence similarity to KU360715 from Iran. The relationship between the strain SRB NIVS-1 and the closest taxonomic species based on 16S rDNA sequences is described in the phylogenetic tree (Figure 5).

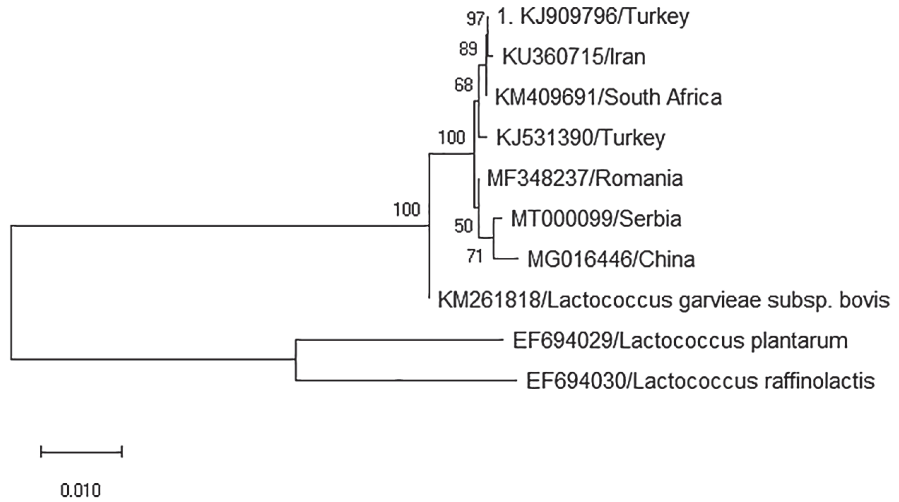


Figure 5. Phylogenetic tree based on 16S rRNA gene sequences showing the position of *L. garvieae* SRB NIVS-1 strain

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.15577609 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

## DISCUSSION

The outbreak of the lactococcosis affecting rainbow trout occurred at a trout aquaculture facility in Zaovine Lake, an artificial lake in the Central-West Serbia, on the Tara Mountain, created on the Beli Rzav River as a reservoir for the Bajina Bašta II reversible hydro power plant. This outbreak with a total mortality of around 40% lasted for three weeks. Rainbow trout mortality due to *L. garvieae* infection can be up to 60%, depending on the water temperature, stress for fish and strain type of the bacterium (Shahi et al, 2018). The water temperature during the outbreak was consistently higher than 14°C (with highest temperature of 20°C). Such high temperature was a critical environmental condition for the appearance of the disease with a high mortality (Pereira et al., 2004, Castro et al. 2017). Clinical findings during the course of the disease, including characteristic symptoms as hyperpigmentation and bilateral exophthalmia, were similar to those previously described in other studies (Evans et al. 2009, Algöet et al.2009, Austin and Austin, 2016).

Pure colonies of the gram positive streptococcus, non-motile, negative for oxidase and catalase, with properties typical for the *Lactococcus garvieae*, as previously described by Collins et al. (1983), were recovered from eye and kidney samples from the diseased rainbow trout. *L. garvieae* strains are phenotypically homogeneous, regardless of their geographic location or the aquatic host from which they were isolated (Buller, 2014). The biochemical properties of the isolated bacteria from rainbow trout were analogous to those described by Austin & Austin (2016). The taxonomy based on a molecular level was used to establish the phylogenesis and taxonomic position of the bacterium. Preferred methods for the identification of *L. garvieae* are MALDI-TOF MS and 16s rRNA gene PCR (Heras Cañas et al. (2015). It is important to emphasize that this pathogen causes serious economic losses due to increased mortality rates (up to 50%), decreasing the growth rates. The appearance of sick fish makes them unmarketable (Vendrell et al 2006). *L. garvieae* is a major fish pathogen of rainbow trout in Europe. To our knowledge, this is the first report of *L. garvieae* associated with trout diseases in Serbia. However, it is likely that the lactococcosis in rainbow trout will become more frequent in the future.

## CONCLUSION

This study confirmed that *L. garvieae* was the etiological agent of a hemorrhagic septicemia in farmed rainbow trout and that the lactococcosis of rainbow trout caused by *L. garvieae* emerged in Serbia. The occurrence could

be attributed to the significant increase in water temperature during summer months, since water temperature is described as the most important environmental factor in the development of the *L. garvieae* infections in trout. In addition, variations in water temperature can affect fish immune response against bacterial infection. Lactococcosis is a limiting problem for rainbow trout culture in many South European countries. After the first occurrence in Spain and Italy, the pathogen and the associated disease has spread rapidly throughout the South Europe, and further to the South-Eastern part of the continent, with disease outbreaks in Greece, Bulgaria and Turkey. In the affected countries, lactococcosis is a major threat to trout culture, especially during awarm period. The rapid spread of the pathogen is a result of the multiple routes of dissemination and transmission of this pathogen. This includes direct spread though the movement of infected fish or asymptomatic carriers and transmission via contaminated water (Savvidis et al., 2007). Since *L. garvieae* have the ability to adapt and survive in many environmental conditions including a wide range of pH, temperatures, salinity concentrations and nutrient sources (Kusuda et al. 1991), the occurrence of the disease in Serbia is a warning for the neighboring countries with trout aquaculture. It is evident that this agent spreads to the new geographic areas, causing the disease with high mortality in susceptible population. The source of outbreak is not known, but since the disease is present in the region and susceptible species are in a river basin which is shared between countries, under adequate conditions, further spread of the causative agent and concomitant disease is inevitable. Due to the ability of *L. garvieae* to colonize multiple, diverse environments, and because it causes infection in a broad range of different hosts, it is reasonable to expect further spread of the bacterium and the disease. Since vaccination is considered the best option to control lactococcosis in rainbow trout, we hope that an appropriate strategy to prevent this infection on Serbian trout farms will be available in the future.

## ACKNOWLEDGEMENT

This study was financially supported by a grant from the Ministry of Education, Science and Technology Development of the Republic of Serbia.

## Author's contributions:

VR, OR, NZ and MS made contributions to concept and design of the study, they collected data and drafted the manuscript. JMZ and BS carried out the molecular diagnostic tests and prepared the alignment of nucleotide

sequences and conducted the molecular genetic analysis. LJV carried out the data analysis. KN revised the manuscript critically and together with VR prepared the final draft of the manuscript. All the authors read and approved the final manuscript.

## Competing Interests

The authors declare that they have no competing interests.

## REFERENCES

1. Aguado-Urda M., Gibello A., Blanco M.M., Garayzábal J.F., López-Alonso V., López-Campos G.H. 2013. Global Transcriptome Analysis of Lactococcus garvieae Strains in Response to Temperature. PLoS One, 8(11): e79692. doi: 10.1371/journal.pone.0079692.
2. Aguado-Urda M., López-Campos G.H., Gibello A., Cutuli M.T., López-Alonso V., Fernández-Garayzábal J.F., Blanco M.M. 2011. Genome sequence of Lactococcus garvieae 8831, isolated from rainbow trout lactococcosis outbreaks in Spain. Journal of bacteriology, 193: 4263–4264. doi: 10.1128/JB.05326-11.
3. Algöet M., Bayley A.E., Roberts E.G., Feist S.W., Wheeler R.W., Verner-Jeffreys D.W. 2009. Susceptibility of selected freshwater fish species to a UK Lactococcus garvieae isolate. Journal of Fish Diseases, Oct .32(10):825-34. doi: 10.1111/j.1365-2761.2009.01058.x.
4. Austin B. and Austin A.D. 2016. Bacterial fish pathogens: Diseases of farmed and wild fish, Springer International Publishing, 6th edition, ISBN 978-3-319-32674-0.
5. Avci H., Aydoğan A., Tanrikul T.T., Birincioğlu S.S. 2010. Pathological and microbiological investigations in rainbow trout (Oncorhynchus mykiss Walbaum, 1792) naturally infected with Lactococcus garvieae. Kafkas Univ Vet Fak Derg 16 (Suppl-B): S313-S318, 2010 DOI:10.9775/kvfd.2010.2452
6. Buller N. 2014. Bacteria and Fungi from fish and other aquatic animals, Bentley, Western Australia: CABI Publishing, 2nd Ed, ISBN 9781845938055. doi: 10.1079/9781845938055.0000.
7. Castro R., Eguera-Brito M.R., López-Campos G.H., Blanco M.M., Aguado-Urda M., Fernández-Garayzábal J.F., Gibello A. 2017. How does temperature influences the development of lactococcosis? Transcriptomic and immunoproteomic in vitro approaches. Journal of Fish Diseases, 40, 10, 1285-1297. doi: 10.1111/jfd.12601.

8. Ceschia G., Giorgetti E., Mazzolini E., Danielis L., Passera A. 1998. Vaccination against streptococcosis in farmed rainbow trout (Oncorhynchus mykiss). Journal of Applied Ichthyology, 14:185-187.
9. CLSI. 2019. Performance Standards for Antimicrobial Susceptibility Testing. 29th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.
10. Collins M.D., Farrow J.A., Phillips B.A., Kandler O. 1983. Streptococcus garvieae sp.nov. and Streptococcus plantarum sp. nov. Journal of General Microbiology, 129, 3427–3431. doi: 10.1099/00221287-129-11-3427.
11. Diler O., Altun S., Adiloglu A.K., Kubilay A., Isikli B. 2002. First occurrence of streptococcosis affecting farmed rainbow trout (Oncorhynchus mykiss) in Turkey. Bulletin- European Association of Fish Pathologists, 22:21-26.
12. Evans J.J., Klesius P.H., Shoemaker C.A. 2009. First isolation and characterization of Lactococcus garvieae from Brazilian Nile tilapia, Oreochromisniloticus (L.), and pintado, Pseudoplathy stomacorruscans (Spix & Agassiz). Journal of Fish Diseases, 32, 943–951. doi:10.1111/j.1365-2761.2009.01075.x.
13. Eldar A. and Ghittino C. 1999. Lactococcus garvieae and Streptococcus iniae infections in rainbow trout (Oncorhynchus mykiss): two similar but different diseases. Diseases of Aquatic Organisms, 1999, 36, 227-231. doi: 10.3354/dao036227.
14. Eyngor M., Zlotkin A., Ghittino C., Prearo M., Douet D.G., Chilmonczyk S., Eldar A. 2004. Clonality and diversity of the fish pathogen Lactococcus garvieae in Mediterranean countries. Applied and Environmental Microbiology, 70 (9): 5132-5137. doi: 10.1128/AEM.70.9.5132-5137.2004.
15. Fortina M.G., Ricci G., Foschino R., Picozzi C., Dolci P., Zeppa G., Cocolin L., Manachini P.L. 2007. Phenotypic typing, technological properties and safety aspects of Lactococcus garvieae strains from dairy environments. Journal of Applied Microbiology, 103, 445-453. doi: 10.1111/j.1365-2672.2006.03265.x.
16. Foschino R., Nucera D., Volponi G., Picozzi C., Ortoffi M., Bottero M.T. 2008. Comparison of Lactococcus garvieae strains isolated in northern Italy from dairy products and fishes through molecular typing. Journal of Applied Microbiology, 105, 652-662. doi: 10.1111/j.1365-2672.2008.03780.x.
17. Gibello A, Galán-Sánchez F, Blanco M.M., Rodríguez-Iglesias M, Domínguez L, Fernández-Garayzábal J.F. 2016. The zoonotic potential of Lactococcus garvieae: an overview on microbiology, epidemiology, virulence factors and relationship with its presence in foods. Research in Veterinary

Science, 109: 59–70. doi: 10.1016/j.rvsc.2016.09.010.
18. Heras Cañas V., Pérez Ramirez M.D., Bermudez Jiménez F., Rojo Martin M.D., Miranda Casas C., Marin Arriaza M., Navarro Marí J.M. 2015. Lactococcus garvieae endocarditis in a native valve identified by MALDI-TOF MS and PCR-based 16s rRNA in Spain: A case report. New Microbes and New Infections, 5: 13–15. doi: 10.1016/j.nmni.2015.02.001 .
19. Kumar S., Stecher G., Li M., Knyaz C., Tamura K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution, 35:1547-1549. doi: 10.1093/molbev/msy096.
20. Kusuda R., Kawai K., Salati F., Banner C.R., Fryer J.L. 1991. Enterococcus seriolicida sp. nov., a fish pathogen. International journal of systematic bacteriology, 41(3):406-9. doi: 10.1099/00207713-41-3-406.
21. Meyburgh C.M., Bragg R.R., Boucher C.E. 2017. Lactococcus garvieae: an emerging bacterial pathogen of fish. Diseases of Aquatic Organisms, 123(1):67-79. doi: 10.3354/dao03083.
22. Nakai T., Sugimoto R., Park K.H., Matsuoka S., Mori K, Nishioka T., Maruyama K. 1999. Protective effects of bacteriophage on experimental Lactococcus garvieae infection in yellowtail. Diseases of Aquatic Organisms, 37, 33-41. doi: 10.3354/dao037033.
23. Palacios M.A., Zamora M.J., Vazquez J, Zamora E, Duran A. 1993. Streptococcosis in rainbow trout (Oncorhynchus mykiss) in Spain. Boll Soc It Patol Ittica, 13, 11-16.??? (časopis)
24. Pereira F., Ravelo C., Toranzo A., Romalde J. 2004. Lactococcus garvieae, an emerging pathogen for the Portuguese trout culture. Bulletin of the European Association of Fish Pathologists, 24, 274-279.
25. Raissy M. and Moumeni M. 2016. Detection of antibiotic resistance genes in some Lactococcus garvieae strains isolated from infected rainbow trout. Iranian Journal of Fisheries Sciences, 15 (1): 221-229.
26. Reimundo P., Pignatelli M., Alcaraz L.D., D'Auria G., Moya A., Guijarro J.A. 2011. Genome sequence of Lactococcus garvieae UNIUD074, isolated in Italy from a lactococcosis outbreak. Journal of bacteriology, 193: 3684–3685. doi: 10.1128/JB.05210-11.
27. Savvidis G.K., Anatoliotis C., Kanaki Z., Vafeas G. 2007. Epizootic outbreaks of Lactococcosis disease in rainbow trout, Oncorhynchus mykiss (Walbaum), culture in Greece. Bulletin- European Association of Fish Pathologists, 27: 223–228.
28. Shahi N., Mallik S.K, Sahoo M., Chandra S., Singh A.K. 2018. First report on characterization and pathogenicity study of emerging Lactococcus garvieae infection in farmed rainbow trout, Oncorhynchus mykiss (Wal-

baum), from India. Transboundary and Emerging Diseases, 65 (4): 1039-1048 doi:10.1111/tbed.12843.
29. Tejedor J.L., Vela A.I., Gibello A., Casamayor A., Domínguez L., Garayzábal J.F. 2011. A genetic comparison of pig, cow and trout isolates of Lactococcus garvieae by PFGE analysis. Letters in Applied Microbiology, 53, 6, (614-619). doi: 10.1111/j.1472-765X.2011.03153.x.
30. Vendrell D., Balcázar J.L., Ruiz-Zarzuela I., de Blas I., Gironés O., Múzquiz J.L. 2006. Lactococcus garvieae in fish: a review. Comparative Immunology Microbiology and Infectious Diseases, 29:177–198. doi: 10.1016/j.cimid.2006.06.003.

Received: 01.04.2020.
Accepted: 19.06.2020.

Original research article
UDC 598.252.3:591.69(497.113)
https://doi.org/10.46784/e-avm.v13i1.100

# THE FIRST STUDY OF THE ENDOPARASITIC FAUNA OF MUTE SWANS (*CYGNUS OLOR*) IN THE NORTHERN PART OF SERBIA

Biljana Đurđević*[1], Ivan Pavlović[2], Marko Pajić[1], Milena Samojlović[1], Miloš Pelić[1], Jelena Petrović[1], Vladimir Polaček[1]

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

## Abstract

In the Northern part of Serbia, which is part of Pannonian Basin, mute swan (*Cygnus olor*) population has notably increased over the last few decades. Like other birds from *Anatidae* family, mute swans are a host of numerous endoparasite species. The aim of this study was to acquire the data on identification and prevalence of endoparasites in mute swans in the Republic of Serbia, as that information is lacking. Individual faecal samples of sixty-eight adult mute swans were examined for the presence of endoparasites. Coprological examination was performed using flotation and sedimentation technique with saturated ZnSO4 solution. The samples were collected from December 2016 to March 2017, during epizootic of highly pathogenic avian influenza H5N8 in the Republic of Serbia. Dead mute swans were collected from twelve different locations in the Northern part of Serbia. Altogether, 39.7% of the examined fecal samples contained different parasites. The endoparasitic fauna was divers and included 3 species of nematodes, 2 cestodes species, one trematode and one protozoan species. Nematodes were the most prevalent helminthes and among these the most frequent nematode species found was *Heterakis dispar* (17.6%), followed by *Echinuria uncinata* (5,8%) and *Ascaridia* spp. (4,4%). Four mixed infections were found, where double infection was the most prevalent. No endoparasites were found in faecal samples of mute swans collected from the locations Sombor and Titel. As this study covers a small population of mute swans, more detailed studies need to be carried out on a larger population

[1]* Corresponding author: biljana@niv.ns.ac.rs

in order to gain an insight into the diversity and prevalence of endoparasites in the Republic of Serbia.

**Key words:** coprological examination, endoparasites, mute swans, nematodes

# PRVO ISTRAŽIVANJE FAUNE ENDOPARAZITA KOD LABUDOVA GRBACA (*CYGNUS OLOR*) NA PODRUČJU SEVERNOG DELA SRBIJE

Biljana Đurđević*[1], Ivan Pavlović[2], Marko Pajić[1], Milena Samojlović[1], Miloš Pelić[1], Jelena Petrović[1], Vladimir Polaček[1]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Republika Srbija
[2] Naučni institut za veterinarstvo Srbije, Beograd, Republika Srbija

## Kratak sadržaj

U severnom delu Republike Srbije koji pripada Panonskom basenu, populacija labudova grbaca (*Cygnus olor*) značajno je porasla tokom poslednjih decenija. Kao i većina drugih ptica iz porodice *Anatidae*, labudovi grbci su domaćini različitih endoparazitskih vrsta. U literaturi ne postoje podaci o endoparazitima labudova u Republici Srbiji, stoga je cilj ovog istraživanja bio identifikacija i prevalenca endoparazita kod ove vrste. Parazitološki je ispitano šezdeset osam pojedinačnih uzoraka fecesa poreklom od odraslih labudova grbaca. Koproloski pregled je izvršen metodom flotacije i sedimentacije zasićenim rastvorom ZnSO4. Uzorci su prikupljeni tokom epizootije visoko patogene avijarne influence H5N8 u Republici Srbiji, u periodu od decembra 2016. do marta 2017. godine. Uginuli labudovi prikupljeni su sa dvanaest različitih lokacija u severnom delu Srbije. Različite parazitske vrste detektovane su u 39.7% pregledanih uzoraka fecesa. Identifikovane su tri vrste nematoda, dve vrste cestoda, jedna vrsta trematoda i jedna vrsta protozoa. Nematode su utvrđene kao helminti sa najvećom prevalencom, a najučestalije detektovane vrste bile su *Heterakis dispar* (17.6%), zatim *Echinuria uncinata* (5.8%) i *Ascaridia* spp. (4.4%). Utvrđene su četiri mešovite infekcije, gde je dvostruka infekcija bila najčešći nalaz Paraziti nisu detektovani iz uzoraka fecesa poreklom od labudova sa lokacija Sombor i Titel. Uzimajući u obzir činjenicu da je u ovom

istraživanju obuhvaćena mala populacija labudova grbaca u Republici Srbiji, potrebno je sprovesti detaljnije studije na većoj populaciji, kako bi se dobio sveobuhvatniji uvid u raznolikost i rasprostranjenost endoparazita.

**Ključne reči**: koprološki pregled, endoparaziti, labudovi grbci, nematode

## INTRODUCTION

It is known than mute swan (*Cygnus olor,* Gmelin, 1789) population started to colonize wetlands in Northern parts of Serbia in the early 1980s (Hulo, 1997). Today there is no precise data about the recent size of the mute swan population, but it is known that in the last three decades they mostly overwinter in Bačka Podunavlje, Northern part of Serbia. Also, there is no detailed survey on all breeding sites or the number of breeding pairs in whole country. However, it is known that the mute swan population in the Republic of Serbia has increased during the last three decades (Tucakov, 2005).

Mute swans are large herbivorous birds, belonging to Anseriformes order, Anatidae family. The Anseriformes order is primarily associated with water and wetland habitats, while the Anatidae are typical waterfowl. Waterfowl characteristic to aggregate in large numbers during breeding season or winter migration can lead to the transfer of disease-causing organisms, including different parasites. Parasitic diseases of waterfowl are common, but they do not cause high mortality rate. However, parasites can contribute to increased mortality in cases of other disease outbreaks (Olsen, 2009).

It is shown that diet and feeding habits play a key role to the parasite fauna in birds. Also, habitat can play an important role in creating parasite assemblages in birds. Some host species that have wider geographic distribution tend to have more diverse parasite community structure, including waterfowl and migratory birds, which distinguish them from non-migratory bird species (Koprivnikar and Leung, 2015). However, it is still not clear which factors most significantly affect the diversity and composition of parasite assemblages in migratory birds and whether this distinguishes them from non-migratory birds (Leung and Koprivnikar, 2016). In Europe, the three different swan species can be found: Mute Swan (*Cygnus olor*), Whooper Swan (*Cygnus cygnus*) and Bewick's Swan (*Cygnus columbianus bewickii*). Out of the three European swan species, mute swans are the most sedentary, and their movements rarely

exceed tens of kilometres (Scott and Rose, 1996; Waldenström et al., 2017).

To our knowledge, there are no literature data about the biodiversity of parasites in mute swans in the Republic of Serbia. Considering its partially migratory behaviour and their strong association with aquatic habitats, we found it very important to establish the extensity and prevalence of endoparasitic infections in mute swan populations in Northern part of Serbia.

## MATERIAL AND METHODS

Uncommon high morbidity and mortality of mute swans were observed in Northern part of Serbia between December 2016 and March 2017. During that period, dead mute swans were collected from twelve different locations (Figure 1) and submitted to the Scientific Veterinary Institute „Novi Sad", in order to determine the cause of death. Most of dead mute swans were found along the shores of Danube armlet. These areas are characterized by shallows and inlets of the Danube River, which are a temporary home for thousands of migratory and resident aquatic birds. All swans were examined by a full necropsy according to a standard protocol and gross lesions were recorded. Due to the emergence of highly pathogenic avian influenza H5N8 outbreaks in the autumn of 2016 in most European countries, as well as a large percentage of mortality in wild aquatic birds, there was a suspicion of this disease. Different tissue samples (lungs, heart, kidney, intestine, spleen, pancreas and brain) were collected from each bird for detection of avian influenza virus and other differential laboratory investigations including histopathology and parasitology. The gastro-intestinal tracts of all 68 mute swans were removed and cut into parts. The intestines and gizzards were further carefully slit open and examined in detail. Faecal samples from distal part of the intestine of each bird were collected, labelled, placed in clean plastic containers and stored until use at 4°C. Coprological examination was performed in parasitology laboratory at Institute of Veterinary Medicine of Serbia, Belgrade using flotation and sedimentation technique with saturated ZnSO4 solution. Adult parasites were examined using light microscopy after being clarified in lactophenol. The identification of the parasites was based on morphological criteria and was carried out following the keys of Anderson (2000), Cole and Friend (1999), Taylor et al., (2007) and Rysavy and Cerna (1988).


Figure 1. Geographical distribution of tested mute swans in the Northern part of Serbia.

## RESULTS

All dead mute swans were found to be positive for the highly pathogenic avian influenza strain (HPAI) H5N8. Generally, all swans were in good body condition during necropsy, with thick subcutaneous and cavitary fat, with mild or no external lesions, which is considered normal in the winter season. Gross pathology included hyperemia, hemorrhages, necrosis in most of visceral organs and the lesions were characteristic for HPAI infection (Božić et al., 2016; Božić et al., 2018).

Out of 68 mute swans examined, infection with endoparasites occurred in 39.7% of mute swans (27/68). The eggs of the following helminths classes were detected: Cestodes, Nematodes, Trematodes and one protozoan species. Four mixed infections were established, where double infection was the most

prevalent and detected in 19.1% of the examined swans. Among the observed helminthes, the predominant parasites were nematodes. Out of the three identified nematode, *Heterakis dispar* was the most prevalent, found in 12 birds (17.6%), followed by *Echinuria uncinata* in 4 birds (5.8%) and *Ascaridia* spp. in 3 birds (4.4%). Two cestoda were identified: *Dilepis undula* in one mute swan (1.4%), and *Cladogynia bulbocirrosus* in 3 birds (4.4%). One trematode species was found, *Apatemon gracilis* in one swan (1.4%). Finally, only one protozoan parasite was found – renal coccidia *Eimeria christianseni* in 3 examined mute swans (4.4%). No intestinal parasites were found in faecal samples of mute swans collected from Sombor and Titel. The parasites found in mute swans and their prevalence is presented in the Table 1.

Table 1. Extensity and prevalence of endoparasites of tested mute swans in the Northern part of Serbia.

| Location | Number of birds examined | Parasite | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cestodes | | Nematodes | | | Trematodes | Protozoa |
| | | ***Dilepis undula*** | ***Cladogynia bulbocirrosus*** | ***Echinuria uncinata*** | ***Heterakis dispar*** | ***Ascaridia* spp.** | ***Apatemon gracilis*** | ***Eimeria christianseni*** |
| Zasavica | 1 | 1/1 | - | - | - | - | - | - |
| Titel | 1 | - | - | - | - | - | - | - |
| Kanal DTD | 17 | - | - | 1/17 | 2/17 | - | - | - |
| Žabalj | 8 | - | - | - | - | 1/6 | - | - |
| Futog | 3 | - | 2/3 | - | 2/3 | - | - | - |
| Palić | 10 | - | - | - | 4/10 | - | - | - |
| Bač | 3 | - | 1/3 | - | - | - | 1/3 | - |
| Kovilj | 10 | - | - | 2/10 | 3/10 | 1/10 | - | - |
| Srbobran | 1 | - | - | - | - | 1/1 | - | - |
| Višnjićevo | 3 | - | - | - | 1/3 | - | - | - |
| Sombor | 2 | - | - | - | - | - | - | - |
| Beograd | 7 | - | - | 1/7 | - | - | - | 3/7 |
| **Total** | **68** | **1** | **3** | **4** | **12** | **3** | **1** | **3** |

(-) – not detected

## DISCUSSION

Free-ranging wild birds, primarily migratory birds, are capable of transmitting parasitic diseases to greater geographical distances, due to their interference with other non-migratory birds. Mute swans in this study were collected from wintering areas for migratory birds, so the cohabitation with these birds presumably contributes to the composition and structure of the parasitic fauna. Research on parasitic fauna of mute swans is mostly conducted in Europe, New Zealand, and Canada (Papazahariadou et al., 1994; Papazahariadou et al., 2008; Pennycott, 1998; Manno et al., 2016; Seegar 1979; Jennings et al., 1961; Sanford, 1978). There are some studies in the Republic of Serbia regarding parasitological examinations in zoo birds of the Anatidae family (Ilić et al., 2018), but to our knowledge, there is no literature data regarding parasitic fauna of mute swans. This report is the first description of endoparasitic fauna in mute swans located in the Northern part of Serbia.

A total of 39.7% of mute swans in this study were positive for parasite infection and nematodes were the most prevalent helminthes. Detected parasitic helminths were from Cestodes, Nematodes and Trematodes classes, and only one protozoan species was diagnosed - renal coccidia *Eimeria christianseni*. Few birds were harboring multiple nematode species and among them a species from Heterakidae family, *Heterakis dispar*, was the most commonly found (17.6%). The same nematode species was detected in small intestine of black necked swans from southern America (Gonzalez-Acuna et al., 2010). Adult worms of the *Heterakis* genus generally live in the lumen of the ceca of birds. Three species are known to be prominent in poultry: *H. gallinarum, H. dispar*, and *H. isolonche* (Park and Shin, 2010), but some species from *Heterakis* spp. genus such as Dalmatian Pelican, Grey Heron, Sea Gulls and Little Owl are also found in wild birds (Papazahariadou et al., 2008). According to the data from Serbian zoos, heterakiosis was diagnosed in 12.74% and 2.56% of the captive birds examined mainly as mixed infection with coccidiosis, capillariosis, askaridiosis and trichostrongylidosis (Ilić et al., 2018).

The nematode *Echinuria uncinata* was detected in 5.8% of mute swans. This nematode was reported earlier in mute swans originating from Northern Greece (Papazahariadou et al. 1994) as well as in another Greek study (Papazahariadou et al., 2008). When *E. uncinata* was first recorded in New Zealand (Clark, 1979), its pathogenicity was indicated and it is regarded as the most devastating parasite of waterfowl in Russia. In general, this species is considered to be highly pathogenic to anatid birds (Silveira et al., 2006). However, in the present work, such condition was not observed and overall prevalence was low.

In the current study, other detected nematode included undefined species from genera *Ascaridia* spp. in 4.4% of mute swans. Ascarids do not normally cause severe pathogenic effects. However, they can cause clinical disease and even death in a case of high intensity of infection (Papini et al., 2012). Ascaridiosis were diagnosed with the overall prevalence of 10.78% and 10.25% in captive birds from Serbian zoos (Ilić et al., 2018), as well as 6.8% of examined birds from Italian zoo (Papini et al., 2012).

Literature data confirms findings of many other nematodes in mute swans and other wild swans that were not diagnosed in our research, comprising species from *Amidostomum* genera (*Amidostomum anseris*), *Capillaria* (*Baruscapillaria obsignata*, *Eucoleus dispar*) (Papazahariadou et al., 2008; Tamaru et al., 2015; MacNeill, 1970), *Acuaria* (*Acuaria uncinate*), and *Tetrameres* (*Tetrameres fissispina*) (Jennings et al., 1961).

In the current study, two cestoda species were identified: *Dilepis undula* in one mute swan and *Cladogynia bulbocirrosus* in 3 examined swans (4.4%). Typically, *Dilepis undula* is a parasite of passeriform birds. Some authors recorded it in blackbirds (*Turdus merula*, *Turdus viscivorus*), hooded crows (*Corvus cornix*), Euro-Asian sparrows (*Passer montanus*) and starlings (*Sturnus vulgaris*) in southern Bulgaria (Marinova et al., 2013). It was also identified in blackbirds in Poland (Rzad et al., 2014).

The cestode *Cladogynia bulbocirrosus* was detected in 3 examined swans (4.4%). To our knowledge, the only literature data on the prevalence of this cestode species in swans was reported in three black-necked swans (*Cygnus melancoryphus*) in Vienna Zoological Gardens (Gonzalez-Acuna et al., 2010). According to the data from Egypt, species from *Cladogynia* genus - *Cladogynia phoeniconaiadis* were reported in ducks in low prevalence (2.7%) (Aboulaila et al., 2011). Also, cestodes of the *Cladogynia* genus were found incidentally in free-ranging flamingos in Mexico (Aguirre et al. 1991) and low prevalence was recorded in birds in *Phoenicopteridae* family (Papazahariadou et al., 2008).

In mute swans from this study, only one trematode species was detected - *Apatemon gracilis* in one swan. *Apatemon gracilis* is an intestinal trematode that was frequently reported in ducks in certain geographic areas of Europe and it was also found in the intestine of various wild birds. Ducks are its main hosts (Liu et al., 2018). The life cycle of this fluke requires two intermediate hosts: the first is a freshwater snail and the second includes frogs and freshwater fish in addition to snails. A final host is infected by feeding on the second intermediate host containing metacercariae. The low prevalence of this fluke in our study may be related to cold winter, bad weather conditions and probably absence of intermediate hosts. Reports of trematode-induced mortalities of

swans are scarce. Some authors described lethal ulcerative hemorrhagic enteritis in mute swans caused by some trematode species such as *Echinoparyphium recurvatum* and *Sphaeridiotrema globules* (Roscoe and Huffman, 1982).

As for protozoan species, one species was diagnosed - renal coccidia *Eimeria christianseni*. Low prevalence of renal coccidia could probably be explained due to a small number of mute swan kidneys examined (10.3% of the tested swans). Renal coccidiosis is caused by protozoal parasites that infect the kidneys and associated tissues. Most of the coccidia that infect the tissues in most bird species belong to *Eimeria* spp. As with most other parasitic infections, this infection is not synonymous with clinical or apparent disease. Asymptomatic infections are far more common than those that are severe and cause mortality (Cole and Friend, 1999). However, in some cases these parasites can cause serious health problems (Giacomo et al. 1997; Pennycott et al. 1998).

## CONCLUSION

The present survey on endoparasitoses is a valuable initial research work which gives an insight into the endoparasitic fauna of mute swans in the Republic of Serbia. Even though the assessment included statistically a small number of animals, it showed that the endoparasitic infections are present in the Northern part of Serbia, and that they are diverse and include nematodes, cestodes, trematodes and protozoa. The results will surely be valuable for preparing further research plans in this field.

## ACKNOWLEDGEMENT

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Projects number TR 31011, TR 31084 and TR31071.

## Author's contributions:

BĐ drafted the manuscript, carried out literature research, performed necropsy and sample collection; IP carried out the parasitological examination; MP participated in the design of the study; MS, MP and JP did the reviewing, editing and supervision; VP revised manuscript critically and gave the final approval of the version to be published.

## Competing Interests

Authors declared no conflict of interests regarding the present paper.

## REFERENCES

1. Aboulaila M., El-Bahy N., Hilali M., Yokoyama N., Igarashi I. 2011. Prevalence of the enteric parasites of ducks from Behera governorate, Egypt. The journal of protozoology research, 21, 2, 36-44. doi: 10.32268/jprotozoolres.21.2_36.
2. Aguirre A.A., Cook R.S., McLean R.G., Quan T.J., Spraker T.R. 1991. Occurrence of potential pathogens in wild Caribbean flamingos (Phoenicopterus ruber ruber) during a lead poisoning die off in Yucatan, Mexico. Journal of Zoo and Wildlife Medicine, 22, 4, 470–475.
3. Anderson R. C. 2000. Order Ascaridida. In Nematode parasites of vertebrates, their development and transmission. 2nd edition, Cabi, Wallingford, UK, 245-348. doi: 10.1079/9780851994215.0001.
4. Božić B., Pajić M., Petrović T., Pelić M., Samojlović M., Polaček V. 2016. Pathologic changes in swans infected with highly pathogenic avian influenza (H5N8) virus. Archives of Veterinary Medicine, 9, 2, 77-86. doi:10.46784/e-avm.v9i2.91.
5. Božić B., Polaček V., Vučićević I., Vidanović D., Vasković N., Prodanov-Radulović J., Aleksić-Kovačević S. 2018. Morphological differences of pancreatic lesions in mute swans and hens naturally infected with highly pathogenic avian influenza virus H5N8. Acta Veterinaria-Beograd, 68, 2, 217-223. doi: 10.2478/acve-2018-0018.
6. Clark W.C. 1979. Echinuria australis n. sp. and E. uncinata (Nematoda: Spirurida), parasites of ducks in New Zealand. New Zealand journal of zoology, 6, 1, 7-12. doi: 10.1080/03014223.1979.10428343.
7. Cole R. A. and Friend M. 1999. "Parasites and Parasitic Diseases (Field Manual of Wildlife Diseases)". Other Publications in Zoonotics and Wildlife Disease. 15. https://digitalcommons.unl.edu/zoonoticspub/15.
8. Giacomo R., Stefania P., Ennio T., Giorgina V.C., Giovanni B., Giacomo R. 1997. Mortality in black siskins (Carduelis atrata) with systemic coccidiosis. Journal of Wildlife Diseases, 33, 1, 152-157. doi: 10.7589/0090-3558-33.1.152.
9. Gonzalez-Acuna D., Moreno L., Cicchino A., Mironov S., Kinsella, M. 2010. Checklist of the parasites of the black-necked swan, Cygnus melanocoryphus (Aves: Anatidae), with new records from Chile. Zootaxa, 2637, 55–68. doi: 10.11646/zootaxa.2637.1.3.

10. Hulo I. 1997. Migration of birds from the families Gaviidae, Podicipitidae and Anatidae on the lake Palic between 1981 and 1996. Ciconia, 6, 51-72.
11. Ilić T., Becskei Z., Gajić B., Özvegy J., Stepanović P., Nenadović K., Dimitrijević S. 2018. Prevalence of endoparasitic infections of birds in zoo gardens in Serbia. Acta parasitologica, 63, 1, 134-143. doi: 10.1515/ap-2018-0015.
12. Jennings A.R., Soulsby E.J.L., Wainwright C.B. 1961. An outbreak of disease in mute swans at an Essex Reservoir. Bird Study, 8, 1, 19-24. doi: 10.1080/00063656109475984.
13. Koprivnikar J. and Leung T.L.F. 2015. Flying with diverse passengers: greater richness of parasitic nematodes in migratory birds. Oikos, 124, 4, 399–405. doi: 10.1111/oik.01799.
14. Leung T.L.F. and Koprivnikar J. 2016. Nematode parasite diversity in birds: the role of host ecology, life history and migration. Journal of Animal Ecology, 85, 6, 1471-1480. doi: 10.1111/1365-2656.12581.
15. Liu D., Zhuo Z., Tao J., Xu J. 2018. A case report of Apatemon gracilis (Szidat, 1928) infection in domestic geese in mainland China. Turkish Journal of Veterinary and Animal Sciences, 42, 2, 139-142. doi: 10.3906/vet-1709-22.
16. MacNeill A.C. 1970. Amidostomum anseris infection in wild swans and goldeneye ducks. The Canadian Veterinary Journal, 11, 8, 164-167.
17. Manno C., Blake D., Ghisleni G., Tecilla M., Macaluso G., Puleio R., Monteverde V., Loira, G.R. 2016. Cardiac filariosis in migratory mute swans (Cygnus olor) in Sicily. International Journal of Health, Animal Science and Food Safety, 3, 1, 20–27. doi: 10.13130/2283-3927/6796.
18. Marinova M.H., Georgiev B.B., Vasileva G.P. 2013. Checklist of Cestodes (Platyhelminthes: Cestoda) of Waterfowl (Aves: Anseriformes) in Bulgaria. Acta zoologica Bulgarica, 65, 4, 537-546.
19. Olsen G.H. 2009. Bacterial and parasitic diseases of Anseriformes. Veterinary Clinics: Exotic Animal Practice, 12, 3, 475-490. doi: 10.1016/j.cvex.2009.07.004.
20. Papazahariadou M, Georgopoulou J, Jordanides P, Antoniadou-Sotiriadou K. 1994. Incidents of death in swans (Cygnus olor). Bulletin of the Hellenic Veterinary Medical Society 45:51–54
21. Papazahariadou M., Diakou A., Papadopoulos E., Georgopoulou I., Komnenou A., Antoniadou-Sotiriadou K. 2008. Parasites of the digestive tract in free-ranging birds in Greece. Journal of Natural History, 42, 5-8, 381-398. doi: 10.1080/00222930701835357.
22. Papini R., Girivetto M., Marangi M., Mancianti F., Giangaspero, A. 2012. Endoparasite infections in pet and zoo birds in Italy. The scientific world journal, 2012, Article ID 253127, doi: 10.1100/2012/253127.

23. Park S.I. and Shin S.S. 2010. Concurrent Capillaria and Heterakis infections in zoo rock partridges, Alectoris graeca. The Korean journal of parasitology, 48, 3, 253-257. doi: 10.3347/kjp.2010.48.3.253.
24. Pennycott T.W. 1998. Lead poisoning and parasitism in a flock of mute swans (Cygnus olor) in Scotland. Veterinary record, 142, 1, 13-17. doi: 10.1136/vr.142.1.13.
25. Pennycott T.W., Ross H.M., McLaren I.M., Park A., Hopkins G.F., Foster G. 1998. Causes of death of wild birds of the family Fringillidae in Britain. Veterinary Record, 143, 6, 155–158. doi: 10.1136/vr.143.6.155.
26. Roscoe D.E. and Huffman J.E. 1982. Trematode (Sphaeridiotrema globulus)-induced ulcerative hemorrhagic enteritis in wild mute swans (Cygnus olor). Avian Diseases, 26, 1, 214-224. doi: 10.2307/1590046.
27. Rysavy B. and Cerna Z. 1988. First record of Eimeria christianseni Walden, 1961 in swans in Czechoslovakia. Folia Parasit 35,3, 280.
28. Rzad I., Sitko J., Salamatin R., Wysocki D. 2014. Helminth community structure study on urban and forest blackbird (Turdus merula L.) populations in relation to seasonal bird migration on the south Baltic Sea coast (NW Poland). Helminthologia, 51, 2, 117-129. doi: 10.2478/s11687-014-0219-6.
29. Sanford S.E. 1978. Mortality in mute swans in Southern Ontario associated with infestation with the thorny-headed worm, Polymorphus boschadis. The Canadian Veterinary Journal, 19, 8, 234-236.
30. Scott D.A. and Rose P.M. 1996. Atlas of Anatidae populations in Africa and Western Eurasia. Wetlands International Publication No 41, Wetlands International, Wageningen, The Netherlands, ISBN: 1900442108.
31. Seegar W.S. 1979. Prevalence of parasitic heartworms in swans in England. Wildfowl, 30, 30, 147-150.
32. Silveira E.F.D., Amato J.F., Amato S.B. 2006. Echinuria uncinata (Rudolphi) (Nematoda, Acuariidae) em Netta peposaca (Vieillot) (Aves, Anatidae) na América do Sul. Revista Brasileira de Zoologia, 23, 2, 520-528. doi: 10.1590/S0101-81752006000200027.
33. Tamaru M., Yamaki S., Jimenez L.A., Sato H. 2015. Morphological and molecular genetic characterization of three Capillaria spp. (Capillaria anatis, Capillaria pudendotecta, and Capillaria madseni) and Baruscapillaria obsignata (Nematoda: Trichuridae: Capillariinae) in avians. Parasitology research, 114, 11, 4011-4022. doi: 10.1007/s00436-015-4629-2.
34. Taylor M.A., Coop R.L., Wall R.L. 2007. The laboratory diagnosis of parasitism In Veterinary parasitology. 3rd edition, Blackwell Publishing Ltd, Oxford, UK, 1825-1920.

35. Tucakov M. 2005. Numbers and seasonal activity of the mute swan (Cygnus olor) on the Kolut fishpond (NW Serbia). Ring, 27, 2, 221-226. doi: 10.2478/v10050-008-0024-z.
36. Waldenström J., Kuiken T., Wille, M. 2017. Narrative overview on wild bird migration in the context of highly pathogenic avian influenza incursion into the European Union. EFSA Supporting Publications, 14(10), 1283E. doi:org/10.2903/sp.efsa.2017.EN-1283

Received: 22.04.2020
Accepted: 16.06.2020.

Original research article

UDC 636.4.09:578.833.3
https://doi.org/10.46784/e-avm.v13i1.235

# CLASSICAL SWINE FEVER VIRUS DETECTION IN FETAL SWINE TISSUES BY IMMUNOHISTOCHEMISTRY

Vladimir Polaček*[1], Biljana Đurđević[1], Tamaš Petrović[1], Jasna Prodanov-Radulović[1], Milena Samojlović[1], Ivana Vučićević[2], Sanja Kovačević-Aleksić[2]

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

## Abstract

The classical swine fever virus has the ability to cross the placental barrier, resulting in the infection of fetuses, which may consequently lead to persistent infection in piglets. The aim of this study was to report the lesions in fetuses naturally infected with CSFV during late gestation and clarify the nature of infected cells and the distribution of viral antigen in different tissues. A total of twenty-nine fetuses aged 82, 83 and 95 gestational days originating from three naturally CSFV infected sows were examined in this study. In all tested sows and their fetuses CSFV was detected using RT-PCR method. Immunohistochemistry method was used to detect viral antigen and monoclonal antibody WH303 was used on formalin fixed tissue samples of brain, spleen, heart, tonsils, kidney, ileocecal valve and umbilical cord. The most common lesions in the majority of fetuses were hyperemia, petechial haemorrhages in the skin, lymph nodes and kidneys. With the exception of myocardium, CSF viral antigen was detected in all the examined tissues. WH303 positive cells included endothelial cells, monocytes, macrophages and lymphocytes. The largest number of positive cells was found in kidneys in all of the examined fetuses. Reticular cells, macrophages, lymphocytes and endothelial cells in the spleen were also intensely and widely stained in most of the fetuses. These results showed that CSFV antigen can be detected in formalin-fixed, paraffin-embedded fetal tissue specimens originating from naturally CSFV infected sows by using monoclonal antibody WH303. Fetal kidneys proved to be a very useful organ for diag-

[1]* Corresponding author: vlade@niv.ns.ac.rs

nosis of the CSF virus. Having that in mind, it is assumed that persistently infected piglets may shed a high amount of viral particles through urine. However, further research is needed to confirm this hypothesis.

**Key words:** classical swine fever virus, fetuses, kidney, immunohistochemistry

# DETEKCIJA VIRUSA KLASIČNE KUGE SVINJA U FETALNIM TKIVIMA PRASADI PRIMENOM IMUNOHISTOHEMIJSKE METODE

Vladimir Polaček*[1], Biljana Đurđević[1], Tamaš Petrović[1], Jasna Prodanov-Radulović[1], Milena Samojlović[1], Ivana Vučićević[2], Sanja Kovačević-Aleksić[2]

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

## Kratak sadržaj

Virus klasične kuge svinja poseduje mogućnost prelaska placentarne barijere, što može dovesti do infekcije fetusa i posledično do nastanka perzistentne infekcije kod prasadi. Cilj ovog istraživanja bio je utvrđivanje lezija koje nastaju kod fetusa prirodno inficiranih virusom klasične kuge svinja tokom kasne faze gestacije, kao i prirodu inficiranih ćelija i distribuciju virusnog antigena u različitim tkivima fetusa. Ukupno je ispitano dvadesetdevet fetusa starosti 82, 83 i 95 dana gestacije, poreklom od tri prirodno inficirane krmače virusom klasične kuge svinja. Prisustvo virusa potvrđeno je kod svih ispitanih krmača i njihovih fetusa upotrebom RT-PCR metode. Za imunohistohemijsku detekciju virusnog antigena u tkivnim isečcima mozga, slezine, srca, tonzila, bubrega, ileoceklane valvule i pupčane vrpce primenjeno je monoklonko antitelo WH303. Kod većine ispitanih fetusa ustanovljena je hiperemija i petehijlna krvavljenja na koži, limfnim čvorovima i bubrezima. Virusni antigen je detektovan u svim ispitanim tkivima fetusa, izuzev tkiva srca. Detektovane WH303 pozitivne ćelije obuhvatale su endotelne ćelije, monocite, makrofage i limfocite. Najveći procenat pozitivnih ćelija na virusni antigen utvrđen je u bubrezima kod svih ispitanih fetusa. Pored toga, veliki broj pozitivnih ćelija dokazan je u

retikularnim, limfoidnim i endotelnim ćelijama slezine kod većine fetusa. Rezultati dobijeni u ovom istraživanju pokazuju da se upotrebom monoklonskog antitela WH303 može detektovati antigen virusa klasične kuge svinja u parafinskim isečcima tkiva fetusa prasadi poreklom od prirodno inficiranih krmača. Pored toga, utvrđeno je da su fetalni bubrezi veoma pogodan materijal za dijagnostiku virusa klasične kuge svinja. Na osnovu ovih nalaza postavljena je hipoteza da perzistentno inficirana prasad mogu izlučivati velike količine virusnih čestica putem urina, međutim, potrebna su dodatna istraživanja kako bi se potvrdila ova hipoteza.

**Ključne reči**: virus klasične kuge svinja, fetusi, bubreg, imunohistohemija

## INTRODUCTION

Classical swine fever (CSF) is one of the most important viral diseases in domestic pigs and wild boars (Blome et al., 2017). The causative agent, *Classical swine fever virus* (CSFV), belongs to the *Pestivirus* genus within the *Flaviviridae* family (Edwards et al., 2000). Other members of this genus are *Bovine viral diarrhea virus 1* and *2* (BVDV-1 and BVDV- 2), *Border disease virus* (BDV) and a growing number of unclassified and so-called atypical pestiviruses (Blome et al., 2017). The disease has a severe socio-economic impact on industrial pig production, small-scale pig keepers and tremendous impact on animal health and is therefore notifiable to the World Organization for Animal Health (OIE) (Edwards et al., 2000).

Different forms of the disease have been observed, ranging from subacute, acute or chronic forms, with lesions varying from mild to severe. In general, infections with more virulent strains typically result in acute hemorrhagic disease while infections with less virulent strains usually lead to chronic or subclinical forms (Muñoz-González et al., 2015). In countries with endemic outbreaks, like Serbia, the disease usually has an acute or subacute course, with classical clinical signs of infection in unvaccinated population (Prodanov-Radulović et al., 2014). When infection occurs during pregnancy, the virus can also infect the fetus due to its ability to pass the placental barrier which in turn might lead to persistent infection in piglets (Van Oirschot and Terpstra, 1977). The outcome of transplacental infection depends on many factors, including the stage of gestation and virulence of the virus. It is known that infection during early pregnancy leads to abortions, mummification and

stillbirth. However, when infection occurs in the second or third trimester of pregnancy, especially between 50$^{th}$ and 70$^{th}$ gestational day, an immunotolerance phenomenon can be induced and persistently infected offspring are born (Moennig et al., 2003). Those piglets seem to be healthy and could survive for several months but they eventually die due to the so-called „late onset" form of the CSF. During that period, they shed high viral loads which are sufficient for transmission (Cabezón et al., 2017). This may cause an uncontrolled spread of CSF and significant losses in large swine population. Due to uncharacteristic profiles of CSF clinical symptoms in pregnant sows, this may lead to delayed identification of potential new sources of infection. Therefore, very mild clinical symptoms might easily be overlooked (the so-called "carrier-sow syndrome"). This has an impact on the efficacy of vaccines and may complicate control in endemically affected countries (Blome et al., 2017). In the literature, there is little information about the pathogenesis of the transplacental infection detected on the field, during eradication campaign. Most data regarding transplacental virus transmission are obtained under experimental conditions (Muñoz-González et al., 2015; Dewulf et al., 2011; Van Der Molen and Van Oirsch, 1981; Von Benten et al., 1980).

Considering the significance of the transplacental infection and possible persistent infection in offspring regarding the disease control and eradication, the aims of this study were the following: reporting the lesions in naturally infected fetuses with CSFV; detecting CSFV in formalin-fixed, paraffin-embedded fetal tissues by immunohistochemistry, as well as to determining the tissue and cellular distribution of CSFV in pig fetuses to better understand the pathogenesis of naturally occurring disease.

## MATERIAL AND METHODS

### *Material*

Twenty-nine fetuses originating from three sows naturally infected with CSFV were morphologically and immunohistochemically examined in this study. All fetal samples were collected during big CSF outbreaks in Serbia, in 2006. Veterinary regulatory measures included stamping out policy of all affected pigs on farms and backyard productions. All sows were of landrace breed, non-vaccinated, reared in rural backyard holdings and were randomly chosen. Blood samples were taken from each sow for viral detection. All sows were virologically negative for African swine fever, porcine reproductive respiratory syndrome, and Aujeszky's disease. Fetuses were obtained by removing the uterus from each sow after euthanasia. The first sow was in the 83$^{rd}$ day

of gestation and had 6 fetuses; the second sow was in 82nd day of gestation and had 11 fetuses, and finally the third sow was in 95th day of gestation with 12 fetuses. Necropsies of all fetuses were performed and gross lesions were recorded. From each swine fetus, the following organs were sampled: brain, kidney, spleen, umbilical cord, intestine, tonsils, ileocecal valve and heart. Tissue samples of kidney, spleen and tonsils were pooled for molecular detection of CSF viral RNA. All tissue samples were fixed in 10% neutral buffered formaldehyde for 48 hours for immunohistochemistry, and embedded in paraffin according to standard laboratory procedures. The samples from four CSF negative pig fetuses were used as control. Tissue samples from two 45-days-old pigs naturally infected with CSFV were used as positive controls.

## *Methods*

### *Detection of CSFV RNA genome*

Conventional, gel based reverse transcriptase-polymerase chain reaction (RT-PCR) test was applied to detect genomic RNA of CSFV in unclotted blood of sows and fetal tissue samples. Total RNA was extracted by TRIzol® reagent (ThermoFisher Scientific, USA) according to manufacturer's instruction. Briefly, 750 µl of TRIzol® reagent was mixed with 250 µL of sample. After 10 min, 200 µL of chloroform was added, mixed and the suspension was centrifuged for 15 min at 14,000 g at 4°C. The RNA containing aqueous phase was removed and precipitated with 500 µL of isopropanol, maintained at room temperature for 10 min, and centrifuged for 10 min at 14,000 g. The RNA pellet was washed with 500 µL of 75% cold ethanol, centrifuged for 5 min at 14,000 g, then dried and resuspended in 50 µL of PCR clean water, and stored at -70°C until examination or was immediately included in RT-PCR. The obtained RNA extracts were further amplified by using primers for E2 region of the CSFV genome gp55-U: 5'-ATA TAT GCT CAA GGG CGA GT-3' (sense, position in genome of the Alfort strain is 3378-3397) and gp55-L: 5'-ACA GCA GTA GTA TCC ATT TCT TTA-3' (antisense, position in genome of the Alfort strain is 3685-3662) described by Katz et al. (1993). One-step RT-PCR amplification was done using commercial kit Qiagen OneStep RT-PCR kit chemistry (QIAGEN, Germany) according to manufacturer's instruction, with small modifications. Briefly, the amplification reaction was carried out at a volume of 25 µL containing 13.5 µL of nuclease-free water, 5 µL of 5 x PCR buffer, 1 µL of dNTP mix (containing 10 mM of each dNTP), 0.25 µL of stock solution of 100 µM of each primer, 1 µL of one step RT-PCR enzyme mix and 4 µL of RNA template. As a positive control in reaction, Alfort 187 strain of

CSFV was used. The amplification conditions (Thermocycler Gradient, Eppendorf, Germany) were as follows: reverse transcription stage at 50°C for 30 min, followed by an initial PCR activation step at 95°C for 15 min, 40 cycles of 95°C for 30 s, 55°C for 60 s, 72°C for 60 s, and a final extension at 72°C for 10 minutes. Amplified products were detected and visualized by electrophoresis on 1.5 agarose gel stained with ethidium bromide.

### *Immunohistochemistry*

To demonstrate CSFV envelope glycoprotein E2 in fetal tissues, the commercially available monoclonal mouse anti-CSFV antibody WH303 (APHA Scientific, UK; catalogue number RAE0826) was used on fixed samples. IHC staining kit applied in this study was Novolink Polymer Detection Systems, Novocastra (Leica Biosystems, USA). Tissue sections were dewaxed and rehydrated in xylene and graded series of alcohol. Antigen retrieval was achieved by heating the sections in a microwave oven (560W) for 21 minutes in a citric buffer (pH 6.0), as previously described by Polaček et al., (2007). Endogenous peroxidase activity was abolished by incubation of the sections with "Peroxidase block" for 5 minutes at room temperature. After two washes in PBS for 5 minutes, all slides were incubated with "Protein block" for 5 minutes. After this step, all slides were washed two times in PBS for 5 minutes and incubated overnight at 4°C in a humidified chamber with primary antibody diluted 1:50 in PBS. After incubation with primary antibody overnight, the slides were given two 5-minute rinses with PBS. "Post-primary antibody" was applied like the secondary antibody for 30 minutes at room temperature. After two washes in PBS for 5 minutes, all slides were incubated with "Novolink™ Polymer" for 30 minutes. Then, all tissue sections were rinsed two times with PBS, and gently rocked. The final reaction was produced by incubating the sections with DAB working solution (50µL of DAB Chromogen was added to 1ml of Novolink™ DAB Substrate Buffer) for 5 minutes. After that, all slides were rinsed in tap water, and counterstained with Mayer's haematoxylin. PBS was used in place of specific primary antibodies as negative controls.

## RESULTS

### *Results of molecular testing*

The results of RT-PCR test confirm that the RNA of the CSFV genome was detected in all blood samples of sows and in all tested fetal tissue samples.

### *Gross lesions*

Some of the detected gross lesions were characteristic of CSF, but not present in all examined fetuses. The most common finding in the majority of fetuses was hyperemia, and it was seen in the skin, submandibular lymph nodes, spleen, tonsils, and the brain (Figure 1a). Petechiae were observed in the skin of the dorso-lateral aspect of neck and abdomen in 19 fetuses (Figure 1b). Some fetuses had petechial haemorrhages in the renal cortex and urinary bladder. In few cases, lymph nodes were swollen and hemorrhagic. There were no macroscopic pathological distinctions in the severity of lesions between fetuses originating from different sows.

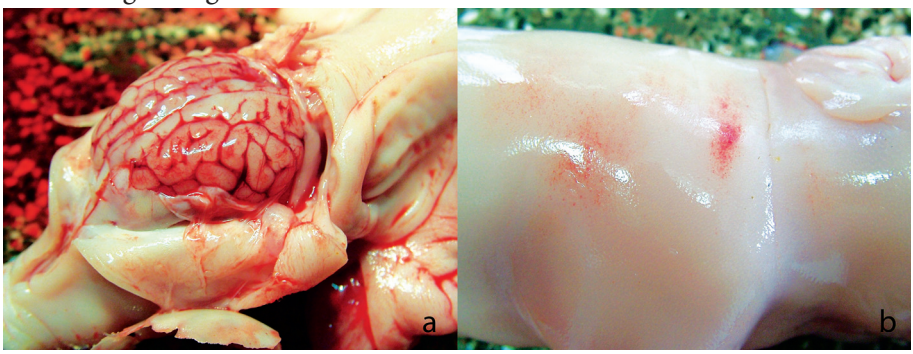


Figure 1. (a) Hyperemia in fetal brain; (b) Petechial hemorrhages in the skin

### *Distribution of CSF viral antigen*

The results of immunohistochemical staining are summarized in Table 1. The monoclonal antibody WH303 specific for CSFV glycoprotein E2 gave a positive staining reaction in tonsils, spleen, kidneys, ileocecal valve, brain, umbilical cord, but no immunoreactivity was detected in the myocardium. Positive cells typically exhibited a dark brown reaction on the membrane. With the exception of myocardium, in all the examined tissues, WH303 positive cells included endothelial cells, monocytes, macrophages and lymphocytes. The most immunopositive tissues were observed in fetuses originating from the first sow (83 gestational days). No differences in the amount or distribution of immunoreactive products were observed between fetuses from the second and third sow (82 and 95 gestational days, respectively).

In all the examined fetuses, the largest number of positive cells was found in kidneys, and a numerous positive duct epithelial, endothelial and mononuclear cells were detected (Figure 2a and 2b). In the spleen, specific immu-

noreactivity was observed in reticular cells, macrophages, lymphocytes and endothelial cells (Figure 2c). In tonsils, specific immunopositivity was detected in the crypt-epithelial cells, macrophages and lymphocytes (Figure 2d). Immunoreactive endothelial cells were observed in umbilical arteries of the umbilical cord (Figure 3a). In the ileocecal valve, viral antigen was detected in crypt epithelial cells and a small number of intraepithelial lymphocytes (Figure 3b). In the brain, virus antigen was found in glial cells, endothelial cells, and in the cells of the mononuclear inflammatory perivascular and meningeal infiltrates (Figure 3c and 3d). No immunoreactivity was observed in neurons.

Table 1. Immunohistochemistry (IHC) results for 29 pig fetuses naturally infected with CSFV

| Fetus number | Age (days of gestation) | Brain | Kidney | Spleen | Umbilical cord | Tonsils | Heart | Ileocecal valve |
|---|---|---|---|---|---|---|---|---|
| | | IHC | IHC | IHC | IHC | IHC | IHC | IHC |
| **1** | 83 | + | + | / | + | + | - | + |
| **2** | 83 | + | + | + | - | - | - | - |
| **3** | 83 | + | + | + | + | + | - | + |
| **4** | 83 | + | + | + | + | + | - | + |
| **5** | 83 | + | + | + | + | + | - | + |
| **6** | 83 | - | + | + | + | + | - | + |
| **7** | 82 | + | + | + | + | + | - | + |
| **8** | 82 | - | + | - | - | - | - | - |
| **9** | 82 | + | + | + | - | + | - | + |
| **10** | 82 | - | + | + | / | - | - | - |
| **11** | 82 | - | + | + | - | - | - | - |
| **12** | 82 | - | + | - | - | + | - | + |
| **13** | 82 | - | + | + | + | - | - | + |
| **14** | 82 | + | + | + | - | - | - | + |
| **15** | 82 | - | + | + | - | + | - | - |
| **16** | 82 | - | + | - | + | + | - | + |
| **17** | 82 | + | + | - | - | - | - | - |
| **18** | 95 | - | + | + | - | + | - | - |
| **19** | 95 | + | + | + | - | - | - | + |
| **20** | 95 | - | + | - | - | - | - | - |
| **21** | 95 | - | + | + | - | - | - | - |
| **22** | 95 | - | + | - | + | + | - | + |
| **23** | 95 | + | + | - | - | - | - | - |
| **24** | 95 | - | + | + | - | - | - | - |
| **25** | 95 | - | + | + | - | - | - | + |
| **26** | 95 | + | + | - | - | + | - | + |
| **27** | 95 | - | + | + | + | - | - | - |
| **28** | 95 | - | + | - | - | - | - | - |
| **29** | 95 | - | + | + | - | - | - | + |
| **Total** | 29 | 12 | 29 | 19 | 10 | 13 | 0 | 16 |

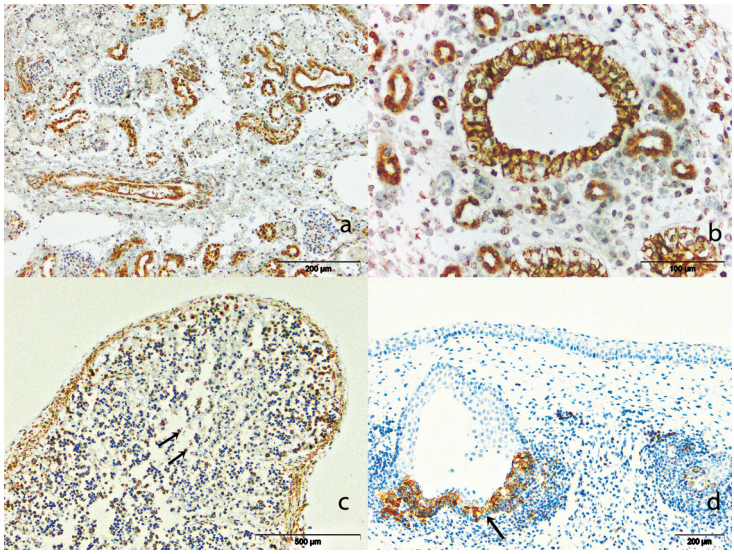


Figure 2. WH303 monoclonal antibody immunoreactivity in fetal tissues: (a, b) Kidney. CSFV antigen positive cells are detected in endothelial cells, tubulocytes and duct epithelial cells; (c) Spleen. CSFV antigen positive cells distributed mostly in the cortex. Scattered positive macrofages detected in the red pulp (arrow); (d) Tonsil. CSFV antigen positive cells detected in the crypt epithelium (arrow)

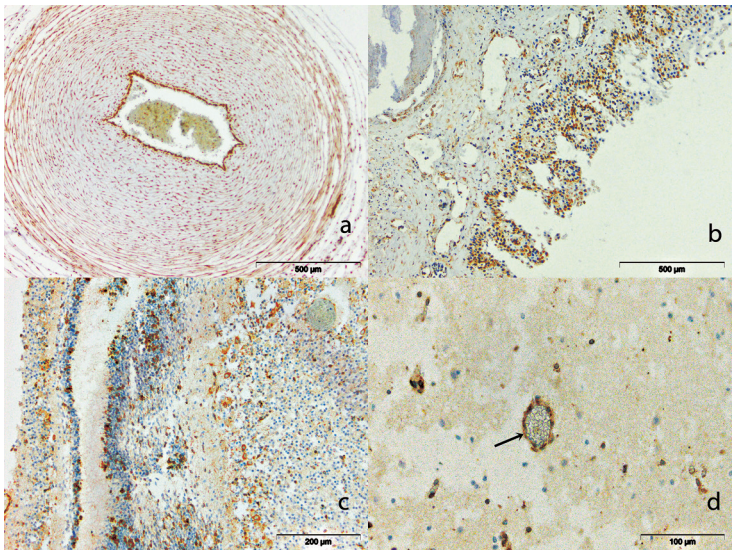


Figure 3. WH303 monoclonal antibody immunoreactivity in fetal tissues: (a) Umbilical cord. CSFV antigen positive cells distributed in vascular endothelium; (b) Ileocecal valve. CSFV antigen positive cells detected within crypt epithelium; (c,d) Brain**.** CSFV antigen positive cells detected in glial cells, meningeal infiltrates and endothelial cells (arrow)

## DISCUSSION

Although other research groups reported analyses of CSFV in different swine tissues by immunohistochemistry, to our best knowledge none of the previous examinations have provided analysis of fetal swine tissues under natural conditions, like the present study. These data are important from the point of controlling CSF disease, considering that transplacentally infected piglets can play a role especially for constant virus shedding and circulation in the swine population for a long period of time. The literature data on immunopathogenesis and tissue tropism of CSFV in infected fetuses is scarce, so it is difficult to compare this type of study with our results. The main reason for that may be strict eradication strategies implemented in most European countries in the past. Although many countries are officially free from this devastating disease, in many countries outside Europe the disease is still epidemic or endemic. According to the official OIE reports for the last 5 years (2016-2020), CSF was detected in countries of South America (Brazil, Colombia, Bolivia, Ecuador, Peru, Haiti), and Asia (China, Nepal, Thailand, Singapore, Philippines, Malaysia, Japan, Russia) (https://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap). In the Republic of Serbia, the last outbreak was reported in 2010 and the control of CSF was carried out for decades by C strain mass vaccination until the end of 2019. All CSF viruses isolated in the Republic of Serbia in 2006 when this study was performed were members of subgroup 2.3 (Milićević et al., 2013).

The results of this retroactive study showed that CSFV antigen can be detected in formalin-fixed, paraffin-embedded fetal tissue samples originating from naturally CSFV exposed sows by using monoclonal antibody WH303. A murine monoclonal antibody WH303 detects the CSF virus specific antigenic domain on the major envelope glycoprotein (E2), and binds to all strains of CSF virus, but not to other pestiviruses. The availability of reliable diagnostic tests is crucial for effective CSF control, and different diagnostic laboratory test procedures for confirmation of the viral antigen presence are available. Among them, immunohistochemistry proved to be a very reliable and suitable method for viral antigen detection in tissue samples, as well as for studying the pathogenesis of both natural and experimental CSFV infections (Belák et al., 2008; Polaček et al., 2007; 2014; 2016; Sánchez-Cordon et al., 2003; Choi and Chae, 2003a; De las Mulas et al., 1997). Besides that, the importance of diagnostic IHC is further emphasized by the observation of Belák et al., (2008) where this test gave positive results long (even seven days) before the appearance of the first clinical symptoms. As mentioned above, the delayed diagnosis may cause

an uncontrolled spread of CSF and consequently lead to heavy losses in swine population. Considering that CSFV infected pregnant sows have atypical and discrete clinical symptoms and that their offspring are a potential reservoir of the disease, we found it very important to perform this study of the tissue distribution of CSFV in pig fetuses in order to study virus biology and possibly confirm some answers regarding pathogenesis.

The pathological lesions in the acute and chronic form of CSF are well-documented (Moennig et al., 2003; Choi and Chae, 2003b; Knoetig et al., 1999; Belák et al., 2008). In the acute form, lesions are usually accompanied by secondary pathogens, but in general they included widespread petechial haemorrhages and ecchymosis, especially on skin, lymph nodes, epiglottis, bladder and kidneys, as well as infarctions of the margin of the spleen. In piglets with a congenital form of CSF infection, pathological changes were less typical and usually included cerebellar hypoplasia, microcephaly, pulmonary hypoplasia, hydrops and other malformations. This underlines that pathological mechanism involved are different from those responsible for postnatal infections (Liess 1987). In the present study, these findings were not detected in fetuses; however, the most common findings were hyperaemia in most organs, as well as petechial haemorrhages in the skin, lymph nodes and kidneys which coincide with the acute form of the disease. Although persistently infected offspring may be clinically normal at birth, it may take several months before they develop lesions and other disturbances that lead to death (Van Oirschot and Terpstra, 1977).

CSF viral distribution in different tissues depends on strains of the virus (highly, moderately or low virulent strain), age of pigs, duration of infection, and susceptibility of the breed (Choi and Chae, 2003a). The immunohistochemical detection of CSFV antigen in tissues of both naturally and experimentally infected pigs over time is well documented (Polaček et al., 2014; Liu et al., 2011; Gómez-Villamandos et al., 2006; Risatti et al., 2005; Choi and Chae, 2003a; De las Mulas et al., 1997). Previous studies have demonstrated that CSFV has a particular affinity for cells of the immune system, phagocytes of the macrophage and monocyte lineage (reticulo-endothelial cells), epithelial and vascular endothelial cells (Feng et al., 2012; Ressang, 1973; Cheville and Mengeling, 1969). In pig fetuses, CSF viral antigen was found predominantly in vascular endothelium, mononuclear cells and epithelial cells and these data are in accordance with those from a previous study. However, immunohistochemical staining of tissues in previous studies showed viral antigen distribution mainly in the cytoplasm of infected cells, while membranous expression was specific in fetal tissues. The reason for different cellular locations of

WH303 in fetuses compared to mature pigs is not known. However, it may be likely associated with some morphogenetic events in fetal development.

In our study, the general course of tissue tropism for the 7 examined tissue samples (from high to low) was as follows: kidney, spleen, ileocecal valve, tonsil, brain, umbilical cord and heart. The heart was the only organ without immunoreactivity in this study, and this is in agreement with previous studies where it was found that the myocardium is not considered as replication site for CSF virus in pigs (Belák et al., 2008; De las Mulas et al., 1997). In general, muscle cells are not considered the sites of CSFV replication (Liess 1987). However, Liu et al., (2011) had shown that low viral content could be detected in the heart of CSF infected pigs using real-time PCR method. This could be explained by the fact that real-time PCR is believed to be more sensitive than IHC method.

Recent work by our group demonstrated that the fetal kidneys were very useful organ for IHC diagnosis of the CSF virus, since out of all examined fetuses the highest level of viral antigen was detected in this organ. Previous studies also indicate that CSFV antigen positive cells in kidneys are a common finding, although the number of positive cells could be low (De las Mulas et al., 1997; Belák et al., 2008). In contrast, Choi and Chae (2003b) have shown that CSFV nucleic acid could be detected in kidneys by in situ hybridization, but they failed to detect viral antigen by immunohistochemistry. These authors explained this discrepancy by the fact that CSF disease process associated with glomerulonephritis is related in some way to the accumulation of viral nucleic acid/antibody complexes, and not complete CSFV particles. Another explanation was that CSFV infected cells may express small amount or no viral antigen on the cellular surface and this could result in failure to detect viral antigen by immunohistochemistry. Nevertheless, some authors preferred kidney tissue samples for diagnosis of CSF by direct immunofluorescence using frozen sections (Pearson, 1992; Terpstra, 1991).

It is shown that in transplacentally, persistently infected piglets, the infection severely affects tissues comprising the immune system, including severe depletion of lymphocytes in thymus and secondary lymphoid organs (Van Der Molen and Van Oirsch, 1981) and viral antigen was widespread in lymphoid tissues (Polaček et al, 2007). In our study, the viral antigen was widely detected in spleen followed by tonsils, which is in accordance with these studies. However, the main route of entry of CSFV in postnatal infection is oronasal and the tonsils are the primary replication site of the virus. In the present study, viral antigen was also detected in tonsils in a certain number of examined fetuses, which might mean that the virus gains access from the bloodstream to tonsils as the secondary replication site.

As mentioned above, CSFV is found to be highly susceptible to infecting vascular endothelial cells (Bensaude et al., 2004) and they are primary target cells for the virus. Dysfunction of blood vessels plays an important role in the pathogenesis of CSF and it was proved that CSFV plays a pathophysiological role in vascular dysfunction through its effect on oxidative stress (He et al., 2014; Bensaude et al., 2004). In the recent study, viral antigen was detected in blood vessels of the brain, spleen, kidneys, as well as in umbilical cord, due to transplacental passage. Some other swine viruses also replicate in umbilical cord due to their ability to cross the placenta of the pregnant sows, as previously described in influenza infection (Khatri and Chattha, 2015) and PRRS virus (Lager and Halbur, 1996).

CSFV, as well as *Bovine viral diarrhea virus* (both viruses of the same genus - *Pestivirus*) are characterized by severe clinical and histopathologic changes in the intestine. The CSFV also has a pathogenic effect on gut-associated lymphoid tissue (GALT) and infection takes place initially in monocytes-macrophages and lymphocytes in lymphoid tissue (Sánchez-Cordón et al., 2003). In the present study, epithelial cells of ileocecal valve crypts and cells in the lumen of these crypts revealed a slightly positive reaction, and immunopositive signal was detected in half of the examined fetuses.

Nervous tissue is considered as one of the target tissues for CSFV, and histological lesions in the brains of pigs with chronic CSFV infection are similar to those in pigs experimentally inoculated with a low-virulence CSFV strain (Choi and Chae, 2003). Previous studies reported viral antigen detection in neurons, glial cells, endothelial cells, and cell infiltrate during CSF infection (Pan et al., 1993; De las Mulas et al., 1997; Gómez-Villamandos et al., 2006; Polaček et al., 2008). A similar viral antigen distribution has been detected in our study, with the main exception of IHC positive neurons, although infection of neurons is reported in natural outbreaks of CSF (Pan et al., 1993; Trautwein, 1988). This difference may be due to a more prolonged course of the natural infection studied in mature pigs.

## CONCLUSION

Regarding the control of this viral disease, the results presented herein indicate the importance of the kidneys in the pathogenesis of transplacental CSFV infection, considering that the highest amount of viral antigen was detected in this fetal organ and all examined fetal kidneys were immunopositive for the antigen of the CSFV. Having that in mind, it is assumed that persistently infected piglets may shed high amount of viral particles through urine. However, further research is needed to confirm this hypothesis.

## ACKNOWLEDGEMENT

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Projects number TR 31011 and TR 31084.

## Author's contributions:

VP and BĐ drafted the manuscript, carried out literature research and immunohistochemical examination; TP carried out the virological examination; JPR participated in the design of the study; MS did the reviewing and editing; IV participated in the immunohistochemical examination and in manuscript revision. SKA was involved in the study design, she revised manuscript critically and gave the final approval of the version to be published.

## Competing Interests

Authors declared no conflict of interests regarding the present paper.

## REFERENCES

1. Belák K., Koenen F., Vanderhallen H., Mittelholzer C., Feliziani F., De Mia G.M., Belák S. 2008. Comparative studies on the pathogenicity and tissue distribution of three virulence variants of classical swine fever virus, two field isolates and one vaccine strain, with special regard to immunohistochemical investigations. Acta Veterinaria Scandinavica, 50, 34. doi: 10.1186/1751-0147-50-34.
2. Bensaude E., Turner J.L., Wakeley P.R., Sweetman D.A., Pardieu C., Drew T.W., Wileman T., Powell P.P. 2004. Classical swine fever virus induces proinflammatory cytokines and tissue factor expression and inhibits apoptosis and interferon synthesis during the establishment of long-term infection of porcine vascular endothelial cells. Journal of General Virology, 85(Pt 4):1029–1037. doi: 10.1099/vir.0.19637-0.
3. Blome S., Staubach C., Henke J., Carlson J., Beer M. 2017. Classical Swine Fever-An Updated Review. Viruses, 9(4), 86. doi: 10.3390/v9040086.
4. Cabezón O., Colom-Cadena A., Muñoz-González S., Pérez-Simó M., Bohórquez J.A., Rosell R., Marco I., Domingo M., Lavín S., Ganges L. 2017. Post-natal persistent infection with classical swine fever virus in wild boar: A strategy for viral maintenance? Transboundary Emerging Diseases, 64: 651-655. doi: 10.1111/tbed.12395.

5. Cheville N.F and Mengeling W.L. 1969. The pathogenesis of chronic hog cholera (swine fever). Histologic, immunofluorescent and electron microscopic studies. Laboratory investigation: a journal of technical methods and pathology, 20, (3), 261-274.
6. Choi C. and Chae C. 2003a. Localization of Classical Swine Fever Virus from Chronically Infected Pigs by In Situ Hybridization and Immunohistochemistry. Veterinary Pathology, 40:107–113. doi: 10.1354/vp.40-1-107.
7. Choi C. and Chae C. 2003b. Glomerulonephritis associated with classical swine fever virus in pigs, Veterinary Record 153, 20-22. doi: 10.1136/vr.153.1.20.
8. Dewulf J., Laevens H., Koenen F., Mintiens K., De Kruif A. 2001. An Experimental Infection with Classical Swine Fever Virus in Pregnant Sows: Transmission of the Virus, Course of the Disease, Antibody Response and Effect on Gestation. Journal of Veterinary Medicine, Series B, 48, 583-591. doi:10.1111/j.1439-0450.2001.00467.x.
9. Edwards S., Fukusho A., Lefevre P.C., Lipowski A., Pejsak Z., Roehe P., Westergaard J. 2000. Classical swine fever: The global situation. Veterinary microbiology, 73 (2-3), 103–119. doi: 10.1016/S0378-1135(00)00138-3.
10. Feng L., Li X.Q., Li X.N., Li J., Meng X.M., Zhang H.Y., Liang J.J., Li H., Sun S.K., Cai X.B., Su L.J., Yin S., Li Y.S., Luo T.R. 2012. In vitro infection with classical swine fever virus inhibits the transcription of immune response genes. Virology Journal, 9, 175. doi: 10.1186/1743-422X-9-175.
11. Gómez-Villamandos J.C., de Leániz I.G., Núñez A., Salguero F.J., Ruiz-Villamor E., Romero-Trevejo J.L., Sánchez-Cordón P. J. 2006. Neuropathologic Study of Experimental Classical Swine Fever. Veterinary Pathology, 43(4), 530–540. doi: 10.1354/vp.43-4-530.
12. He L., Zhang Y., Fang Y., Liang W., Lin J., Cheng M. 2014. Classical swine fever virus induces oxidative stress in swine umbilical vein endothelial cells. BMC veterinary research, 10, 279. doi:10.1186/s12917-014-0279-3.
13. De las Mulas J.M., Ruiz-Villamor E., Donoso S., Quezada M., Lecocq C., Sierra M.A. 1997. Immunohistochemical detection of hog cholera viral glycoprotein 55 in paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation, 9, 10-16. doi: 10.1177/104063879700900103.
14. Liu J., Fan X.Z., Wang Q., Lu X., Zhao Q.Z., Huang W., Zhou Y.C., Tang B., Chen L., Zou X.Q., Sha S., Zhu Y.Y. 2011. Dynamic distribution and tissue tropism of classical swine fever virus in experimentally infected pigs. Virology Journal, 8,201. doi: 10.1186/1743-422X-8-201.
15. Katz J.B., Ridpath J.F., Bolin S.R. 1993. Presumptive diagnostic differentiation of hog cholera virus from bovine viral diarrhoea and border disease

viruses by using a cDNA nested-amplification approach. Journal of Clinical Microbiology, 31, 565-8.
16. Khatri M., Chattha K.S. 2015. Replication of influenza A virus in swine umbilical cord epithelial stem-like cells. Virulence, 6 (1), 40-9. doi: 10.4161/21505594.2014.983020.
17. Knoetig S.M., Summerfield A., Spagnuolo-Weaver M., McCullough K.C. 1999. Immunopathogenesis of classical swine fever: role of monocytic cells. Immunology, 97(2), 359-366. doi:10.1046/j.1365-2567.1999.00775.x.
18. Lager, K.M. and Halbur P.G. 1996. Gross and Microscopic Lesions in Porcine Fetuses Infected with Porcine Reproductive and Respiratory Syndrome Virus. Journal of Veterinary Diagnostic Investigation, 8(3), 275–282. doi:1177/104063879600800301.
19. Liess B. 1987. Pathogenesis and epidemiology of hog cholera. Annals of Veterinary Research, 18,139-145.
20. Milićević V., Radojičić S., Valčić A.M., Ivović V., Maksimović-Zorić J., Radosavljević V. 2013. Detection and genotyping of classical swine fever virus isolates in Serbia. Acta Veterinaria - Beograd, Vol. 63, No. 2-3, 191-200. doi: 10.2298/AVB1303191M.
21. Moennig V., Floegel-Niesmann G., Greiser-Wilke I. 2003. Clinical Signs and Epidemiology of Classical Swine Fever: A Review of New Knowledge. The Veterinary Journal, 165, 1, 11-20. doi:10.1016/S1090-0233(02)00112-0.
22. Muñoz-González S., Ruggli N., Rosell R., Pérez L.J., Frías-Leuporeau M.T., Fraile L., Montoya M., Cordoba L., Domingo M., Ehrensperger F., Summerfield A., Ganges L. 2015. Postnatal Persistent Infection with Classical Swine Fever Virus and Its Immunological Implications. PLoS ONE, 10(5): e0125692. doi: 10.1371/journal.pone.0125692.
23. OIE: WAHIS Interface, Disease information. Available at: https://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap.
24. Pan I.C., Huang T.S., Pan C.H., Chern S.Y., Lee S.H., Lin Y.L., Huang B.Y., Lin C.C., Li N.J., Lin J.P., Yang Y.H., Chiu S.Y., Chang J.S., Hue D.K., Lee H.C., Chang C.N. 1993. The skin, tongue and brain as favorable organs for hog cholera diagnosis by immunofluorescence. Archives of Virology, 131,475–481. doi: 10.1007/BF01378648.
25. Pearson J.E. 1992. Hog cholera diagnostic techniques. Comparative Immunology, Microbiology & Infectious Diseases, 15, 213-219. doi: 10.1016/0147-9571(92)90094-8.
26. Polaček V., Božić B., Prodanov-Radulović J., Petrović T., Vučićević I., Becskei Ž., Aleksić-Kovačević S. 2016. Immunohistochemical Detection of

Classical Swine Fever Virus in Different Fetal Tissues of Naturally Infected Sows. In *Abstract Book*, 34th Annual Meeting of the ESVP & 27th Annual Meeting of the ECVP, 7-10.09.2016, Bologna, Italy, 217.

27. Polaček V., Vasković N., Prodanov J., Knežević M., Aleksić-Kovačević S. 2008. Immunohistochemical Detection of E2 Protein (gp55) of Classical Swine Fever Virus in Brain and Ileocoecal Valve of Experimentally Infected Pigs. In *Abstract book,* 26th Annual Meeting of European Society of Veterinary Pathology, Dubrovnik, Croatia, 156.
28. Polaček V., Prodanov-Radulović J., Došen R., Petrović T., Becskei Z., Aleksić-Kovačević S. 2014. Expression of E2 (gp 55) glycoprotein of classical swine fever virus in lymphoid tissue and brain of experimentally infected piglets with different immunological status. Acta Veterinaria-Beograd, 64, 2, 213-225. doi:10.2478/acve-2014-0020
29. Polaček V., Prodanov J., Lazić S., Petrović T., Rašić Z., Aleksić-Kovačević S. 2007. Immunohistochemical detection of B and T lymphocytes in mandibular lymph nodes of experimentally infected piglets with classical swine fever virus,. Acta Veterinaria-Beograd, 57, 2-3, 199-208. doi: 10.2298/AVB0703199P.
30. Prodanov-Radulović J., Došen R., Polaček V., Petrović T., Stojanov I., Ratajac R., Valčić M. 2014. Classical swine fever: active immunisation of piglets with subunit (E2) vaccine in the presence of different levels of colostral immunity (China strain). Acta Veterinaria-Beograd, 64 (4): 493-509. doi: 10.2478/acve-2014-0046.
31. Ressang A.A. 1973. Studies on the pathogenesis of hog cholera. II. Virus distribution in tissue and the morphology of the immune response. Zentralblatt Veterinarmedizin Reihe B, 20 (4), 272-288.
32. Risatti G.R., Borca M.V., Kutish G.F., Lu Z., Holinka L.G., French R.A., Tulman E.R., Rock D.L. 2005. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. Journal of virology, 79 (6), 3787–3796. doi:10.1128/JVI.79.6.3787-3796.2005.
33. Sánchez-Cordón P.J., Romanini S., Salguero F.J., Ruiz-Villamor E., Carrasco L., Gómez-Villamandos J.C. 2003. A Histopathologic, Immunohistochemical, and Ultrastructural Study of the Intestine in Pigs Inoculated with Classical Swine Fever Virus. Veterinary Pathology, 40 (3), 254–262. doi: 10.1354/vp.40-3-254.
34. Terpstra C. 1991. Hog cholera: an update of present knowledge. The British veterinary journal, 147, 397-406. doi: 10.1016/0007-1935(91)90081-W.
35. Trautwein G. 1988. Pathology and pathogenesis of the disease. In: *Classical Swine Fever and related viral diseases*. Ed. Liess B., Martinus Nijhoff Publishing, Boston, 27–54.

36. Van Der Molen E.J. and Van Oirsch J.T. 1981. Congenital Persistent Swine Fever (Hog Cholera) I. Pathomorphological Lesions in Lymphoid Tissues, Kidney and Adrenal, Zentralblatt Veterinarmedizin Reihe B, 28, 89-101.
37. Van Oirschot J.T. and Terpstra C.A. 1977. A congenital persistent swine fever infection. I. Clinical and virological observations. II. Immune response to swine fever and unrelated antigens. Veterinary Microbiology, 2, 121–42. doi: 10.1016/0378-1135(77)90004-9
38. Von Benten K., Trautwein G., Richter-Reichhelm H.B., Liess B., Frey H.R. 1980. Experimental transplacental transmission of hog cholera virus in pigs. III. Histopathological findings in the fetus. Zentralblatt Veterinarmedizin Reihe B, 27, (9-10), 714–724, doi: 10.1111/j.1439-0450.1980.tb02026.x.

Received: 10.05.2020.
Accepted: 26.05.2020.

Short communication UDC 637.3:579.842.1/.2(497.11)
https://doi.org/10.46784/e-avm.v13i1.104

# CONTRIBUTION TO THE KNOWLEDGE ABOUT THE PRESENCE AND ROLE OF *ENTEROBACTER GERGOVIAE* IN SENSORY CHARACTERISTICS OF DAIRY PRODUCTS

Katarina G. Mladenović[1]*, Mirjana Ž. Grujović[1], Ljiljana R. Čomić[1]

[1] University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Kragujevac, Republic of Serbia

## Abstract

*Enterobacter gergoviae* KGPMF 20 was found in traditionally made cheese from Sokobanja (South-eastern Serbia). In this paper, the characteristics of the species were evaluated by investigation of adhesion to different solvents and co-aggregation ability with other species. Moreover, its enzymatic activity was evaluated by using spectrophotometric method, with the intention to detect the role of the isolate in the sensory characteristic of cheese. The results of enzymatic activity indicated that *E. gergoviae* KGPMF 20 has low, almost no enzymatic activity. It could be concluded that this isolate did not affect the sensory characteristic of cheese.

**Key words:** *Enterobacter gergoviae* KGPMF 20, cheese originating from Serbia, enzymatic activity

[1] Corresponding author: katarina.mladenovic@pmf.kg.ac.rs

# DOPRINOS SAZNANJIMA O PRISUSTVU I ULOZI *ENTEROBACTER GERGOVIAE* U ORGANOLEPTIČKIM KARAKTERISTIKAMA MLEČNIH PROIZVODA

Katarina G. Mladenović[1*], Mirjana Ž. Grujović[1], Ljiljana R. Čomić[1]

[1] Univerzitet u Kragujevcu, Prirodno-matematički fakultet, Odeljenje za biologiju i ekologiju, Kragujevac, Republika Srbija

## Kratak sadržaj

*Enterobacter gergoviae* KGPMF 20 pronađen je u tradicionalno proizvedenom siru iz Sokobanje (jugoistočna Srbija). U ovom radu, procenjene su karakteristike vrste ispitivanjem sposobnosti adhezije sa različitim rastvaračima i sposobnosti koagregacije sa drugom vrstom. Enzimska aktivnost vrste je procenjena spektrofotometrijskom metodom, sa namerom da se utvrdi uticaj vrste u organoleptičkim karakteristikama sira. Rezultati enzimske aktivnosti pokazali su da *E. gergoviae* KGPMF 20 pokazuje slabu enzimsku aktivnost. Na osnovu rezultata, može se zaključiti da vrsta nema uticaj na organoleptičke karakteristike sira.

**Ključne reči:** *Enterobacter gergoviae* KGPMF 20, sir iz Srbije, enzimatska aktivnost

## INTRODUCTION

Cheese from Sokobanja (Southeastern Serbia) is made from unpasteurized cow milk. Since potentially pathogenic bacteria are not eliminated by high temperatures during the pasteurization process, they may be found in cheese. *Enterobacter gergoviae* is a member of the Enterobacteriaceae family and it was isolated from the cheese originating from Sokobanja (Mladenovic et al., 2018). *Enterobacter gergoviae* can be isolated from fresh cow's milk (Wahyuni and Budiarso, 2009), and from traditional cheese as well (Ethiopian cottage cheese) (Melkamsew et al., 2012).

Based on its occurrence in dairy products, the objective of this study was to investigate *E. gergoviae* KGPMG 20 isolated from traditionally produced cheese from Sokobanja (Serbia). The ability of adhesion in the presence of solvents, co-aggregation ability with *Enterococcus faecalis* KGPMF 49, and the potential to produce extracellular enzymes were examined.

## MATERIALS AND METHODS

The tested cheese was produced in a traditional way, in countryside households around Sokobanja (Southeastern Serbia). The methods of production, sampling, and analyses of chemical characteristics of cheese were described in Mladenović et al. (2018). *E. gergoviae* species KGPMG 20 was found in cheese. Biochemical characteristics and identification of the species were described in Mladenović et al. (2018).

### *The adhesion to solvents*

The adhesion to solvents of *E. gergoviae* KGPMG 20 was measured in accordance with the method described in Rosenberg (1980), with some modifications (Bellon-Fontaine et al., 1996; Kos et al., 2003). After incubating the bacteria in TSB for 24 h, the bacteria were centrifuged at 5000 rpm for 15 minutes, and then washed twice and suspended in 0.1 M $KNO_3$ (pH 6.2) to approximately 108 CFU $mL^{-1}$. The absorbance of the bacterial suspension was measured at 600 nm ($A_0$). 1 mL of solvent was added to 3 mL of bacterial suspension. After 10 minutes of incubation at room temperature, the two-phase system was mixed using a vortex for 2 minutes. The aqueous phase was removed after 20 minutes of incubation at room temperature and its absorbance was measured at 600 nm ($A_1$). The percentage of bacterial adhesion in the presence of solvent was calculated as follows:

$$\text{Adhesion } \% = (1-A_1/A_0) \times 100$$

Three different solvents were tested in this study: xylene (Sineks, Belgrade, Serbia), which is a polar solvent; chloroform (Alkaloid, Skoplje, Macedonia), a monopolar and acidic solvent, and ethyl acetate (Zorka, Sabac, Serbia), a monopolar and basic solvent. Only bacterial adhesion to xylene demonstrated cell surface hydrophobicity or hydrophilicity (Ocaña and Nader-Macías, 2002). According to Ocaña and Nader-Macías (2002), the percentage of hydrophobicity is expressed as 0 - 35% - low hydrophobicity; 36 - 70% - medium hydrophobicity; 71 - 100% - high hydrophobicity.

### *The co-aggregation ability*

The co-aggregation of *E. gergoviae* KGPMF 20 with *Enterococcus faecalis* KGPMF 49 isolated from the same Sokobanja cheese was examined. *E. faecalis*

KGPMF 49 was isolated from the same cheese (Muruzović et al., 2018). The co-aggregation was monitored by using a modified procedure described in Ocaña and Nader-Macías (2002). Overnight bacterial cultures were centrifuged at 5000 rpm for 15 minutes, and then washed twice in PBS buffer (Alfa Aesar GmbH & Co, Karlsruhe, Germany) followed by resuspension in 4 mL of the same buffer and the cell number was approximately $10^8$ CFU $mL^{-1}$. 2 mL of each suspension of both bacteria whose coaggregation was monitored was mixed well on a vortex. After mixing, 200 µL from the surface of the suspension was transferred to a microtube containing 1800 µL of PBS, and the absorbance values were read at 600 nm ($A_0$). The same procedure was repeated after 2 h (At). The percentage of coaggregation of the species was calculated in the following way:

$$\text{Coaggregation } \% = (A_0\text{-}A_t)/A_0 \text{ x}100$$

### *The preparation of fermentation liquid and detection of enzymatic activity*

In order to investigate the enzymatic activity, it is necessary to obtain fermentation liquids of the isolate. 100 µL of overnight bacterial culture was separately inoculated in 10 mL of TSB (Torlak, Belgrade, Serbia) and MH (Torlak, Belgrade, Serbia). Inoculated broths were incubated for 24 h at 37°C. After the incubation, the samples were centrifuged at 10.000 rpm/30 min/ 4°C. Then, the supernatant, which represented the fermentation liquid, was separated. Fermentation liquid was kept in the refrigerator at 4°C until the experiment was performed. Two broths were used as they had a different composition and there was a possibility that they could affect the enzymatic activity of bacteria.

After the preparation of fermentation liquid, the enzymatic activity was evaluated. The acid and alkaline invertase activity, the activity of alkaline phosphatase, α-amylase activity, proteolytic activity and the total concentration of protein were all determined by the methods described in Jakovljević (2014).

## RESULTS AND DISCUSSION

In this paper, the adhesion and co-aggregation ability, as well as the enzymatic activity of *E. gergoviae* KGPMF 20 were demonstrated for the first time. The isolate originated from Sokobanja cheese which was produced in the households around Sokobanja (southeastern Serbia), from fresh and unpasteurized cow's milk. According to the results from Mladenović et al. (2018), *E.*

*gergoviae* KGPMF 20 is a gram-negative, oxidase negative and catalase-positive bacteria. It showed the fermentation ability of glucose and lactose with production of acid and gas, while the ability of $H_2S$ production (hydrogen sulphide) form triple sugar was not detected. It formed a light pink colony on HiChrome coliform agar. The investigated isolate was sensitive to antibiotics (streptomycin, chloramphenicol and tetracycline).

The adhesion ability of *E. gergoviae* KGPMF 20 was detected in the presence of chloroform (13.85%), and ethyl acetate (13.89%). The species demonstrated no ability of adhesion in the presence of xylene.

Adequate hydrophobic/hydrophilic properties of microorganisms can contribute to beneficial processes such as degradation of hydrocarbons or biodegradable polyesters during milk fermentation (Obuekwe et al., 2009). Hydrophobic microorganisms may have the ability to form a biofilm to various abiotic and biotic surfaces (Krasowska and Karel, 2014). According to Tresse et al. (2006), cellular hydrophobicity is crucial for biofilm formation. Del Re et al. (2000) and Giaouris et al. (2009) indicated that bacteria with a hydrophobic surface have a higher binding affinity for epithelial cells and solid surfaces. Bacterial biofilm can lead to bacterial resistance to antibiotics and thus contributes to the pathogenicity of one species. Bacterial adhesion in the presence of xylene is an indicator of hydrophobicity or hydrophilicity of cell surface. The ability to adhere in the presence of two other solvents, chloroform and ethyl acetate is an indicator of the ability of bacterial cell as a donor of base or acid electron acceptors (Bellon-Fontaine et al., 1996). Based on the results, it can be concluded that *E. gergoviae* KGPMF 20 had low hydrophobicity. It showed no ability of adhesion to xylene, meaning that it had a low potential to attach and form biofilm in abiotic surfaces.

The co-aggregation of *E. gergoviae* KGPMF 20 and *E. faecalis* KGPMF 49 was investigated for the first time in this study. The percentage of co-aggregation between these bacteria was 14.2%. Enterobacteria and enterococci are a member of the normal flora of the human gastrointestinal tract (Silva et al., 2012; Pugin et al., 2017) and of the dairy products made from unpasteurized cow's milk (Muruzović et al., 2018; Mladenović et al., 2018). Therefore, their interaction needs to be investigated and evaluated.

The enzymatic activity of *E. gergoviae* KGPMF 20 was investigated in two different broths and the results are shown in Table 1. The results indicated that small activity of amylase and alkaline phosphatase was observed, while the activity of acid and alkaline invertase was not detected (except for the very small activity of acid invertase in MH). The protease activity was observed only in TSB.

Table 1. The enzymatic activity of *E. gergoviae* KGPMF 20

| *E. gergoviae* KGPMF 20 | | |
|---|---|---|
| Proteins and enzymes | Type of media | Absorbance |
| Protease (IU/mL) | TSB | 7.6 |
| | MH | / |
| Total proteins (mg/mL) | TSB | 0.13 |
| | MH | 0.04 |
| Acid invertase (IU/mL) | TSB | / |
| | MH | 0.05 |
| Alkaline invertase (IU/mL) | TSB | / |
| | MH | / |
| Amylase (IU/mL) | TSB | 0.05 |
| | MH | / |
| Alkaline phosphatase (IU/mL) | TSB | 0.11 |
| | MH | 0.06 |

/ - not detected

According to the results of the screening method, *E. gergoviae* KGPMF 20 does not have the proteolytic or lipolytic activity (Mladenović et al., 2018). According to a study by Kamaladevi et al. (2014), *E. gergoviae* isolated from the areas of Vaippar, Thoothukudi District and Tamil Nadu in India demonstrated the ability to produce lipase. In this paper, it was shown that *E. gergoviae* KGPMF 20 had a very low or no enzymatic activity, so it had no role in sensory properties of cheese.

In the Sokobanja cheese, the presence of the *Enterobacter* genus with only *E. gergoviae* KGPMF 20, was determined (Mladenović et al., 2018). Based on the available literature, it was found that the appearance and description of *Enterobacter* sp. is scarce compared to the description and presence of other members of the *Enterobacteriaceae* family (Tabla et al., 2016; Mladenović et al., 2018).

## CONCLUSION

Based on the results from this study, *E. gergoviae* KGPMF 20 can interact with other species isolated from cheese. The species had a low hydrophobicity and showed no adhesion ability in the presence of xylene. It produces a very low concentration of extracellular enzymes, so it had no effect on the sensory

characteristics of cheese. Previous results on this isolate showed its sensitivity to antibiotics. Therefore, the appearance of *E. gergoviae* in cheese is probably the result of a spontaneous transfer from an animal to fresh milk.

## ACKNOWLEDGMENTS

This work was supported by the Serbian Ministry of Education, Science and Technological Development (No. 451-03-68/2020-14/200122).

### Authors' contributions

KM made substantial contributions to basic idea, conception and design, acquisition of samples and data, analysis of the data and interpretation of results; MG was involved in drafting of the manuscript, revising it critically for important intellectual content, and LjČ gave the final approval of the manuscript to be published.

### Competing interests

The authors declare that they have no competing interests.

## REFERENCES

1. Bellon-Fontaine M.N., Rault J., van Oss C.J. 1996. Microbial adhesion to solvents: a novel method to determine the electron donor/electron-acceptor or Lewis acid-base properties of microbial cells. Colloids and Surfaces, 7, 1-2, 47–53. doi:10.1016/0927-7765(96)01272-6.
2. Del Re B., Sgorbati B., Miglioli M., Palenzona D. 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology, 31, 438–442. doi:10.1046/j.1365-2672.2000.00845.x.
3. Giaouris E., Chapot-Chartier M.P., Briandet R. 2009. Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. International Journal of Food Microbiology, 131, 1, 2–9. doi:10.1016/j.ijfoodmicro.2008.09.006.
4. Jakovljević D.V. 2014. Biochemical characteristics of selected fungal species as a function of detergent biodegradation. Doctoral dissertation, Faculty of Science, Kragujevac, Serbia (in Serbian).

5. Kamaladevi B., Prabhavathi P., Sankareswaran M., Anbalagan S., Radhakrishnan N., Prabhu D. 2014. Screening and medium optimization of lipase producing bacteria from saltpan. Research Journal of Chemical and Environmental Sciences, 2, 72–77.
6. Kos B., Šušković J., Vuković S., Simpraga M., Frece J., Matošić S. 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology, 94, 981–987. doi:10.1046/j.1365-2672.2003.01915.x.
7. Krasowska A. and Karel S. 2014. How microorganisms use hydrophobicity and what does this mean for human needs? Frontiers in Cellular and Infection Microbiology 4, Article 112. doi: 10.3389/fcimb.2014.00112.
8. Melkamsew A.T., Pal M., Beda H.A. 2012. Bacteriological study on coliform organisms from Ethiopian traditional cheese west show zone. African Journal of Microbiology Research, 3, 3, 188–191. doi:10.5829/idosi.ijmr.2012.3.3.64213.
9. Mladenović K., Muruzović M., Žugić Petrović T., Stefanović O. Čomić Lj. 2018. Isolation and identification of *Enterobacteriaceae* from traditional Serbian cheese and their physiological characteristics. Journal of Food Safety, 38, 1, Article ID e12387. doi:10.1111/jfs.12387.
10. Muruzović M., Mladenović K., Žugić Petrović T., Čomić Lj. 2018. Characterization of lactic acid bacteria isolated from traditionally made Serbian cheese and evaluation of their antagonistic potential against *Enterobacteriaceae*. Journal of Food Processing and Preservation, 42, 4, Article ID e13577. doi:10.1111/jfpp.13577.
11. Obuekwe C., Al-Jadi Z.K., Al-Saleh E. 2009. Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. International Biodeterioration and Biodegradation, 63, 273–279. doi:10.1016/j.ibiod.2008.10.004.
12. Ocaña V. and Nader-Macías M.E. 2002. Vaginal lactobacilli: self- and coaggregation ability. British Journal of Biomedical Science, 59, 4, 183–190. doi: 10.1080/09674845.2002.11783657.
13. Pugin B., Barcik W., Westermann P., Heider A., Wawrzyniak M., Hellings P. 2017. A wide diversity of bacteria from the human gut produces and degrades biogenic amines. Microbial Ecology in Health and Disease, 28, Article ID 1353881. doi:10.1080/16512235.2017.1353881.
14. Rosenberg M., Gutnick D., Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters, 9, 1, 29–33. doi:10.1111/j.1574-6968.1980.tb05599.x.

15. Silva N., Igrejas G., Gonçalves A., Poeta P. 2012. Commensal gut bacteria: distribution of *Enterococcus* species and prevalence of *Escherichia coli* phylogenetic groups in animals and humans in Portugal. Annals of Microbiology, 62, 449–459. doi:10.1007/s13213-011-0308-4.
16. Tabla R., Gómez A., Simancas A., Rebollo J.E., Molina F., Roa I. 2016. *Enterobacteriaceae* species during manufacturing and ripening of semi–hard and soft raw ewe's milk cheese: Gas production capacity. Small Ruminant Research, 145, 123–129. doi:10.1016/j.smallrumres.2016.11.008.
17. Tresse O., Lebret V., Benezech T., Faille C. 2006. Comparative evaluation of adhesion, surface properties, and surface protein composition of *Listeria monocytogenes* strains after cultivation at constant pH of 5 and 7. Journal of Applied Microbiology, 101, 1, 53–62. doi:10.1111/j.1365-2672.2006.02968.x.
18. Wahyuni A.E.T.H. and Budiarso T.Y. 2009. Detection of *Enterobacter sakazakii* and another *Enterobacter* sp. from Dairy Cow's Milk in Boyolali and Sleman. Animal production, 74–77.

Submitted: 23.04.2020.
Accepted: 28.06.2020.

Case report

UDC 636.7:591.463.1
https://doi.org/10.46784/e-avm.v13i1.54

# ACCIDENTAL FINDING OF *DIROFILARIA REPENS* IN DOG DURING THE QUALITY CONTROL OF SEMEN– CASE REPORT

Jelena Apić[1*], Tomislav Barna[1], Marina Žekić Stošić[1], Aleksandar Milovanović[1], Bojan Lukić[2], Aleksandar Potkonjak[3], Sara Savić[1]

[1] Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia
[2] PVA „Moj Veterinar", Kać, Republic of Serbia
[3] University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Republic of Serbia

## Abstract

A nine-year-old male dog of Doberman breed was taken to the laboratory for animal reproduction of the Scientific Veterinary Institute „Novi Sad" for semen quality control. The sample of the ejaculate was taken without difficulty; however, the sample contained a significant amount of fresh blood (total ejaculate volume was 5 ml). The assessment of semen quality from such sample was not possible because blood components mask the spermatozoa. Nevertheless, the sample of ejaculate drop was placed for microscopic observation and analysis. In the semen, a lot of blood cells and only a few spermatozoids (mostly not moving) were found; however, something else was observed - the presence of a live motile organism, long-shaped, looking very much like a larval stage of a parasite *Dirofilaria sp.* Next day, the blood sample was taken for the analysis for dirofilariosis. The result of the ELISA test for *Dirofilaria immitis* antigen was negative indicating the absence of adult worms of *Dirofilaria immitis*. The result of modified Knott test showed the presence of larval stages of *Dirofilaria repens.*

**Key words:** male dog, semen quality**,** *Dirofilaria repens,* Knott test

[1] Corresponding author: jelena.a@niv.ns.ac.rs

# SLUČAJAN NALAZ *DIROFILARIA REPENS* TOKOM KONTROLE KVALITETA SEMENA PSA – PRIKAZ SLUČAJA

Jelena Apić[1*], Tomislav Barna[1], Marina Žekić Stošić[1],
Aleksandar Milovanović[1], Bojan Lukić[2], Aleksandar Potkonjak[2], Sara Savić[1]

[1] Naučni institut za veterinarstvo „Novi Sad“, Novi Sad, Republika Srbija
[2] PVA „Moj Veterinar“, Kać, Republika Srbija
[3] Univerzitet u Novom Sadu, Poljoprivredni fakultet, Departman
za veterinarsku medicinu, Novi Sad, Republika Srbija.

## Kratak sadržaj

Mužjak, rase Doberman, starosti devet godina, doveden je u laboratoriju za reprodukciju životinja, Naučnog instituta za veterinarstvo „Novi Sad“, radi redovne kontrole kvaliteta nativnog ejakulata. Procedura uzimanja ejakulata protekla je bez poteškoća. Međutim, dobijeni uzorak obilovao je znatnom količinom sveže krvi (ukupna količina ejakulata je bila 5 ml). Iz takvog uzorka nije moguće utvrditi validan kvalitet spermatozoida/ejakulata, jer krvne ćelije maskiraju spermatozoide. Ipak, iz znatiželje, uzorak kapi ejakulata je postavljen na mikroskopsku predmetnicu i mikroskopiran. U uzorku je pronađeno mnoštvo krvnih ćelija i tek po koji spermatozoid (uglavnom statičan), ali primećeno je nešto drugo – prisustvo žive forme, duguljastog oblika, veoma slično larvalnoj formi parazita *Dirofilaria sp*. Narednog dana, od istog psa je uzet uzorak periferne krvi radi analize na prisustvo diorfilarioze. Rezultati ELISA testa za antigen *Dirofilaria immitis* su bili negativni, što znači da adultnih formi parazita nema. Međutim, Knott testom utvrđeno je prisustvo larve *Dirofilaria repens.*

**Ključne reči:** mužjak, pas, kvalitet semena**,** *Dirofilaria repens,* Knott test

## INTRODUCTION

Today, there is a growing demand for dogs of different breeds with superior genetic traits meaning the good exterior traits (constitution, color, temper, etc.) and health status in order to obtain a better offspring. This especially means the exclusion of hereditary diseases in these animals, such as dysplasia,

blood coagulation disorders, diseases of the eye and the heart and other health problems that can cause a lot of pain to dogs and their owners / breeders, a lot of worry and economic costs.

Quality control of the semen in dogs has been showing increasing tendency among the dog keepers and breeders. More often, dog owners or breeders decide to test the semen of males before natural mating or artificial insemination of bitches. Over the time, dog breeding has become a lucrative business. Therefore, before the purchase of the male dog, some customers require from the breeder/owner to provide the semen analysis results obtained in accredited laboratories. Since 2016, Scientific Veterinary Institute „Novi Sad" (NIV-NS), laboratory for domestic animal reproduction, is accredited for the assessment of animal semen quality, using CASA - Computer Assisted Sperm Analysis. On this occasion, the owner brought a dog to determine the quality of the semen and received a result on dirofilariosis.

Dirofilariosis is a vector borne disease, transmitted by a mosquito bite. Disease reservoirs are dogs and wild canids but the disease can also be transmitted to humans. Climatic, ecological, and many other factors have an influence on *Dirofilaria sp*. transmission. Dogs and wild canids can be definitive hosts for both types of *Dirofilaria sp*. On the other side, less well adapted or "aberrant" hosts are cats, wild felids and other mammalians (Potkonjak et al., 2020). There are two forms of dirofilariosis. One is caused by *Dirofilaria immitis* (Heartworm disease) where the adult worms migrate into the heart of a dog and cause serious problems, very often leading to the death of a dog. The other form caused by *Dirofilaria repens* is much milder form of the disease yet much more difficult to identify (Capelli et al., 2018). The disease caused by *D. repens* has almost no symptoms in dogs. Adult worms are imbedded under the skin and can persist there for a long time producing new larvae. Dogs can express nervousness or itchy feeling, if anything at all. Rarely, some cutaneous manifestations such as pruritus, dermal swelling and subcutaneous nodules can occur, or ocular conjunctivitis can be observed depending on the site where adult *Dirofilaria* are imbedded. It is important to emphasize that *D. repens* infection can be transmitted to people causing a zoonotic form of dirofilariosis (Genchi and Kramer, 2017).

Both forms of dirofilariosis have been identified and reported by many authors in Serbia (Tasić et al., 2003; Savić-Jevđenić et al., 2004; Savić et al., 2012; Spasojević Kosić et al., 2012; Krstić et al., 2017). Human cases of dirofilariosis have also been detected and reported in Serbia (Džamic et al., 2004; Krstić et al., 2017).

## CASE PRESENTATION

Male dog of Doberman breed, 9 years old, was brought in for semen collection and quality control. By observing the general condition of the dog, the weakness of posterior legs was noticed. The dog had a strong libido and the process of taking ejaculate passed without difficulty. However, the ejaculate was full of substantial amount of fresh blood (total ejaculate volume was 5 ml). The assumption was that a small blood vessel was hurt during the process of semen intake. It was explained to the dog owner that it is not possible to assess the semen quality from that sample because the blood cells mask the spermatozoa and white blood cells weaken the sperm. They release substances, which destroy microorganisms that cause infection but also affect the sperm by destroying the sperm membrane, impair sperm movement, and damage sperm DNA. The result obtained from such kind of sample would not be valid. Nevertheless, the sample of ejaculate drop was placed under the microscope for observation and analysis (USB 200i light microscope at 100× magnification - Proiser, Paterna, Spain).

On the next day, blood sample was taken from the dog for analysis to dirofilariosis using modified Knott test and ELISA antigen test for *Dirofilaria immitis*. Modified Knott test was used for the detection of *Dirofilaria sp*. circulating microfilariae (Bazzocchi et al., 2008). Morphological characteristics of microfilariae (cephalic and caudal ends) were used in order to differentiate *D.immitis* and *D.repens* microfilariae of the two *Dirofilaria* species (Genchi et al., 2007). ELISA test was used for the detection of *Dirofilaria immitis* female adults' antigen.

The semen contained a lot of blood cells and just a few spermatozoids (mostly not moving). The whole semen sample was of dark red color resulting from excess blood in the sample. In the same semen sample, something else was seen - a live motile organism, long-shaped, looking very much like a larval stage of a parasite *Dirofilaria sp.* (Figure 1). The owner was immediately notified that his dog is suspected for dirofilariosis and that it would be recommendable to bring the animal for additional testing. The very next day, a blood sample was taken and analysed in the laboratory of Scientific Veterinary Institute „Novi Sad". The result of the analysis performed using ELISA test for *Dirofilaria immitis* antigen was negative, indicating that there were no *Dirofilaria immitis* adult worms present. The result of modified Knott test revealed the presence of larval stages of *Dirofilaria repens* (Figure 2) moving in blood drop under the microscope.

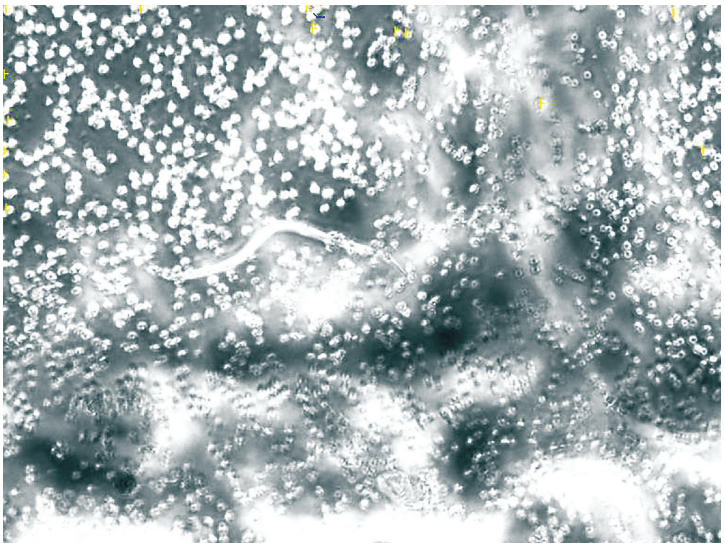

Figure 1. Photo of dog semen quality testing on CASA instrument.

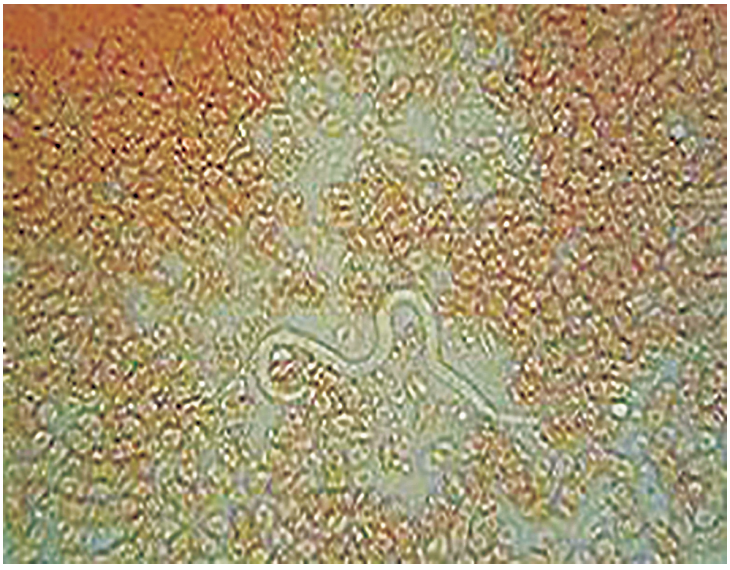

Figure 2. Photo of microscopic examination of dog blood – Knott test, 100× magnification

## DISCUSSION

Previous study on the prevalence of dirofilariosis in pet dogs in Novi Sad has shown the increase of *Dirofilaria immitis* infection and a decrease of infection with *Dirofilaria repens* (Spasojević Kosić et al., 2012, 2014) as compared to the first report on the prevalence of *Dirofilaria repens* infection (Tasić et al., 2008) and mixed infection with both parasites in dogs (Spasojević Kosić et al., 2014). Epidemiological importance of dirofilariosis in dogs caused by *Dirofilaria repens* lays in the zoonotic potential of the disease. The study of Spasojevic et al has shown that seroprevalence of *Dirofilaria repens* was dropping during the period from 2010 to 2016, while that of *Dirofilaria immitis* was rising in the same period (Spasojević Kosić et al., 2016).

A group of authors (Savić-Jevđenić et al., 2004) detected dirofilariosis caused by *D. immitis* in dogs in the region of Novi Sad, while another group of authors (Tasić et al., 2003) established dirofilariosis caused by *D. repens, D. immitis* and *Dipetalonema reconditum* in dogs in the same region. There is no doubt that the region from which the dog from our case study originates is endemic for dirofilariosis. *Dirofilaria repens* in dogs is pretty often neglected. A lot of the owners have only heard about *Dirofilaria immitis* as the causative agent of a heartworm disease because of its „deadly nature" for dogs. As compared with the disease caused by *Dirofilaria immitis*, dirofilariosis caused by *Dirofilaria repens* is most frequently asymptomatic and remains undiagnosed because the dog-owners do not recognize that there is something wrong. Another problem is the lack of commercial test for testing the dogs for the presence of *Dirofilaria repens*. There are no ELISA or fast/snap tests that can be used for *Dirofilaria repens*. Most commonly, the diagnosis is established unintentionally and the only option is to perform modified Knott test on blood sample for the identification or (when possible) a PCR for definite confirmation of the pathogen.

The reports of human ocular and subcutaneous dirofilariosis in Serbia published so far (Džamić et al., 2004) have confirmed the importance of dirofilariosis caused by *Dirofilaria repens* in human medicine in our country. Some of the authors identified adult *D. repens* worms in peritoneal location, that is, in the scrotum of dogs. During castration procedure, they were found protruding out from incised tunica vaginalis. The worm infected dogs had veins congestions, widening of the cavernous spaces of testes and epididymis, as well as thickened and enlarged epididymis along with interductal fibrosis. No significant effects on the process of spermatogenesis were observed (Ravindran et al., 2016). In a dog from our case study, there were no clinical symptoms of

the same kind. However, the general condition of the dog was changed; the dog was nervous, uncomfortable, with week posterior legs. The semen sample showed very few spermatozoids with a very low, almost none activity. At this point, it cannot be stated whether the clinical status of the dog is a consequence of *Dirofilaria repens* infection. The dog will be subjected to therapy and observed during next several months in order to confirm the clinical status.

## AKNOWLEDGEMENT

This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia, grants TR 31084.

## Authors' contributions

JA, SS, BL and AP made contributions to conception and design of the study, involved in data collection and drafting the manuscript. MŽ, AM and TB carried out the acquisition of sample, microscopic and serological tests and data analysis. SS revised the manuscript critically and together with JA prepared the final draft of the manuscript. All authors read and approved the final manuscript.

## Competing interests

The author(s) declare that they have no competing interests.

## REFERENCES

1. Bazzocchi C., Mortarino M., Grandi G., Kramer L.H., Genchi C., Bandi C., Genchi M., Sacchi L., McCall J.W. 2008. Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against *Dirofilaria immitis* in experimantally infected dogs. International Journal of Parasitology, 38, 12, 1401-1410. doi: 10.1016/j.ijpara.2008.03.002.
2. Capelli G., Genchi C., Baneth G., Bourdeau P., Brianti E., Cardoso, Danesi P., Fuehrer H.P., Giannelli A., Ionică AM., Carla M., Modrý D., Montarsi F., Krücken J., Papadopoulos E., Petrić D., Pfeffer M., Savić S., Otranto D., Poppert S., Silaghi C. 2018. Recent advances on *Dirofilaria repens* in dogs and humans in Europe. Parasites Vectors, 11, 663. doi: 10.1186/s13071-018-3205-x.
3. Džamić A.M., Arsić-Arsenijević V., Radonjić I., Mitrović S., Marty P., Kranjčić-Zec I.F. 2004. Subcutaneous *Dirofilaria repens* infection of the eyelid in Serbia and Montenegro. Parasite, 11, 2, 239-40.

4. Genchi C. and Kramer L. 2017. Subcutaneous dirofilariosis (*Dirofilaria repens*): an infection spreading throughout the old world. Parasites Vectors, 10, 2, 517. doi: 10.1186/s13071-017-2434-8.
5. Genchi C., Venco, L., Genchi M. 2007. Guideline for the laboratory diagnosis of canine and feline Dirofilaria infections. In Mappe Parassitologiche 8. Eds. C. Genchi, L. Rinaldi, G. Cringoli, Rolando Editore, Naples, 137-144.
6. Krstić M., Gabrielli S., Ignjatović M., Savić S., Cancrini G., Ranđelović G., Momčilović S., Stojnev S., Otašević S. 2017. An appraisal of canine and human cases reveals an endemic status of dirofilariosis in parts of Serbia. Molecular and Cellular Probes, 31, 37-41. doi:10.1016/j.mcp.2016.08.005.
7. Potkonjak A., Rojas A., Gutierezz R., Nachum-Biala Y., Kleinerman G., Savić S., Polaček V., Pušić I., Haruss S., Baneth G. 2020. Molecular survey of *Dirofilaria* species in stray dogs, red foxes and golden jackals from Vojvodina, Serbia. Comparative Immunology, Microbiology and Infectious Diseases, 68, 101409. doi: 10.1016/j.cimid.2019.101409.
8. Ravindran R., Julie B., Swapna S.A., Jerin F., Lenka D., Nandakumar S., Sabu S.M. 2016. Research Note *Dirofilaria repens* in scrotum of dogs. Tropical biomedicine, 33, 4, 842-846.
9. Savić S., Vidić B., Grgić Ž., Jurišić A., Ćurčić V., Ruzić M., Lolić Z. 2012. Vektorske zoonoze pasa u Vojvodini. Archives of Veterinary Medicine, 5, 1, 77-87. doi:10.46784/e-avm.v5i1.164.
10. Savić-Jevđenić S., Vidić B., Grgić Ž., Milovanović A. 2004. Brza dijagnostika dirofilarioze pasa u regionu Novog Sada. Veterinarski glasnik, 58, 5-6, 693-8.
11. Spasojević Kosić Lj., Lalošević V., Lalošević D., Simin S., Vasić I., Kuruca Lj. 2012. Prevalence of dirofilariosis in pet dogs in Novi Sad. Contemporary agriculture, 61, 3-4, 247-254.
12. Spasojević Kosić Lj., Simin S., Lalošević V., Lalošević D., Kuruca Lj., Nikolić S., Nerac D. 2014: Updating the prevalence of dirofilariosis in pet dogs in Novi Sad, Vojvodina, Serbia, Contemporary agriculture, 63, 4-5, 487-493.
13. Spasojević Kosić Lj., Lalošević V., Simin S., Kuruca Lj. 2016. Dirofilariosis and Angiostrongilosis in pet and hunting dogs in Novi Sad, Vojvodina, Serbia. Archives of Veterinary Medicine, 9, 2, 53-62. doi: 10.46784/e-avm.v9i2.89.
14. Tasić A., Katić Radivojević S., Klun I., Mišić Z., Ilić T., Dimitrijević S. 2003. Prevalencija filarioza pasa u nekim područjima Vojvodine. U Zbornik radova i kratkih sadržaja, 15. Savetovanje veterinara Srbije. Zlatibor, Srpsko veterinarsko društvo, 172.

15. Tasić A., Rossi L., Tasić S., Miladinović-Tasić N., Ilić T., Dimitrijević S. 2008**.** Survey of canine dirofilariasis in Vojvodina, Serbia. Parasitology Research, 103, 1297-1302. doi: 10.1007/s00436-008-1132-z.

Submitted: 16.01.2020.
Accepted: 16.06.2020.

# Note for Contributors

**ARCHIVES OF VETERINARY MEDICINE (AVM)** is a journal of the Scientific Veterinary Institute "Novi Sad" in Novi Sad. The journal publishes original research papers and review articles, short communications and case reports. The journal is devoted to the advancement and dissemination of scientific knowledge concerning veterinary sciences, biotehnology and related academic disciplines. The journal is published in the English language, with an abstract in English and Serbian language. All manuscripts are sent for a review and evaluation. In the case the reviewer suggests additional changes, the manuscript will be sent to the first author with a kind request to change the manuscript. In the case the author does not want to change, appropriate argumentation should be given. Final decision on accepting the manuscript is given by the Editor in Chief or Deputy Editor.

By submitting a manuscript, Authors warrant that their contribution to the Journal is their original work, that it has not been published before, that it is not under consideration for publication elsewhere, and that its publication has been approved by all co-authors, if any, and tacitly or explicitly by the responsible authorities at the institution where the work was carried out. Authors are exclusively responsible for the contents of their submissions, the validity of the experimental results and must make sure they have permission from all involved parties to make the data public. Authors wishing to include figures or text passages that have already been published elsewhere are required to obtain permission from the copyright holder(s) and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the Authors. Authors must make sure that only contributors who have significantly contributed to the submission are listed as authors and, conversely, that all contributors who have significantly contributed to the submission are listed as authors.

Peer review process is managed electronically. Authors have the possibility to provide the contact details of one or two potential peer reviewers, but the final decision on the choice of reviewers is on Editor decision without any obligation to contact any of the Author-recommended peer reviewers. The Editor nominates peer reviewers (at least two). The submitted manuscripts are sent to the reviewers together with the instructions for reviewers and accompanying reviewers' evaluation form. Acceptance of the manuscript is based on the reviews and on the final decisions of the reviewers regarding the acceptability of the manuscript. A reviewer's decision is one of following: "Accepted as it is", "Accepted after minor revision", "Accepted after major compulsatory revision" or "Rejected". If there is marked discrepancy in the decisions between the reviewers, the Editor can send the manuscript to another reviewer for additional comments and further decision, or make the decision based on the existing information.

Names of the reviewers are not transmitted to the author (single blind review). The peer review process is expected, in general, to start within two weeks after the submission of the manuscript. The Corresponding Author must indicate clearly in a separate letter what corrections have been made in response to the reviewers' comments point by point, and giving the relevant new line numbers using *Final show markup* view under Track Changes in MS Word Review ribbon. Corrected manuscripts are expected to be returned within three weeks. Accepted manuscripts are published on **https://niv.ns.ac.rs/e-avm/index.php/e-avm/issue/current** and then selected for a subsequent print issue.

The Journal *Archives of Veterinary Medicine* (AVM) uses open-source software for the management of peer-reviewed academic journals, the *Open Journal System (OJS)*, created by the Public Knowledge Project (PKP), released under the GNU General Public License. Instructions for the submission using OJS 3.0 are available on the Open Journal Systems web page (OJS 3.0 User Guide" © by PKP **https://openjournalsystems.com/ojs-3-user-guide/**).

**General notes**

The manuscript must be written in clear and concise English, and should be checked by a native-English speaker or certified English instructor with a good understanding of scientific terminology. The complete manuscript must be prepared according to instructions given in this section. Please read all instructions carefully. A manuscript that has not been prepared in accordance with the Journal's instructions for authors will not be considered for review.

All manuscripts submitted to *Archives of Veterinary Medicine*, must be accompanied by a Cover letter (see below). Also, authors are required to submit a scanned copy of the signed original, Competing interests, Authors contribution, and License to Publish which can be downloaded from the Journal homepage (see below).

Approval of the ethics committee must be obtained prior to the start of the study and be available upon request. Authors report this approval in their manuscript in the first paragraph of the Materials and Methods section, including the name of the regulatory group, reference number and date of the approval.
*Examples:*

*Ethical approval*: The research related to animal use complied with all the relevant national regulations and institutional policies for the care and use of animals (name of regulatory group, number and date).

*Ethical approval*: No ethical approval was obtained because this study did not involve laboratory animals and only involved non-invasive procedures (e.g. collection of waste tissue after surgery, fecal samples, voided urine etc.)

Registration and/or Login are required to submit items online and to check the status of current submissions. Please note that by signing up and submitting your manuscript to *Archives of Veterinary Medicine* you agree to the Journal's terms and conditions. All correspondence, including notification of the Editor's decision and requests for revision, is done by e-mail. The Journal submission system guides you stepwise through the process of entering your article details and uploading your files. Editable files (Microsoft Word, .doc, or .docx format) are required to typeset your article for final publication. The text of the submitted manuscript should be written in good English language. The Editor reserves the right to amend submitted manuscripts to a suitable standard of formal, written English. Manuscripts are pre-evaluated at the Editorial Office in order to check whether they meet the basic publishing requirements and quality standards, and authors will be notified by email upon receiving their submission. Only those contributions which conform to the following instructions can be accepted for peer-review. Otherwise, the manuscripts will be returned to the authors with comments / suggestions.

Online submission (**https://niv.ns.ac.rs/e-avm/index.php/e-avm**)

- If you already have a username and password for Archives of Veterinary Medicine continue to Login Page (**https://niv.ns.ac.rs/e-avm/index.php/e-avm/login**).
- If you are not registered with this site, you need to fill in and submit the form avalable on Registration Page (**https://niv.ns.ac.rs/e-avm/index.php/e-avm/user/register**).

**Layout of the manuscript, Style and Form**

The paper should be in Word program, Latin characters, size 12 pt., Times New Roman with justified alignment, double spaced. Left and right margins 20 mm, top and foot margins 30 mm, paper size A4. The journal requires continuous line numbering in submitted manuscripts. Number the pages consecutively with the title page being page 1. If special symbols are used, use font Symbol. The manuscript should be submitted on paper size A4 in electronic form. Pagination on the right side, starting from the title page.

The journal publishes the following types of articles: Original research articles, Review articles, Short communications and Case reports.

***Original research articles*** describe the original research, previously unpublished and includes the following sections: Title Page, Abstract with Key words, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgments, References, Tables/Figures (with legends). The writing style should be concise but clear enough to enable readers to reproduce the experimental methodology as described. The article must present a clear hypothesis and data expressed according to an appropriate experimental design. The Original research article should not contain more

than 40 references, and usually have no more than 5000 words.

***Review articles*** represent analysed and discussed summaries of different topics in the area of veterinary medicine, biotechnology and related scientific disciplines which are of broad interest to the target audience of *Archives of Veterinary Medicine*. The authors of a Review articles should be experts on the subject of the review, as proven by their own published peer-reviewed papers and by citations of these papers in the submitted review. It is essential that the authors of a review article are cited as authors of at least five other articles, published in Journals from the SCI list, addressing the same subject as the review article submitted. A review contains: Title Page, Abstract with Key words, Main Text, Conclusion, Acknowledgements, References, Tables/Figures/ Illustrations (with legends). The main text in reviews preferably has less than 6000 words.

***Short communications*** represent the concise but complete descriptions of limited investigations, which will not be included in a later full research article. A short communication usually contains the following sections: Title Page, Abstract with Key words, Introduction, Materials and Methods, Results and Discussion, Conclusion, Acknowledgments, References, Tables/Figures/Illustrations (with legends). A short communication should not contain more than 15 references and preferably has less than 3000 words.

***Case Reports*** are reports describing a novel case (or set of cases) with detailed documentation and diagnostic investigation. Authors are encouraged to describe how the subject of the Case report is novel or unusual in the covering letter that will accompany the submission of the Case report. Case reports usually contain the following sections: Title page, Abstract with Key words, Case presentation, Discussion, Acknowledgements, References, Tables/Figures/Illustrations (with Legends). Case reports usually have no more than 2500 words, and should not contain more than 15 references.

**Title page**

On the title page the following should be written:

- The title of the paper in capital letters, without underlining and abbreviations
- The names of the authors (first name and surname, followed by a comma).
- Above the surname place a number in superscript (1,2,3) that denotes the institution where the author works (affiliation).
- Full name of the institutions should be given below the list of authors (the following order should be considered: university or organization/institution, department, city, and country for all authors).
- One of the authors must be designated as the Corresponding Author (add an asterisk next to the Author's surname). Full contact details including postal address, and e-mail address for the corresponding author must be provided at the bottom of the Title page.

**Abstract**

An Abstract not exceeding 300 words must be provided with and three to eight key words. It should contain and summarize the most important facts from the submitted manuscript. For Original research articles, Short Communications and Case reports the material and techniques used must be mentioned without going into methodological detail, and the most important findings must be summarized. An Abstract for a Review paper *should contained* background, key findings and/or conclusions. Citations, tables and specialist abbreviations are not included in any Abstract.

Authors from Serbia must also submit an Abstract and Key words in Serbian, including the title of the paper, list of authors and their affiliations on a separate page after References. Translation of the Abstract and Key words from English to Serbian language will be performed by the Editorial Office for Authors who are not fluent in Serbian.

**The main Text**

All the text headings should be typed in bold capital letters, and the subheadings, where necessary should be in bold italic with only an initial capital letter. Use short and concise sentences. Name the drugs as their International Nonproprietary Names (so called generic names). If the name of a specific drug is to be stressed, name it together with the producer (in brackets). The names of devices write as used in trade (name of the producer in brackets). When using an abbreviation for the first time, write the words that stand for. Abbreviations cannot be used in the title and summary. Text should not be longer than 8 pages. Avoid long enclosures.

***Tables and Figures***

Tables and Figures (graphs, images, schemes etc.) must not be integrated into the main text. Instead, they must be submitted as separate, supplementary files. Tables and Figures are numbered consecutively using Arabic numerals (Figure 1, Figure 2, Figure 3, Table 1, Table 2, Table 3, *etc.*). Results which can be described as short statements within the text must not be presented as Figures or Tables. Data must not be replicated in Tables and Figures. The titles and legends help make the tables and figures understandable without the reader having to refer to the main text. However, they must also be concise and are not used to re-describe the methodology. Appropriate numbers and titles for tables and numbers and legends (including titles and explanations of markings) for figures are typed single line spaced using Times New Roman, 12 pt, in the main text and placed next to the relevant text in the article to enable the Editors to place them properly.

*Tables* are submitted as one document, with one table per page. Tables should be submitted as editable text and not as images. Each table is prepared on a separate page

and is appropriately numbered with a short, descriptive table title placed above. Tables can include footnotes, which are placed below the table and explain any abbreviations. Use superscript Arabic numbers (1, 2, 3 etc., in the correct order of reading the table, from top left to bottom right) to link each footnote to the appropriate item in the table requiring explanation.

Each *Figure* (graphs, images, schemes etc.) is submitted as a separate file. Uppercase letters A, B, C, etc. should be used to identify parts of multipart figure. Images must be at resolution 300 dpi or higher and allowable formats – JPG, TIFF. For microphotographs, scale bars with appropriate units must be provided. Symbols, arrows or letters used in photographs should contrast with the background. Legends for figures do not appear in the figures. However, when uploading the figures in the submission procedures, please ensure that the files are appropriately identified as Figure 1, Figure 2, etc. Legends for figures include both the figure number and title and explanations of any symbol, line or marking used in the figure and are placed at the appropriate location within the main text.

**Headings**

Headings in the paper are: **INTRODUCTION, MATERIAL and METHODS, RESULTS, DISCUSSION (or RESULTS and DISCUSSION for Short Communications), CONCLUSION and REFERENCES**. For Review articles instead M&M, Results and Discussion section, the Main text section should subdivided by different subheadings describing topics presented/discussed/analysed. For Case studies this part could be the same or replaced by Case presentation part with or without different subheadings.

**INTRODUCTION** points on the most important, i.e. most recent data regarding the topic with a short presentation of the aims of this research. The essence of the problem and the purpose of the study should be pointed to in this section. The key aspects of the published literature and research should be reviewed. A detailed literature survey or a summary of the results should be avoided. The last part of the Introduction must contain the specific aim(s) of the study.

**MATERIAL and METHODS**. Here describe the conditions in the experiment, name the used methods, material and animals. The Ethical statement (or description why ethical statement is not applicable) should be provided at the beginning of the section. This section includes, as appropriate, a description of study design, experimental animals or data about the samples used, analytical methods and statistical analyses. Identify the methods and procedures in sufficient details to allow others to reproduce the study. If methods are widely known, they are not described, but appropriate references must be cited. For new methods, the detailed protocols for the method should be included. Authors must provide references for established methods including sta-

tistical methods. Specify any general computer program used. Identify all drugs and chemicals used with generic or chemical names, doses and route of administration. For diagnostic kits/reagents and instruments used in the study provide manufacturer, product number, city and country where applicable.

**RESULTS**. Results are presented in a logical order and in parallel with the Methods (for every Method, there should be a Result), using text descriptions and Tables and/or Figures without duplicating the results between these formats. To enhance clarity, this section can be divided into subsections, each with a concise subheading in italics and which provides details of findings that are required to support the conclusions made in the manuscript.

**DISCUSSION**. Here give analyses of the obtained results comparing to the results and opinions of other authors, pointing the importance of this research, without giving a conclusion. The Discussion section is not used to summarize current knowledge. The Discussion should clearly identify the main Conclusions of the study. Authors are to provide a clear explanation of the importance and relevance of these Conclusions. Any new information should be distinguished from the previous findings, and relevant hypotheses can be generated.

**CONCLUSION.** Here the authors gives his final conclusions. This part consists of a short integration of Results that refer directly to the stated aim(s) of the study and a statement on the practical implications of the results. The statements and conclusions not supported by data in the presented research should be avoided. This section should not exceed one short paragraph. Do not summarize the Discussion or current knowledge in this section.

**ACKNOWLEDGEMENT.** The source of funding for the study should be stated in this section. Also, those who have made a substantial contribution to the study in terms of design, execution, analysis or manuscript drafting/revision but do not fit the criteria for authorship should be mentioned in this section. It is the responsibility of the Authors to ensure that those being acknowledged have agreed to being named in this part.

**REFERENCES**

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). The author should list the references, preferably the most recent one. Only essential references should be included in the text, and these are restricted to the necessary minimum. Cite in-text references by surname and year in parentheses (e.g. Petrović, 2014). In the case of two authors, use the word "and" (e.g. Petrović and Savić, 2015). The abbreviation "et al." must be used in all cases where more than two authors are cited (e.g. Lazić et al., 2016). Multiple references within parentheses in the text are cited in chronological order (e.g. Petrović, 2014; Petrović and Savić, 2015;

Lazić et al., 2016). In case the same author has more publications in the same year, additional letters are added next to the year (Savić et al., 2008a; Savić et al., 2008b).

In the list of **REFERENCES,** references should be numbered with Arabic numerals, one under the other, written in alphabetical order (use the A-Z sort tool) according to the surname of the first author. In general, the number of references is not limited, but only essential references should be included in the text, and these are restricted to the necessary minimum.

**Examples of references:**

**Articles in journals:**

1. Stojanović D., Maličević Ž., Ašanin R. 2002. The use a new model for the investigation of sepsis. Acta Veterinaria, 52, 2/3, 125-131.
2. Chen J. and McClane B.A. 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infection and Immunity, 83, 2369–2381. doi:10.1128/IAI.03136-14.
3. Williams R.B. 2015. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathology, 34, 159-180. doi: 10.1080/03079450500112195.
4. Bailey M.A., Macklin K.S., Krehling J.T. 2013. Use of a multiplex PCR for the detection of toxin-encoding genes *netB* and *tpeL* in strains of *Clostridium perfringens.* ISRN Veterinary Science, Article ID 865702, 1-4. doi:10.1155/2013/865702.

**Books:**

5. Ficken, M. D. and Wages, D. P. 1997. Necrotic enteritis in Diseases of Poultry, Eds. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, Iowa State University Press, Ames, Iowa, USA, 10th edition, ISBN xxx-xxx-xxxxx-xx-x.

**Chapters in books:**

6. Plumb J.A. and Hanson L.A. 2011. Sturgeon viruses. In *Health maintenance and principal microbial diseases of cultured fishes*. Eds. J.A. Plumb, L.A. Hanson, 3rd edition, Blackwell Publishing, 219-225.

**Articles in proceedings:**

7. Giangaspero A., Marangi M., Pati S., Cafiero M.A., Camarda C., Sparagano O.A.E. 2011. Investigating the presence of acaricide residues in laying hens naturally infected by the red mite *Dermanyssus gallinae*. In *Book of Abstracts*, The 12th Asian food conference 2011, BITEC Bangna, Bangkok, Thailand, 27.
8. Vidanović D., Petrović T., Šekler M., Debeljak Z., Vasković N., Matović K., Plavšić

B., Dmitrić M. 2018. Avian influenza in Serbia: epidemiological situation during 2016–2017. In *Programme and Abstract book*, 11th International Congress for Veterinary Virology, 12th Annual Meeting of EPIZONE, 27-30.08.2018, University of Veterinary Medicine Vienna, Vienna, Austria, 118 (p187).

9. Lazić G., Lazić S., Bugarski D., Grubač S., Lupulović D., Samojlović M., Petrović T. 2018. Human enteroviruses in river water and sewage in Vojvodina. In *Book of Abstracts*, International Scientific Conference "Green economy and environment protection", Belgrade, 23-25. April 2018, edited by Larisa Jovanović, Belgrade, Naučno stručno društvo za zaštitu životne sredine "ECOLOGICA", 95-96. ISBN 978-86-89061-11-6.

**Lows and Regulations:**

10. European Union. 2003. Commission Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official Journal of the European Union, L 268:29. **https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2003R1831:20100901:EN:PDF**

**Citations with organisations as authors:**

11. European Food Safety Authority. 2016. Peer review of the pesticide risk assessment of the active substance benzoic acid. EFSA Journal, 14(12):4657-n/a. **http://dx.doi.org/10.2903/j.efsa.2016.4657.**

**Software:**

12. Statistica (Data Analysis Software System). 2006. v.7.1., StatSoft, Inc., USA (**www.statsoft.com**).

**Web Links:**

13. OIE: Animal Diseases. Available at: **http://www.oie.int/en/animal-health-in-the-world/information-on-aquatic-and-terrestrial-animal-diseases/.** Accessed 07.08.2019.
14. European Centre for Disease Prevention and Control (ECDC). Historical data by year - West Nile fever seasonal surveillance. Available at: https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical Accessed 31.07.2019.

**Cover letter**

All submitted manuscripts must be accompanied by a cover letter which explains why the manuscript is suitable for publication in *Archives of Veterinary Medicine* and declaring that the manuscript has not been submitted or accepted for publication else-

where. Authors should suggest one or two potential unbiased reviewers who are qualified to review the manuscript (reviewers' affiliations with email addresses, and briefly explain why authors think the suggested scientists would be good reviewers). This letter must warrant that all authors have seen and approved the content.

**Authors' contributions**

In order to give appropriate credit to each author of a paper, the individual contributions of authors to the manuscript are specified by initials in this section. An author is generally considered to be someone who has made significant contribution to a published study. All contributors who do not meet the criteria for authorship should be listed in the Acknowledgements section.

**Competing interest**

A competing interest exists when your interpretation of data or presentation of information may be influenced by your personal or financial relationship with people or organizations. Authors are required to complete a declaration of competing interests. All competing interests that are declared will be listed at the end of published articles. Where an author gives no competing interests, the listing will read 'The author(s) declare that they have no competing interests'.

**Note**

A paper that is not in accordance to the aforementioned instructions will not be sent for a review and will be returned to the authors for corrections.

**Copyright**

Once the manuscript is accepted for publication, authors assign a *License to Publish*. Authors retain copyright of the published papers and grant the publisher the right to publish the article, to be cited as its original publisher in case of reuse, and to distribute it in all forms and media. The final proof will be sent to the Corresponding Author, together with a *License to Publish*, which all authors have to sign. When an issue is completed, a hard copy of the issue will be produced as well as electronic copy on the Journal webpage. If the submitted manuscript is not accepted for publication by the journal, all rights to the manuscript will be retained by the author(s).

Articles published in *Archives of Veterinary Medicine* will be Open-Access articles distributed under a **Creative Commons Attribution International License** (CC BY 4.0).

**Address of the journal**

Scientific Veterinary Institute "Novi Sad",
21000 Novi Sad, Rumenački put 20
Tel. 381 (0)21 4895 321
E-mail: **arhiv@niv.ns.ac.rs**
Web: **https://niv.ns.ac.rs/e-avm/**
Account No: 355-1006444-18 Vojvođanska banka,
ID No.: 08608857,
PIB 100236555

**Privacy Statement**

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purpose or to any other party.

# Beleške / Notes

# Beleške / Notes

# Beleške / Notes

# Beleške / Notes

# Beleške / Notes

UDK 619(051) ISSN 1820-9955

**Archives of Veterinary Medicine**
**Arhiv veterinarske medicine**

Vol. 13 No. 1 str. 1-136 Novi Sad, 2020.



Scientific Veterinary Institute „Novi Sad" Novi Sad
Naučni institut za veterinarstvo „Novi Sad" Novi Sad