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FANDING OF PARASITIC NEMATODES OF FISHES PRESENT IN THE MARKET

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Abstract

When placing the fish and fish products on the trade it is necessary to pay attention to the presence of zoonotic parasites that can lead to infection of people especially if the fish is consumed raw or undercooked. Epidemiologically the most important are helminths from the group of *Nematoda*. The most important are *Anisaxis* spp. and *Eustrongylides excisus*. Anisakiasis is a serious zoonotic disease with a dramatic increase in prevalence throughout the world in the last two decades. The larvae are found in marine fish species most frequently in sardines, herring and mackerel. Eustrongylidosis is a disease that occurs primarily in freshwater fish species: catfish, zander and pike, a cause is a parasite of the genus *Eustrongylides* of which the most important species is *Eustrongylides excisus.* In this paper, the nematodes that occur in fish that are present in the market in the Republic of Serbia are present. Investigations of *Anisaxis* spp. were carried out at the Institute of Veterinary Medicine Novi Sad in the period of 2000-2013. Total of 2414 samples of imported marine fish was inspected. In 25 (1.29 %) samples of herrings (*Clupea harengus*) and mackerels (*Scomber scombrus*) was identified *Anisakis* spp. Investigations of *Eustrongylides* spp . were carried out in the period 2010-2013 at the Danube- Tisa -Danube Canal. Samples were collected from zander (*Sander lucioperca*) and European catfish (*Siluris glanis*) in which the presence of nematodes was found in the abdominal cavity, muscles, the lumen of the stomach and gastric wall where the parasites were encapsulated. Individuals of zander were examined during the 2013 at the Veterinary Institute Novi Sad, where the presence of larvae *Eustrongylides* spp.u muscle was detected. In order to avoid infecting people with parasites of fish it is necessary to carry out continuous control and monitoring. Fresh

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fishes and traditional fish products must be inspected for the presence of parasites before they find on the trade. Continuous education is a key factor in combating zoonotic diseases. Avoid consumption of raw or poorly cooked fish is still the best preventive procedure.

Key words: zoonotic nematodes, *Anisakis* spp., *Eustrongylides excisus*, fish, market

NALAŽENJE PARAZITSKIH NEMATODA KOD RIBA NA TRŽIŠTU

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Kratak sadržaj

Prilikom stavljanja u promet i pregleda ribljeg mesa i proizvoda od ribe treba obratiti pažnju na prisustvo zoonotskih parazita koji mogu dovesti do inficiranja ljudi naročito ako se riba konzumira sirova ili termički nedovoljno obrađena. Sa epidemiološkog aspekta najveći značaj imaju helminti iz grupe *Nematoda*. Najznačajnije su *Anisaxis* spp. i *Eustrongylides excisus*. Anisakijaza je ozbiljno zoonotsko obolenje sa dramatičnim porastom prevalence širom sveta u poslednje dve decenije. Larve se mogu naći kod morskih vrsta riba najčešće kod sardine, haringe i skuše. Eustrongilidoza je obolenje koje se javlja kod slatkovodnih riba prvenstveno grabljivica: soma, smuđa i štuke, a izazivaju je paraziti iz roda *Eustrongylides* od kojih je najznačajnija vrsta *Eustrongylides excisus*. U ovom radu prikazane su nematode koje se javljaju kod riba koje su prisutne na tržištu u Republici Srbiji. Istraživanja prisustva *Anisaxis* spp. su sprovedena na Naučnom institutu za veterinarstvo Novi Sad u periodu 2000-2013. Ukupno je pregledano 2414 uzoraka riba iz uvoza od čega je u 25 (1.29%) utvrđen *Anisakis* spp., kod haringe (*Clupea harengus*) i skuše (*Scomber scombrus)*. Istraživanja prisustva *Eustrongylides* spp. su sprovedena u periodu od 2010-2013 godine na kanalu Dunav-Tisa-Dunav. Prikupljeni su uzorci konzumnog smuđa (*Sander lucioperca*) i soma (*Siluris glanis*) kod kojih je utvrđeno prisustvo nematoda u abdomenu, muskulaturi, lumenu želuca i želudačnom zidu gde su paraziti bili inkapsulirani. Jedinke smuđa pregledane se tokom 2013. godine i

na Naučnom Institutu za veterinarstvo Novi Sad gde je utvrđeno prisustvo larvi *Eustrongylides* spp.u muskulaturi. Kako ne bi došlo do zaražavanja ljudi zoonotskim parazitima riba neophodno je sprovoditi stalnu kontrolu i monitoring. Sveže meso ribe i tradicionalni riblji proizvodi pre nego što se nađu u prometu moraju biti pregledani na prisustvo parazita. Kontinuirana edukacija je ključni faktor u borbi sa zoonozama a izbegavanje konzumiranja sirovog ili termički slabo obrađenog ribljeg mesa i dalje najbolja preventivna procedura.

Ključne reči: zoonotske nematode, *Anisakis* spp., *Eustrongylides excisus*, ribe, tržište

INTRODUCTION

Zoonotic nematodes infect freshwater and marine fish species. Pathology normally occurs within the intestines but can affect all organs. Nematodes can infect fish as adults but larval stages of nematodes infecting piscivorous birds, mammals or reptiles, or less frequently predatory fish, can also infect fish species (Roberts and Janovy, 2000). Some nematodes are zoonotic. Among these parasite *Anisakis* spp. has the highest medical importance because of the severe allergic reactions and gastrointestinal symptoms it causes in humans after eating or handling infected fish or crustaceans (Lima dos Santos and Howgate, 2011). These symptoms are especially prevalent in countries where it is common to eat raw or undercooked fish. Anisakiasis a serious zoonotic disease with a dramatic increase in prevalence throughout the world in the last two decades. People who are usually accidental hosts infected when ingest the third larval stage of the parasite that can be found in the internal organs and muscles of a large number of marine and anadromous fish (sardines, herring, salmon, tuna, mackerel, etc.) (Bullini, 1997; Oliva, 1999). Definitive host of *Anisaxis* species are marine mammals commonly dolphins and whales in which intestines are adult forms of the parasite. The first intermediate hosts are marine crustaceans and second intermediate hosts are cephalopods and fishes. *Anisakis simplex* is present in Atlantic, Pacific, Mediterranean Sea, Norwegian Sea and Barents Sea. The highest prevalence of anisakiasis is found in nort Asia and western Europe (Netherlads, Germany, France and Spain) (Chai et al., 2005). In Serbia *Anisakis* can be present in imported marin fish.

The most significant freshwater fish zoonotic nemotods which occurs in Serbia are *Eustrongylides* species (Ljubojević et al., 2012: Novakov et al., 2013). Thay have complex life cycles involving a definitive host and two intermediate hosts. Definitive hosts include aquatic birds mostly from order Ciconiiformes family Ardeidae, Anseriformes, Gaviiformes and Pelecaniformes (Spalding

and Forrester, 1993; Measures 1988). First intermediate hosts for *Eustrongylides* spp. are aquatic oligochaetes (Spalding et al., 1993). Second intermediate hosts are planktivorous and benthivorous fishes that could pass the infection on to fishes (paratenic hosts) and finally on to fish-eating birds (Moravec, 1994). Such exposure is usually common in larger fish species, like channel catfish - *Ictalurus punctatus* or pike-perch - *Sander lucioperca*, which, as predators, become infected with *Eustrongylides* spp. nematodes. In fish, these parasites are conspicuous as long, red, coiled individuals located in the body cavity or embedded in the muscle (Mitchum 1995, Overstreet 2003).

In humans who have consumed raw or undercooked fish, *Eustrongylides* spp. have produced gastritis and intestinal perforation (Deardorff and Overstreet 1991; Cole 1999). Guerin et al., (1982) were the first to report a natural (accidental) human infection with *Eustrongylides* spp.

The goal of this paper is to distinguish the presence of *Anisakis* spp. and *Eustrongylides* spp. nematodes in marine and freshwater fish species that are present in the market and inland waters of Serbia and to indicate the need for adequate preparation of fish meat.

MATERIAL AND METHODS

Investigations of *Anisaxis* spp. were carried out at the Institute of Veterinary Medicine Novi Sad in the period of 2000-2013. Total of 2414 samples of imported marine fish was inspected. Each fish was eviscerated and carefully examined. In same cases muscles of fish were checked by method of artificial digestion.

Diagnostics and investigations of *Eustrongylides* spp. larvae were conducted from 2010-2013. Fifty two fish samples of European catfish *(Siluris glanis)* weighing 250-450 g and twenty one samples of zander (*Sander lucioperca*) were collected from different locations on Danube-Tisa-Danube Canal. Fishes were parasitologically examined through cutting the body and the abdominal part, digestive tract and other ventral organs. Samplaes of zander was also examined at the Institute of Veterinary Medicine Novi Sad during 2013. Collected nematodes were were fixed in 70% ethanol for 24 h and cleared in lactic acid for morphological observation. Relative parametars were measured and identification was performed using Bauer (1987), Moravec (1994) and Anderson (2000) keys.

RESULTS AND DISCUSSION

Anisakis spp was first isolated in the laboratory of Veterinary Institute of Novi Sad in 2003. On a total of 2414 samples of imported marine fishes, in 25 (1.29%) was found *Anisakis* spp. Presence of *Anisakis* spp. in samples of imported fish from 2000-2013 are shown in table 1 and chart 1. Coiled nematodes were present in herring (*Clupea harengus*) and mackerel (*Scomber scombrus*) (Fig. 1). Common to all positive samples is the presence of the viscera, because fishes were not eviscerated after fishing. Number of parasites ranged from 2-3 to 52 nematodes per fish. In two samples (0.08%) the presence of larvae in fish muscles was performed by the method of artificial digestion.

Table 1. Presence of *Anisakis* spp. in samples of imported fish.

Years	Number of inspected fish	Number of positive fish	% of positive fish
2000	12	0	0
2001	12	0	0
2002	17	0	0
2003	66	3	4.54
2004	268	3	1.2
2005	239	4	1.67
2006	212	1	0.41
2007	151	3	2
2008	177	3	1.69
2009	394	5	1.3
2010	440	2	0.45
2011	240	0	0
2012	148	0	0
2013	38	1	2.63

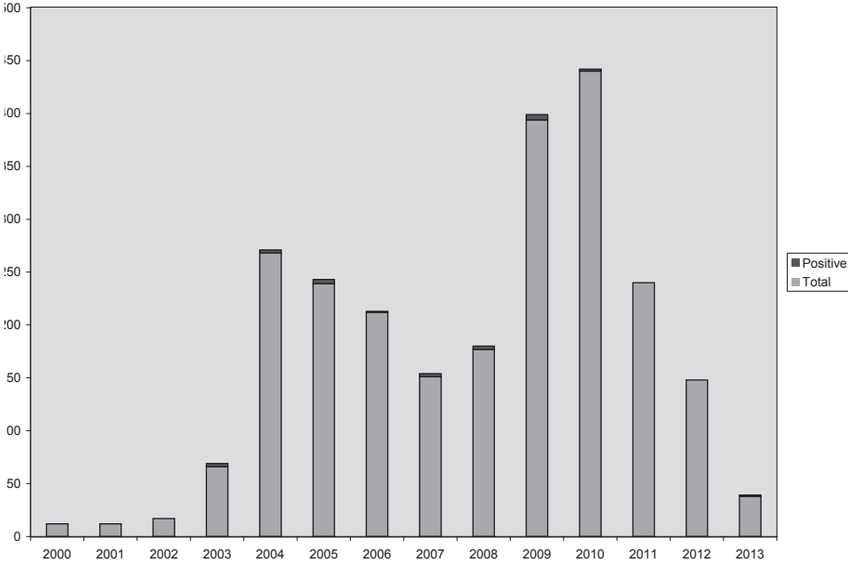

Chart 1. Presence of *Anisakis* spp. in samples of imported fish.

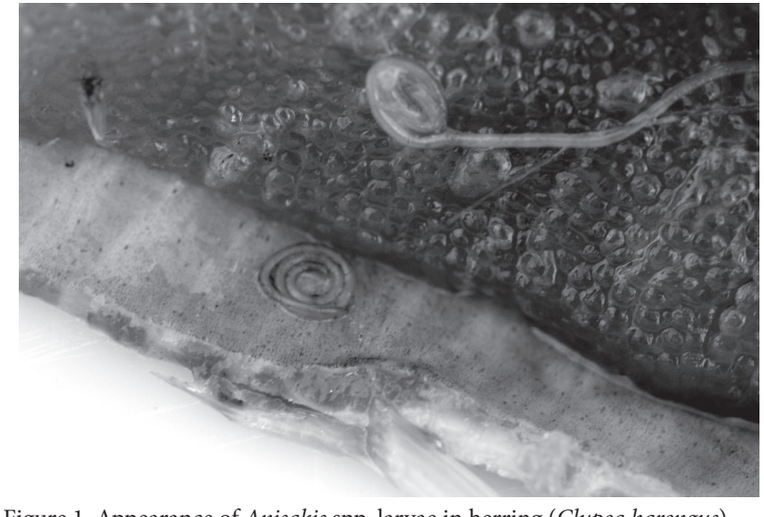

Figure 1. Appearance of *Anisakis* spp. larvae in herring (*Clupea harengus*).

Presence of *Eustrongylides* spp. nematodes was revealed in the abdominal cavity (Figure 2), musculature , in the lumen of the stomach and encapsulated

in stomach in 4 individuals of zander and 6 individuals of European catfish, what represented the prevalence of 14.26%, respectively 11.54%. During the 2013 at the Institute of veterinary medicine of Novi Sad larvae of *Eustrongylides* spp. were detected in the muscles of four samples of zander (Fig. 3). The number of parasites per fish ranged from a few up to the 256. This larva, was robust and pinkish red. Length of body was 27 – 60.5 mm, maximum width 0.49 – 0.58 mm.

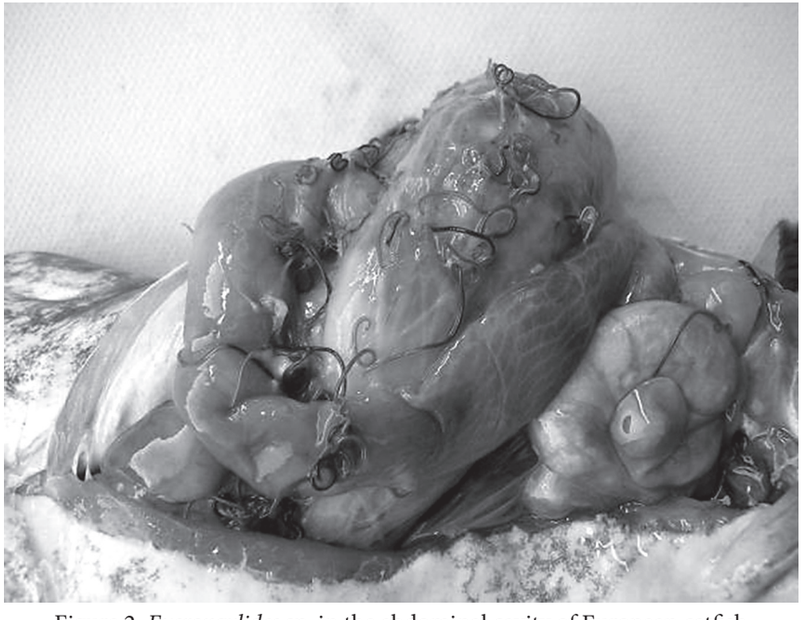

Figure 2. *Eusrongylides* sp. in the abdominal cavity of European catfish (*Siluris glanis*).

Figure 3. *Eusrongylides* spp. in the muscles of zander (*Sander lucioperca*).

Fish parasites such as *Anisakis* spp. and *Eustrongylides* spp. are highly important because they are capable to infect humans (Mohammad et al., 2011). Murrell (2002) suggested several control measures for preventing parasitic infections originating from freshwater, such as environmental control of surface water, hygienic aquaculture, and the control or elimination of the first intermediate hosts. FDA (2001) indicated that the effective methods for killing parasites are freezing, heating, adequate combination of salt content and storage time or hot smoking. On the other hand, brining and cold smoking may reduce the parasite hazard in fish, but they do not eliminate or minimize it to an acceptable level (Murrell, 2002). While health education is a key factor in combating zoonotic infections, experience in various countries has shown that for successful implementation of control measures, it is necessary, as Hughes (1992) points out, to have formal and informal cooperation between medical and veterinary interests at all levels of government, and with the community. According to Okumura et al., (1999) and Chieffi et al., (1992), the recommendation to avoid consumption of raw or poorly cooked fish is still the best preventive procedure.

Archives of Veterinary Medicine, Vol. 6, No. 2, 3-13, 2013.
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CONCLUSION

Zoonotic nematodes can lead to human infection and should pay special attention on them. Fresh fish meat and traditional fish product should be subjected to a visual examination for the purpose of detecting visible nematodes and other parasites before being placed in the trade. The consumption forms and the preparation of the fish food should be modified in a way that hazards to human health due to zoonotic parasites could be avoided. Health education is a key factor in combating zoonotic infections.

LITERATURE

1. Anderson R. C.**:** Nematode Parasites of Vertebrates Their Development and Transmission. 2nd Edition. *CABI Publising*, 2000.
2. Bauer, O. N.**:** Key for determination of freshwater fish parasites of SSSR. *Academy of science SSSR*. Lenjingrad. (Ru), 1987.
3. Bullini L.:Antarctic Communities: Species, Structure, and Survival. UK: Cambridge University Press, 1997.
4. Chai Jong-Y., Murell K.D., Lymberyc J.A.: Fish borne parasitic zoonoses: Status and issues, *International Journal for Parasitology*, 35, 1233-1254., 2005.
5. Chieffi P.P., Gorla M.C.O., Vieira Tores D.M.A.G.: Human infection by Phagicola sp. (Trematoda-Heterophyidae) in the municipality of Registro, São Paulo State, Brazil. *Rev. Inst. Med. Trop. São Paulo*, 32**,** 285-288., 1992.
6. Cole R.A**.:** Eustrongylidosis. In: *Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds* (ed. by M. Friend and J.C. Franson), pp. 223–228. Biological Resources Division, Information and Technology Report 1999– 2001, U. S. Geological Survey, Washington, DC, 1999.
7. Lima dos Santos C.A,M., Howgate P.: Fish born zoonotic parasites and aquaculture: A review, Aquaculture, doi: 10.1016/j.aquaculture. 2011.05.046., 2011.
8. Ljubojević D., Bijelić-Čabrilo O., Novakov N., Ćirković M., Davidov I., Jovanović M.: *Eustrongylides* sp. In freshwater fish species as a potential hazard for humans. Proceedings of the international conference BFSQ, Belgrade, Serbia 2012.
9. Deardorff T.L. and Overstreet R.M.: Seafood-transmitted zoonoses in the United States: the fishes, the dishes, and the worms. In: *Microbiology of Marine Food Products* (ed. by D.R. Ward & C.R. Hackney), pp. 211–265. Van Nostrand Reinhold, New York, 1991.
10. FDA,: Fish and Fisheries Products Hazards and Controls Guidance. 3rd Edition. Food and Drug Administration, Center for Food Safety and Appli-

ed Nutrition, Washington, DC, USA http://www.fda.gov/Food/Guidance Compliance Regulatory Information/Guidance Documents/Seafood/Fish and Fisheries Products Hazards and Controls Guide/default.htm, 2001.
11. Guerin P.F., Marapendi S., MC Grail L.: Intestinal perforation caused by larval Eustrongylides. *Morb. Mort. Week. Rep*., 31, 383-389., 1982.
12. Hughes K.L.: The impact of zoonotic diseases on the workforce and the community. pp. 301–313 in *Zoonoses.* Proceedings 194, Post Graduate Committee in Veterinary Science, University of Sydney, 1992.
13. Measures L.N.: Revision of the genus Eustrongylides Ja¨gerskio¨ld, 1909 (Nematoda: Dioctophymatoidea) of piscivorous birds. *Canadian Journal of Zoology* 66, 885–895., 1988.
14. Mitchum D.L.: Parasites of Fishes in Wyoming. Wyoming Game and Fish Department, Cheyenne, WY. Overstreet R.M. (2003) Presidential address: flavor buds and other delights. *Journal of Parasitology* 89, 1093–1107., 1995.
15. Mohammad R., Iraj M., Mahzad A. M., Behyar J., Bagher A. F., Saeed S.S.: Occurrence and intensity rate of internal Metazoan parasites in *Rutilus frisii kutum* and the first report of *Dioctophyma renale* (Nematoda: *Dioctophymidae*) in Iran. World J Zool, 6(1):91-97., 2011.
16. Moravec F.: Parasitic nematodes of freshwater fishes of Europe, *Kluwer Academic Publishers,* 1994.
17. Murrell K.D.: Fishborne zoonotic parasites: epidemiology of freshwater fishes of Europe, *Kluwer Academic Publishers,* 1994., detection and elimination. Lactic acid bacteria in fish preservation. In: H.A. Bremner (Ed), *Safety and quality issues in fish processing.* Woodhead Publishing Ltd. CRC pres, New York: 114-141. 2002.
18. Novakov N., Bjelić-Čabrilo O., Ćirković M., Ljubojević D., Lujić J., Davidov I., Jovanović M.: Eustrongylidosis of European Catfish (*Siluris glanis*). Bulg. J. Agric.Sci., Supplement 1:72-76., 2013.
19. Okomura M.P.M., Derez A.C.A., Espindola A.: Principais zoonoses parasitárias transmitidas por pescado – revisão. *Rev. Ed. Cont.*, 2, 66-80.(Sp)., 1999.
20. Oliva M.: Matazoan parasites of the jack mackerel Trachurus murphyi in a latitudinal gradient from South America (Chile and Peru). *Parasite*, 6 (3): 223-230. 1999.
21. Overstreet R.M.: Pesidential address: flavor buds and other delights. *Journal of Parasitology* 89, 1093–1107., 2003.
22. Roberts L., Janovy J.,undations of Parasitology: Sixth Edition. Boston: McGraw-Hill Higher Education.,, 2000.
23. Spalding M.G. and Forrester D.J., 1993. Pathogenesis of Eustrongylides

ignotus (Nematode: Dioctophymatoidea) in Ciconiiformes. *Journal of Wildlife Diseases* 29, 250–260., 1993.
24. Spalding M.G., Bancroft T.B. and Forrester D.J.: The epizootiology of eustrongylidosis in wading birds (Ciconiiformes) in Florida. *Journal of Wildlife Diseases* 29, 237–249., 1993.

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Q FEVER EPIDEMIOLOGY AND CONTROL IN DOMESTIC ANIMALS

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Abstract

The significance of each domestic animal in the epidemiology of Q-fever is different in various regions and it depends on the number of animals, level of infection, herd size, type of breeding and the conditions of hygiene. Epizootiological studies in our country show greater prevalence in sheep, than in cattle. Q-fever is maintained in Vojvodina in endemo-epidemic form. In Q-fever management programs for control of in sheep and cattle, serological examination and vaccination of the animals is recommended. Efficiency of the application of these measures should be conducted by applying recommended serological tests and detection of causative agent should be done by PCR method. A study on the comparative examination of efficiency, of different vaccination protocols has not yet been conducted. This would refer primarily to the duration of vaccination program, animal categories that are supposed to be vaccinated and timing for vaccination. A significant decrease in the level of infection was found after vaccination during the first years upon application of vaccination program. When vaccination in heifers was done before pregnancy with vaccines containing *C. burnetii* phase I strains, it was five times less likely that the infection would occur. Vaccination of cows with chronic infection prevents shedding of riketsia via milk, regardless whether the shedding is constant or intermittent. Vaccination represents a new concept of suppression of this zoonozes in the terms of human protection, and also in the terms of creating areas free from Q-fever in endemic regions. General preventive measures applied in the case of Q-fever infection are: movement restriction, separation of ani-

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mals, restriction of milk and wool, adequate hygiene, using the protective clothing, desinfection of equipement and vechicles, removing of placenta particles, removing of manure, quarantine after calving and lambing and not use common grazing. People proffesionally exposed to the risk from infection should be educated about the disease.

Key words: Q-fever, control, vactination, sheep, cattle

EPIDEMIOLOGIJA I MERE KONTROLE Q-GROZNICE KOD DOMAĆIH ŽIVOTINJA

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Kratak sadržaj

Značaj pojedinih vrsta domaćih životinja u epidemiologiji Q-groznice je različit u pojedinim područjima a zavisi od njihove brojnosti stepene zaraženosti, veličine stada, načina uzgoja i zoohigijene. Epizootiološka ispitivanja u našoj zemlji ukazuju na veću prokuženost ovaca nego goveda. Q-groznica se u Vojvodini održava endemoepidemiski. U programu kontrole protiv Q-groznice kod goveda i ovaca predlaže se serološka ispitivanja i vakcinacija životinja. Efikasnost primene ovih mera treba sprovoditi primenom preporučenih seroloških testova, a dokazivanje uzročnika PCR metodom.Međutim, uporedno ispitivanje efikasnosti različitih protokola vakcinacije nije istraženo. To se pre svega odnosi na trajanje programa vakcinacije, koje kategorije životinja i kada treba da budu vakcinisane. Primenom vakcinacije uočeno je značajan pad stepena zaraženosti u prvim godinama primene programa vakcinacije. Primena vakcine od sojeva C.burneti faze I, konstatovano je da je vakcinacija junica pre graviditeta imala pet puta manju verovatnoću da će se zaraziti nego gravidne jedinke. Vakcinacija hronično inficiranih krava sprečava izlučivanje rikecija preko mleka, bilo da se radi o konstantnom ili intermitentnom izlučivanju. Primena vakcinacije dala je novi koncept suzbijanja ove zoonoze ne samo u smislu zaštite ljudi, već i u pravcu stvaranja područja slobodnih od Q-groznice u endemskim regionima Od opštih preventivnih mera primenjuje se: zabrana kretanja, razdvajanje životinja, zabrana korišćenja mleka i vune, adekvatna higijna, nošenje zaštitne odeće, dezinfekcija opreme i vozila, uklanjanje delova posteljice, adekvatno uklanjanje stajnjaka, karantin kod teljenja i jagnjenja i ne koristiti zajedničku ispašu. Osobe profesionalno izložene riziku potrebno je da prođu obuku i da se edukuju o prirodi oboljenja.

Ključne reči: Q-groznica, mere kontrole, vakcinacija, ovce, krave

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INTRODUCTION

Q-fever is an infectious disease which is the most common of all rickettsia caused diseases in the world and also in our country (2,4,8,10,12). This antropozoonozes is characterized with a wide spectrum of hosts and vectors. Primary role of domestic animals is as reservoirs for the infection of humans and therefore it is very important to precisely and in time Q-fever is detected in domestic animals (3,5,6,17). C. burnetii is one of the most resistant of all unsporalizing microorganisms. In tick feces, it can last for 586 days, in dried blood at room temperature it can last for 5 months, in dust 120 days in wool 12-16 months at 4-6 C, and in milk more than 30 days (13).

Sanitation of endemic foci of Q-fever is practically pretty close to impossible because of the high resistance of the causative agent of Q–fever, a small infectious dosage and various epidemiologies. Spreading of the disease via ticks as vectors is not significant, but inhalation of the causative cause together with the dust is. For development of an infection in domestic animals, a primary cycle in the nature is important. Positive reactors are found in sheep, cattle goats, swine, horses, poultry and cats (4, 9,14, 25) . From clinical point of view, Q fever is not a negligible issue in veterinary medicine. The miscarriages have been registered, primarily in the sheep, cattle and goats, but also other reproductive disturbances are present, mastitis, poorly viable offspring, etc.(19, 20, 22). The significance of some domestic animals in epidemiology of Q-fever is different in certain regions and it depends on their number, level of infection, size of the herd, way of breeding and zoo hygiene. The epidemic movement of the Q-fever disease is influenced by other factors such as air flow, rainfall, and density of population and geological characteristics of the terrain (17,24).

Alimentary infections are far less significant compared to the airborne infections (16). The cause of these infections is usually contaminated milk and milk products. Among all food, milk carries the biggest risk of infection. Milk pasteurization is a prophylactic measure for excluding every possible risk of infection for humans.

The problem of prevention from Q-fever is in the lack of specific protection measures and poor efficiency of general preventive measures. Habits of the

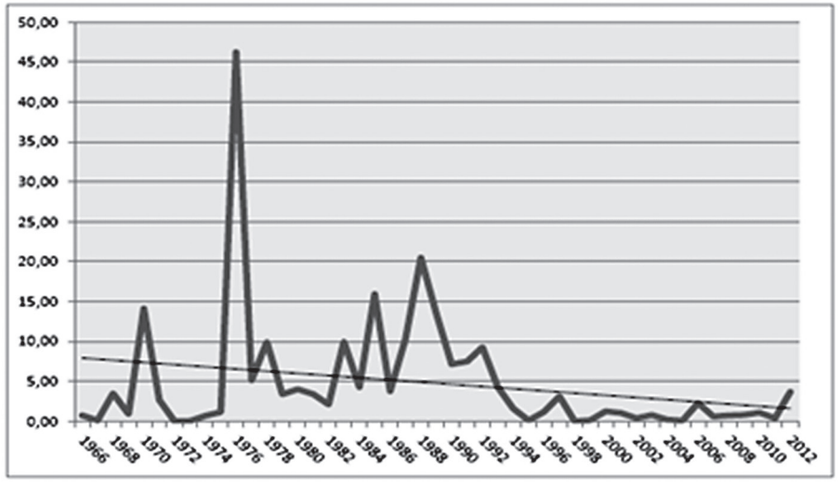
farmer's established long time ago are difficult to change. The basic factors that have negative influence to epidemiologic situation of Q-fever are high level of positive reactors among sheep, nomad way of farming and usage of pastures in sheep breeding.

Situation in AP Vojvodina

Epizootiological study in our country shows that the prevalence in sheep is higher than the prevalence in cattle (11,17,21). Nomad farming has a special significance in the occurrences of epidemics. In Vojvodina, Q-fever is being maintained endemoepidemicaly(15,17).

Results after a study on the presence of Q-fever on cattle farms in Vojvodina show a significant presence (9,5%), while in certain farms the percentage of seroreactors is even over 30% (9,14,25). The highest percentage of seropositive findings was detected in cows which had miscarriages (l9, 4%), and the lowest was found in animals from endemic regions (4, 8%) (19,20). The results from serological analysis of workers at cattle farms with enzootics of Q fever show the presence of high level of risk from the infection with *C.burnetii,* although none of the seropositive workers did not report that he or she was ill from Q fever. Since Vojvodina is an endemic region for Q fever, risk level was analyzed from the infection among users of the raw milk in Vojvodina population (16). A significant difference was not found between the rate of infection in raw milk users (10,81%), compared to the rate of infection in the rest of the population (9,16%). There are literature data on epidemics of Q fever among cattle farm workers, where the infections were in mild form or without clinical symptoms of the disease (2,13,16). The significance of sheep in the epidemiology of Q fever in Vojvodina is analyzed based on a topographic distribution of registered cases of Q fever in humans, results of serological analysis of 1340 sheep blood samples and 1398 blood samples from the residents of villages. Measured with the percent of positive reactors, sheep from individual economies (sector) have a greater epidemiological significance. Seroprevalence in the sheep from individual sector (13,8 %) is significantly higher compared to the seroprevalence in the sheep from farms 9,9 % (X= 4,593). A statistically significant (X=94,287) higher percentage of seropositive animals was found in the regions where Q fever epidemics were registered (31,3 %), compared to the seroprevalence of sheep in other regions (8,3 %). The significance of sheep in the epidemiology of Q fever is confirmed by a significantly higher (X=16,606) seroprevalence in village residents (20,3 %), who are professionally or in some other way in contact with sheep, compared to the residents without the contact with sheep (12,3 %). Sheep using pasture have a greater epidemiological significance. Seroprevalence in the neighbors of the owners that take their sheep to the pasture (28,1 %) is higher than in the owners of stable breeding sheep (11,1 %). The difference is highly significant (X=15,038). In the group of zooantroponoses, Q fever takes a leading position in the region of Vojovdina (17,221,24). The number of reported cases of Q fever in humans in Vojvodina is 95% of all reported cases in Serbia as whole (15). This is why Q fever is considered to be a specific problem of this province.

Graph 1 – Incidence of Q fever in Vojvodina during the period from 1966-2012*



* Institute for public health of Vojvodina: Infectiousdiseases in AP Vojvodina, 2012.

Up to the beggining of 90-es Q fever was a leading zoonozes in Vojvodina. From 1983-1992. an average incidence for Q fever was 10,2/100.000, rangeing from 3,8-20,4/100.000 (15). Large epidemics of Q fever followed the line of nomad sheep flocks movement. Since sheep are the main reservoir of the disease, Q fever was seasonal with about 90% patiens at the end of winter and beggining of the spring, during the lambing season(15). Since 1991. the number of Q fever patients has significantely decreased. What was mainly caused by the abssence of nomad flocks from other regions of Former Yugoslavia. During the last ten years, the incidence is 0,82/100.000. Q fever can now be seen as small family epidemics among domestic animal owners and it does

not have a seasonal character any more. The disease has kept a characteristic demographic distribution with a highest specific incidence for productive population and males. More frequently the disease is found in males because of the higher rate of exposure, but also because of the difference in clinical manifestation of the disease. A study was done on 323 patients with acute infection and it was determined that asymptomatic infections were significantly more frequent in female patients (14,0%) then in male patients (4,3%) (15,17). In a study on the prevelance level for *C. burnetii* in population of AP Vojvodina in the middle 80es, the difference between the level of prevelance in different genders was not found (9,4% for males and 9,3% for females), while the average incidence for Q fever in males was 2,6 times higher (12,3/100.000) compared to the females (4,8/100.000) (15,,17). Even when the incidence is significantly reduced, the number of male patients (1,10/100.000) is 2 times higher compared to females (0,55/100.000)

CONTROL

General preventive measures give insufficient results in suppression and prevention of Q fever infection and specific prevention has no adequate solutions in the whole world. Great resistance of *Coxiella* in the environment and different epidemiology lead to almost impossible sanitation of endemic focus. Within the control program against Q fever the most frequently suggested measures are serological examination and vaccination of animals (2,13,23). One of the measures is removing of positive reactors from the flock and in other animals constant control is needed and separation of seropositive animals. Milk from seropositive cows must be pasteurized. Several authors have determined positive effects after vaccination in infected and non infected animals.

Q fever control in cattle is of a great significance, because of the presence of reproductive failures and because they are a significant source of infection and environment contamination (2,12,13,18,23). In practice, vaccination is recommended in infected herds and flocks, but a study on efficiency of different vaccination protocols has not been done, which is related to length of vaccination program, which categories of animals should be vaccinated and when(18). The latest research show good results on the level of single animal and on the level of flock or herd. Analyzed parameters were excretion of the causative agent, contamination of the environment and health status of the animals with a most important aim to determine measures for prevention and suppression of the infection. After application of vaccination a significant decrease of infection level was found during the first years of the vaccination

program application, with remark that this period should be prolonged. As a key factor to the success, vaccination should be done not only for a short period of time. During the first year of vaccination there is a reduction of clinical symptoms (abortions, infertility), but vaccination period of 3-4 years is necessary in order to stop the shedding of bacteria. Vaccination program for cows and heifer is considered more efficient then program only for heifers. After application of vaccine *C.burnetii* phase I, heifers vaccinated before pregnancy have five times less probability of getting the infection then already pregnant cows(1, 2,13,18). These results justify the application of the vaccination programs for animals in non infected herds and heifers before pregnancy. Besides, vaccination of cows should be done when the level of seroprevalence is low, meaning in herds of cows where the infection did not spread yet. In infected herds, vaccination should be done in all the animals or at least heifers. Vaccination of chronically infected cows prevents shedding of rickettsia through milk, constant or intermittent shedding. Vaccinated animals are considered not infected regardless to the specific antibody titer level. Vaccination does not aim only protection of the animals from infection, but also it eliminates shedding of C*oxiella* with milk (2,13). Efficiency of the application of all control measures including vaccination should be estimated with application of serologic test and PCR methods by systematic sampling of blood, milk, vaginal mucus and feces. Application of vaccination has given a new concept to the suppression of this zoonozes not only in the service of public health but also for creating regions free of Q fever in endemic regions(13).

After vaccination a problem of local reactions on the place of inoculation can appear, in sensitized animals and also in non sensitized ones as we have found in our research. In our study we did not detect rickettsia in milk samples from vaccinated cows 45 days after infection. Non vaccinated animals were continuously shedding rickettsia during the whole period of study. Today there are several vaccines at disposal for application in cows and sheep, such as bivalent vaccine *C.burnetti* and *Ch.psittacci* for sheep. Higher efficiency (300 times) was shown after vaccination with vaccines made of *C.burnetii* phase I, more virulent (has a complete LPS) then vaccine made of a virulent strains in phase II. Strains at disposal are usually Nine Mile strain, and rarely Henzerling strain for vaccine preparation. There are no unique programs for animal's vaccination; it is done related to the epidemiological situation in a certain region. Vaccination as a measure of prevention is used in Australia for sheep and humans exposed to the risk. In Russian federation vaccination is done in certain regions where Q fever appears endemically, where infection foci are present in the nature and in France and Slovakia, depending on the epidemiological

situation in cows and sheep. Vaccination is a measure which will prevent the shedding of *Coxiella* and reduce the risk from spreading rickettsia, what will significantly reduce the risk of infection for humans. This does not mean that during the next year there will be no infections in humans, the disease can be eliminated but not eradicated (2,13).

The aim of vaccination after the lambing season is to prevent new infections during the next lambing in young animals which are not pregnant. A two time vaccination before pregnancy reduces the risk from *C.burnetii* infection, majority of abortions are prevented and shedding of *C.burnetii* after the abortion would be reduced.

According to the literature data, a study on chemiotherapy application was done in infected animals. A possible recommendation for the prevention is usage of tetracycline in sheep, in dose 8mg/1kg, in water, few weeks before lambing (3,5).

General preventive measures that can be applied are the following: prohibition of movement, separation of the animals, prohibition of use of milk and wool, adequate hygiene measures, pest control, wearing of protective clothes, disinfection of equipment and vehicles, removal of placenta parts, adequate removal of manure (storage for 90 days in a roofed storage, afterwards it can be spread on the soil and plow), quarantine when calving and lambing and trade, avoid common grazing.

PREVENTIVE MEASURES

Control of Q fever in domestic animals and prevention of environment contamination with *C.burnetii* are the most important measures in protection of humans from infection (13). Other measures that can significantly reduce the risk from infection are: education of the residents in rural regions about the ways of infection, possible risks and precautions, education of farmers and other professionally exposed people with the aim of establishing a good agricultural practice. These actions can have influence on the reduction of risk for Q fever. Measures of self protection and zoo hygienic measures:

- Personal hygiene during and after working with animals
- Work clothing and shoes
- Use of protective gloves for the removal of postpartum products
- Additional equipment for self protection (mask, goggles) when in contact with materials with high risk (after abortions, during Q fever epidemics, when cleaning the objects for sheep, goat and cattle)
- Safe removal of placenta, aborted and stillbirth animals

- Regular cleaning an disinfection during lambing or calving
- Rodent control and tick control
- Reduce the dust
- Use of quarantine when purchasing new animals
- Determine the causes of abortion and still birth
- Restrict entrance of people and other animals (including dogs and cats) whenever it is possible

Pregnant women, immunocompromised persons and persons with cardiovascular problems (especially if there is a valve failure) should avoid close contact with the animals, particularly during the partial season. It is recommended to use pasteurized milk and use of pasteurized milk for milk products (cheese). There should be a restriction order to objects or space which is of high risk for Q fever in humans and entrance should be allowed only to vaccinated humans.

Immunization of humans with a high risk occupation is a primary preventive step against Q fever (13). There is a certain risk from serious local reactions to vaccine in humans which were exposed to the infection or were vaccinated. That is why before vaccination it is important to:

- Check in anamnesis if the person had Q fever, or had clinical symptoms characteristic for Q fever or was vaccinated
- Analyze blood serum for the presence of antibodies against *C.burnetii* or
- Perform an intradermal test

Every positive result after screening preludes vaccination. Vaccination induces a solid immunity in majority of vaccinated individuals. Good communication on local and regional level and also cooperation between human and veterinary medicine is necessary because of the significance of this disease.

EXPERIENCE FROM HOLLAND

Publicity was informed about the event in Holland when there was a great Q fever epidemic registered in humans (7, 13). It all begun in 2005, and in the period from 2007-2009, the number of patients in one region was 3523 persons and for the whole period, more than 4000 patients for Q fever were found. During the epidemic, in 60% of patients the disease was found in asymptomatic form, 20% of patients were hospitalized and 7 people died (13). In the procedure of revelation the epizootiological background, abortions in goats were found in that region. It was proved that the goats were a source of infection.

The following procedures were applied (7):
- all pregnant animals were removed from all infected farms
- all animals were examined with a serological ELISA test three times
- bulk samples of milk were examined by PCR method two times
- elimination of infected animals
- animals were vaccinated according to the age and size of the herd
- prohibited visitors
- prohibited use of milk
- application of biosafety measures

During this period 35000 pregnant animals were killed and costs were estimated for 6 million Euros. At the same period of time in neighboring countries Germany and Belgium, Q fever was found in humans and animals (13).

During the year 2010, European agency for food safety (EFSA) had an assignment to come out with measures for the estimation of risk factors and estimation of efficiency of measures for Q fever control in EU (13).

In Barcelona, 2011, a Second European meeting on Q fever was held where new scientific knowledge was discussed about epidemiology, pathogenesis, diagnostic methods and control measures for this important zoonozes.

Data in Europe after the epidemic in Holland show great variability in laboratory diagnostic methods that were used, criteria for explaining the results depending on the aim of diagnosis - herd screening, identification of animal that is shedding rickettsia, epidemiological research, and routine diagnostics. One of the conclusions of the Symposium was that professional knowledge is still insufficient, especially in epidemiology, identification of the ways of infection and of potential reservoirs. Regular veterinary surveillance of animals is necessary in order to track the infection and propose an accurate diagnosis in time. Based on the knowledge we have today, a key measure in Q fever control is vaccination of the animals with *C.burnetii* vaccine, phase I.

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LITERATURA

1. Arricau-Bouvery N., Souriau A., Bodier C., Dufour P., Rousset E., Rodolakis A.: Effect of vaccination with phase I and phase II Coxiella burnetii vaccines in pregnant goats. *Vaccine*, 23, 35, 4392-4402, 2005.
2. Behumer D.E., Biberstein E.L., Riemann H.P., Franti C.P., Sawyer M.: Q fever (Coxiella burnetii) Investigation in Dairy Cattle : Persistence of Antibodies After Vaccination. *Am. J. Vet. Res.*, 36 , 781-784, 1975.
3. Berri M.m Crochet D., Santiago S., Rodolakis A.: Spread of Coxiella burnetii infection in a flock of sheep after an episode of Q fever. *Vet.rec.*, 157, 23, 737-740, 2005a.
4. Biberstin E.L., Behymer D.E., Bushnell R., Crenshaw G.,Riemann H.P., Franti C.E.: A survey of Q fever (Coxiella burnetii) in California Dairy Cows. *Am J. Vet. Res.* ; 35, 1577-1582, 1974.
5. Blain S.: Contagious diseases of ruminants: management of Q-fever in goats. *Summa, Animali da Reddito*, 2, 3, 59-63, 2007.
6. Korany M. Johnson KM. A survaey of Q fever antibodies in a high risk population in Panama. *Am J Trop. Med Hyg*, 29, 5,1007-11, 1980.
7. Karagiannis I., Schmmer B., van Lier A., Timen A., Schneeberger P., van Rotterdam B., de Bruin A., Wijkmans C., Rietveld A., van Duynhoven Y.: Investigation of a Q fever outbreak in a rural area of the Nitherlands. *Epidemiol. And Infect*. 137, 9 1283-1294, 2009.
8. Macellaro A., Akesson A., Norlander L.: A survey of Q-fever in Sweden. *Eur. J. Epidemiol,* 9, 2 , 213-6. 1993.
9. Rašeta B., Galić M., Šeguljev Z.: Q-groznica kod domaćih životinja u Vojvodini sa posebnim osvrtom na oboljenja ljudi. U: Zbornik radova, 4.Kongres mikrobiologa Jugoslavije, Beograd, 347, 1981,
10. Rašeta (Vidić) B., Mihajlović B.: Q-groznica kod domaćih životinja u SAP Vojvodini.*Vet. glasnik*, 27 , 9, 695-703, 1983.
11. Rašeta(Vidić) B., Galić M., Pavlović R., Šeguljev Z.: Raširenost Q-groznice kod ovaca u Vojvodini. U: Zbornik radova, 25.Naučni sastanak mikrobiloga, epidemiologa i infektologa Jugoslavije, Pula, 299-301, 1983.
12. Schliesser Th., Schmidt U. : Zur Varbriatung und Bedeuting des Q-fibers bei Schaf und Rind. berisht. d. 8. kong. Dt. Vet. med. Gas., Berlin-Hamburg, 238-242. 1970.
13. Scientific opinion: Scientific Opinion on Q fever: *EFSA journal*, 8, 5, 1595, 2010.
14. Šeguljev Z., Vuković B., Stefanović S., Vidić B., Boboš S., Bačić M.: Izučavanje Q-groznice na farmama goveda u SAP Vojvodini.*Vet. glasnik*, 42, 11-12, 729-733, 1988.

15. Šeguljev Z., Vuković B., Vidić B., Bačić M.: Zoonoze u Vojvodini. U: Zbornik radova, Međunarodni Naučni skup Zaštite životne sredine i poljoprivrede, EKO'93, Novi Sad, 1993.
16. Šeguljev Z., Vidić B., Vuković B., Jerant-Patić V., Boboš S.: Rizik koji predstavlja infekcija izazvana sa C.burnetii za korisnike sirovog mleka u ishrani. *Vet. glasnik*, 48 , 3-4, 299-301, 1994.
17. Šeguljev Z., Vidić B., Šajgalik M.: The importance of sheep in the epidemiology of Q-fever in Vojvodina. *Folia veterinaria*, 41, 3-4, 107-111, 1997.
18. Vidić B., Boboš S., Lazić S., Bojović G.: Q-groznica u krava - imunoprofilaksa i njen uticaj na izlučivanje C.burnetii u mleku.*Vet. glasnik*, 44 , 1,27-32, 1990.
19. Vidić B., Mihajlović B., Galić M., Pavlović R., Boboš S.: Findings of antibodies for C.burnetti with cows having clinical indications of Q-fever. *Acta Vet*. 40 , 1, (27-30), 1990.
20. Vidić B., Boboš S., Lazić S., Grgić Ž., Hristovski M., Zorić M.: Q-Fever (Coxiella burnetii)in cows with reproductive disorders. 1-7 Mac.*Vet. Review*, 24, 1-2 1-7, 1995.
21. Vidić B., Šeguljev Z., Vuković B., Grgić Ž.: Importance of sheep in epidemiology od Q-Fever. Mac. *Vet. Review*, 25, 1-2, 1996.
22. Vidić B., Boboš S., Grgić Ž., Orlić D., Bugarski D.: Sheddings of Coxiella burnetii through milk and its influence on the mammary gland. In: Proceedings, 1.Middle-European Congress for buiatrics, Balatonfured, Hungary, 316-322. 1999.
23. Vidić B., Bjelajac B., Šeguljev Z.: Q-grounica: epizootiologija, dijagnostika i kontrola = Q-fever: epizootiologija, diagnostic and control. *Vet. žurnal Republike Srpske*, 1, 1/2, 38-42, 2001.
24. Vidić B., Šeguljev Z., Grgić Ž., Savić-Jevđenić S.: Seroepidemiology of Q-fever in sheep.In: Microbiologia Balkanica, 3nd Balkan Confrence of Microbiology, Istanbul, Turkish Microbiological Society, 460, 2003.
25. Vidić B., Ašanin R., Boboš S., Bugarski D.: Q-fever (Coxiella burneti) investigations in dairy cows. In: Prvi Simpozijum o zoonozama sa međunarodnim učešćem, Sarajevo, Udruženje mikrobiologa BiH, 62. 2005.

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STUDY OF THE EQUINE VIRAL ARTERITIS SEROCONVERSION AT HORSE STABLES IN THE TERRITORY OF VOJVODINA

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Abstract

The paper presents the results of the study on equine viral arteritis presence in five horse stables in the territory of Vojvodina. The research encompassed analysis of clinical manifestation of the disease and seroconversion to equine viral arteritis throughout a two-year period (2012 and 2013). Analysis of clinical manifestations of the disease was performed according to the anamnestic data obtained from staff veterinarians on the stud farms. Prevalence of equine viral arteritis was determined by testing 204 blood serum samples from 102 horses. Sera samples were collected twice at one-year interval, i.e. in 2012 and 2013, and examined by virus neutralization test. Seropositive results as well as seroconversion were obtained on one of the five investigated horse stables. At the stable with seropositive animals, 27 horses were serologically tested at one-year interval. Out of the total of 27 animals, antibody positive sera were obtained in 16 and 17 horses in 2012 and 2013, respectively. Antibody titre values ranged from 1 to 10 $\log_2$, with average values of 3.11 and 3.15 $\log_2$ in 2012 and 2013, respectively.

Key words: equine viral arteritis virus, horse stables, seroprevalence, seroconversion, virus neutralization test

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ISPITIVANJE SEROKONVERZIJE NA VIRUS VIRUSNOG ARTERITISA KONJA U ERGELAMA NA PODRUČJU VOJVODINE

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Kratki sadržaj

U radu su prikazani rezultati ispitivanja prisustva bolesti virusnog arteritisa konja u 5 ergela na teritoriji Vojvodine. Ispitivanja su vršena sa aspekta kliničkih manifestacija bolesti i serokonverzije na virus virusnog arteritisa konja tokom dve godine (2012. i 2013.). Analiza kliničke manifestacije bolesti vršena je na osnovu anamnestičkih podataka, dobijenih od veterinarskog osoblja ergela. Utvrđivanje virusnog arteritisa konja vršeno je ispitivanjem 204 uzorka krvnih seruma, metodom serum neutralizacije, prikupljanih od istih konja (102) tokom 2012. i 2013. godine, odnosno u intervalu od godinu dana. Seropozitivan nalaz, kao i serokonverzija, utvrđen je na jednoj, od pet analiziranih ergela. Na ergeli u kojoj je utvrđen seropozitivan nalaz, odnosno serokonverzija, serološki su ispitana 27 grla u intervalu od godinu dana. Od 27 serološki ispitanih konja, pozitivan nalaz utvrđen je kod 16 konja 2012., a 2013. godine kod 17 konja. Utvrđena vrednost titra antitela iznosila je od 1 do 10 $\log_2$. Prosečna vrednost titra antitela 2012. iznosila je 3,11, a 2013. godine 3,15 $\log_2$.

Ključne reči: virus virusnog arteritisa konja, ergele, seroprevalenca, serokonverzija, serum neutralizacioni test

INTRODUCTION

Equine viral arteritis (EVA) is an infectious disease that can affect all ungulate species, as well as all horse categories. The disease is quite common worldwide causing severe health problems and considerable economic losses. The causal agent of the disease is the virus (*Equine arteritis virus*–EAV) from the family *Arteriviridae*. EAV is an enveloped, spherical *virus* of 50-70 nanometres (nm) in diameter possessing single-stranded ribonucleic acid (RNA)

genome of 12.7 kb (*Balasuriya et al. 1999*). The virus was isolated for the first time in 1953 in *Bucyrus*, *Ohio* state, U.S.A. (*Nowotny, 2001*). The name of the virus, arteritis, is based on the characteristic pathogenic activity observed in blood vessels. The virus replicates in muscle cells and endothelial cells of small blood vessels, predominantly arteries, and may lead to the range of inflammatory processes.

According to data from the literature (*Holyoak et al, 2008*), the disease has been reported in horse populations in all continents: North and South America, Europe, Asia, Australia and Africa. Only Iceland and Japan are considered EVA-free. A research that dates back to the 60ies and 70ies of the last century has established the following seroprevalence rates of EAV: Switzerland 11.3%, England 2.3%, Netherlands 14% and Germany 1.8%.

However, the investigation dated 1998 revealed seroprevalence rates of some 20% in Germany and about 2% in non-vaccinated horses in the U.S.A. (*Holyoak et al 2008*). In Austria, the EAV seroprevalence ranges between 10 and 12%; however, on some stud farms the rate reaches even 100% (*Nowotny 2001*). Seroprevalence rate in Anatolia (central Turkey) is 23.4% (*Bulut et al. 2012*).

So far, the rate of EAV seroprevalence in the Republic of Serbia has not been confirmed, but the results reported by several authors (Petrović et al., 2002; Urošević et al., 2003; Lazić, 2013) indicated the presence of EAV infection in the country. In the Republic of Serbia, the research studies on EVA infection has been conducted only since the beginning of this century, i.e. in 2002, encompassing only imported horses in quarantines. More recently, the disease has been gaining more attention and numerous horse breeders have put efforts to initiate serological testing, foremost of breeding animals.

Infected horses shed the virus in nasal and ocular discharge, urine, aborted foetuses and sperm (stallions). The virus can cause a range of health disorders in horses, including interstitial pneumonia, panvasculitis with consequent oedema predominantly in eye lids, abdomen, distal parts of the limbs, preputium and scrotum, thrombosis associated with bleeding, necrosis in lymph nodes and kidneys, abortions and inflammation of accessory sex glands. Sometimes, the infection may pass without developing any health status disorders (*Del Piero, 2000*). Infected stallions are potential natural reservoir of the virus. If the virus affects accessory sex glands, stallions remain infected for life and frequently shed the virus in the semen (*Del Piero, 2000; Nowotny, 2001; Holyoak et al 2008)*. Testing the possibility of virus spreading through the semen and its detection in the semen was the topic of interest of numerous researches. Development of modern laboratory diagnostic methods, such as molecular biology

assays, gave rise to more intense research in this field, which have confirmed that seropositive stallions shed the EAV in their semen (*Ramina et al., 1999; Balasuriya et al., 2002; Mankoč et al., 2002; Guthrie 2003; Chenchev et al., 2010*)

MATERIAL AND METHODS

Samples

Blood samples were collected in five horse stables from the territory of Vojvodina Province. The horse stables were designated with letters A – E. The sampling encompassed the same horse population and was performed twice at one-year interval, i.e. in 2012 and 2013. Samples were collected by venipuncture of the *Vena jugularis* into Vacutainer tubes, and serum separation was performed after spontaneous coagulation. Anamnestic data on health status during the past 3-year period were recorded for each horse subjected to blood sampling. Thus, 102 blood samples of horses were obtained in 2012, and the same number of samples in 2013 so, in total 204 blood serum samples from five horse stables were collected and examined on anti-EAV antibodies presence.

The breed structure of horses at the examined farms was rather diverse, yet several breeds were dominant at particular farms: Lipizzan breed at farms A and E, English pureblood and half-blood breed at farms B and C and Lipizzan and English pureblood and half-blood as well as Nonius breed at farm D. At all investigated stud farms, the breeding systems were in accordance with common practices of horse farming. The stallions are housed in individual boxes, as well as highly pregnant mares or pospartum mares. Other horse categories are raised in groups. Throughout the year, the horses from all farms participate at various competitions, such as galopp races or parade competitions.

Serum samples analysis

Identification of anti-EAV antibodies in blood serum samples was performed by the method of virus neutralization, according to procedure set out by *OIE Terrestrial Manual, Chapter 2.5.10, 2008*. Virus neutralization was performed by using rabbit-kidney *cell line* RK-13 and *Bucyrus* strain of EAV. According to the OIE Terrestrial Manual, antibody titre values ≥2 $\log_2$ are considered as seropositive result. Animals that do not have a certified history of vaccination against EVA are considered infected, i.e. such stallions are considered potential source of infection.

RESULTS AND DISCUSSION

To provide a clear overview of our findings, the obtained results are presented in Tables. The present study encompassed 102 horses (averagely one fourth) out of the total of 355 horses (in 2012) and 386 horses (in 2013) that were raised in 5 examined horse stables (Table 1.). Presence of anti-EAV antibodies was obtained in animals from one horse stable (E), where 62 and 72 horses were raised during 2012 and 2013, respectively. At this farm, 27 horses were examined during the two aforementioned years. Presence of anti-EAV antibodies was confirmed in 16 (59.26%) examined horses in 2012 and in 17 (62.96%) examined horses in 2013.

Table 1. Overview of examined horses according to the farm and EVA antibody finding

No.	Horse stables	No. of horses in stables	No. of examined horses in stables	Result	
				Positive	Negative
1	A	34	12	0	12
2	B	82	23	0	23
3	C	78	20	0	20
4	D	120	20	0	20
5	E	72	27	16* (59.26%) 17** (62.96%)	11* (40.74%) 10** (37.04)
Total		386**	102	16* (15.67%) 17** (16.67%)	86* (84.31%) 85** (83.33%)

* 2012
** 2013

Equine arteritis virus infection has been established only on farm E. Thus, the analysis of the results obtained on this stud farm can illustrate the effects of this infection on the health status of horses, incidence of the infection within different horse categories as well as the potential for virus dissemination on the horse farm.

According to anamnestic data obtained from staff veterinarians and stud farm manager, during the past 3- and 4-year periods clinical symptoms indicating equine viral arteritis were not observed. Abortions were recorded only sporadically and were mainly associated with twin-embryo pregnancies. Frequently, mares from other stables were introduced to the stud farm for breeding, mostly lacking the documents of serological testing for sexually transmitted infectious agents.

The EVA antibody titre values established during 2012 and 2013 in all investigated horses are presented in Table 2. The analysis of the results indicate virus circulation within the stable, and that the most probable route of virus spread was sexual transmission. Analysis of mating schedule revealed that seropositive stallions were used for mating, therefore an increased number of mares become infected with this virus. The number of seropositive horses increased for one animal in 2013, i.e. from 16 seropositive animals in 2012 to 17 seropositive animals in 2013. The established antibody titre values did not vary significantly; however, the average EAV antibody titre value were somewhat higher in 2013 (3.15 $\log_2$) as compared to 2012 (3.11 $\log_2$) indicating permanent circulation of the virus on this farm. Still, the seroprevalence to EAV dropped from 25.81% in 2012 to 23.61% in 2013, which is due to the increased number of horses (10 animals) on farm E in 2013.

Table 2. Overview of anti-EAV antibody finding in horse blood sera on farm E in 2012 and 2013

No.	Sample No.	Horse category	Antibody titre ($\log_2$)	
			2012	2013
1	1-21751	Stallion	7	5
2	2-01452	Stallion	0	0
3	3-09426	Stallion	2	2
4	4-22154	Stallion	9	9
5	6-22912	Stallion	0	0
6	16-02828	Stallion	2	3
7	6-03179	Gelding	0	0
8	15-00128	Gelding	0	0
9	8-13745	Mare	0	0
10	9-23714	Mare	0	0
11	10-11623	Mare	4	4
12	11-16872	Mare	4	4
13	12-14794	Mare	4	4
14	13-02702	Mare	5	5
15	31-03459	Mare	0	7
16	17-33372	Mare	5	5
17	19-03036	Mare	5	6
18	20-02936	Mare	1	3
19	21-24452	Mare	5	5
20	22-17332	Mare	6	6
21	23-02961	Mare	7	7
22	24-34861	Mare	0	0
23	25-27196	Mare	6	3
24	26-05112	Mare	10	7
25	27-14166	Mare	0	0
26	29-02567	Mare	2	0
27	30-02830	Mare	0	0
Average antibody titre value			3.11	3.15

Assessment of seropositive results to EAV on stud farm E according to horse category (Table 3.) reveals an increase in number of seropositive animals in the category of mares, which, according to the obtained anamnesis data, most probably results from mating with seropositive stallions. EAV antibody titre value in the mare marked 31-03459 was 7 $\log_2$ in 2013, whereas the same animal was seronegative in 2012 (Table 2.). This mare was mated by a seropositive shedder stallion during mating season 2012/2013, thus supporting the suspicion of sexual transmission of this viral infection in the stable.

Table 3. Number of horses with positive anti-EAV antibody finding on farm E in 2012 and 2013, according to horse categories

Category	Number of examined animals	Number of anti-EAV antibody positive animals	
		2012	2013
Stallions	6	4	4
Geldings	2	0	0
Mares	19	12	13
Total	27	16	17

Sexual transmission and dissemination of the infection in stud farm E can be better understood by analyzing the distribution of established anti-EAV antibody titre values as well as the mating schedule records. Mating of seropositive stallions during several seasons results in persistence and dissemination

of the infection in this stud farm. Distribution of the established antibody titre values according to horse categories is presented in Tables 4. and 5. for 2012 and 2013, respectively.

Table 4. Distribution of the established anti-EAV antibody titre values according to horse categories in stud farm E in 2012

Category	No. of exam. animals	Antibody titre (log_2)											Average antibody titre value
		0	1	2	3	4	5	6	7	8	9	10	
Stallions	6	2	-*	2	-	-	-	-	1	-	1	-	3.33
Geldings	2	2	-	-	-	-	-	-	-	-	-	-	0
Mares	19	6	1	1	-	3	4	2	1	-	-	1	3.37
Total	27	10	1	3	-	3	4	2	2	-	1	1	3.11

* - negative antibody finding

Table 5. Distribution of the established anti-EAV antibody titre values according to horse categories in stud farm E in 2012

Category	No. of exam. animals	Antibody titre (log_2)											Average antibody titre value
		0	1	2	3	4	5	6	7	8	9	10	
Stallions	6	2	-*	1	1	-	1	-	-	-	1	-	3.17
Geldings	2	2	-	-	-	-	-	-	-	-	-	-	0
Mares	19	6	-	-	2	3	3	2	3	-	-	-	3.47
Total	27	10	-	1	3	3	4	2	3	-	1	-	3.15

* - negative antibody finding

However, despite positive anti-EAV antibody findings, analysis of general health status of both stallions and mares during 2012 and 2013 revealed neither health status disorders nor failed fertilizations and abortions. The foaling occurred within normal time period, and foals did not manifest any health disorders. These data indicate that EAV infection can persist without health disorders manifestation, which is in accordance with the data reported by other authors (*Del Piero, 2000; Urošević et al., 2003; Lazić et al., 2013*).

Analysis of the results obtained in this research revealed that they well

correspond with the data from the literature. High seroprevalence on some horse stables reported by Nowotny *(2001)* and several other researchers was due to an unobstructed virus transmission within the farm. Virus shedding in the semen of seropositive stallions is frequently reported by numerous authors (*Mankoč et al. 2002; Chenchev et al. 2010)*, thus seroconversion in mares inseminated with semen originating from seropositive stallions was expected result at the stable E.

CONCLUSIONS

Out of the 5 horse stables with in total 355 horses (2012) and 386 horses (2013) encompassed by this research, EAV infection was confirmed at one stable. In that seropositive stable the established antibody titre values did not vary significantly; however, the average EAV antibody titre value were somewhat higher in 2013 (3.15 log_2) as compared to 2012 (3.11 log_2) indicating permanent circulation of the virus on this farm. According to the obtained results, we may conclude that sexual transmission was the route of virus transmission and dissemination in this stud farm.

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LITERATURA

1. Balasuriya U.B., Hedges J.F., Nadler S.A., Mccollum W.H., Timoney P.J., Maclachlan N.J.: Genetic stability of equine arteritis virus during horizontal and vertical transmission in an outbreak of equine viral arteritis. *Journal of General Virology*, 80, 1949–1958, 1999.
2. Balasuriya U.B., Leutenegger M.Ch., Topol B.J., McCollum H.W., Timoney P.J., Maclachlan N.J.: Detection of equine arteritis virus by real-time TagMan reverse transcription-PCR assay. *Juranal of Virological Methods*, 101, 21-28, 2002.
3. Bulut O., Yavru S., Yapici O.K.M., Avei O.: The Serological Investigation of Equine Arteritis Infection in Central Anatolia of Turkey. *Journal of Animal and Veterinary Advances*, 11, 7, 924-926, 2012.

4. Chenchev I., Polychronova L., Chakarova S., Genova I.: The real time PCR for detection of Equine Arteritis Virus. *Arhiv veterinarske medicine*, 3, 1, 3-11, 2010.
5. Del Piero F.: Equine Virus Arteritis. *Veterinary Patholgy*, 37, 287-296, 2000.
6. Guthrie A.J, Howell P.G, Hedges J.F, Bosman A.M, Balasuriya U.B, McCollum W.H, Timoney P.J, Maclachlan N.J.: Lateral transmission of equine arteritis virus among Lipizzaner stallions in South Africa. *Equine Veterinary Journal*, 35, 596–600, 2003.
7. Holyoak R.G., Balasuriya U.B., Broaddus C.C. Timoney P.J.: Equine viral arteritis: Current status and prevention. *Theriogenology*, 70, 3, 403-414, 2008.
8. Kosec M., Hostnik P.: Detection of Equine Arteritis Virus in semen of seropositive stallions. *Veterinarska Novina* 28, 2, 213-219, 2002.
9. Lazić S., Lupulović D., Lazić G., Gavrilović P., Bugarski D., Petrović T.: Research on incidence and effects of viral arteritis on health status of horses at stables. In: Proceedings, 10th International Symposium Modern Trends in Livestock Production, Belgrade, 2 - 4 October, 2013, Institute for Animal Husbandry, Beograd, Institute for Animal Husbandry, 2013, Str.1252-1259
10. Mankoč S, Barlič-Maganja D, Grom J. Toplak I., Klobučar M., Nowotny N.: Virusni arteritis konja. U: Zbornik radova i kratkih sadržaja, 13. Savetovanje veterinara Srbije, 2001, 33-36.
11. OIE Terrestrial Manual: Chapter 2.5.10, 2008.
12. Ramina A., Valle D.L., De Mas S., Tisato E., Zuin A., Renier M., Cuteri V., Valente C., Cancellotti M.F.: Detection of equine arteritis virus in semen by reverse transcriptase polymerase chain reaction-ELISA. *Comparative Immunology, Microbiology & Infectious Diseases*, 22, 187-197, 1999.
13. Urošević M., Nowotny N., Đuričić B., Trailović R. D., Petrović T., Savić-Jevđenić S.: Indirektno dokazivanje prisustva virusnog arteritisa konja (EAV) kod pastuva u jednom zapatu, *Savremena poljoprivreda* Novi Sad, 52, 3-4, 503-507, 2003.

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DEFINITION OF bactericidal action in disinfectants TO *Mycobacterium tuberculosis*

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Abstract

One of the main directions in the system of prophylaxis and fight against tuberculosis of animals belongs to the disinfection. For each disinfectant, it is important to have the following: good solubility in water, to own the wide spectrum of antimicrobial action, not to reduce antimicrobial activity in the presence of organic matters and hard water, not to be toxic or low-toxic for people and animals, not to have an unpleasant smell, not to damage the decontaminated objects, to be proof at storage, accessible for transporting and application, and also economic when exposed in the environment. The purpose of the research was, to study a bactericidal action in new disinfectant, in relation to Mycobacterium. The first stage of study was on the bactericidal properties in potential disinfectant with atypical mycobacteria, type of *M. fortuitum* № 122. For this purpose preparations were done by following the "Javelle-Kleyd" and "Geksadekon" instructions. It was concluded that crop and biological methods have bactericidal activity in relation to Mycobacteria tuberculosis, bovine kind with disinfectants "Javelle-Kleyd" in concentration 0,1 % and exposure of 30 minutes and 1 hour and "Geksadekon" in concentration 3 % and exposure time of 5 hours. "Javelle-kleyd" and "Geksadekon" disinfectants in certain modes, are possible to apply for prophylactic and forced disinfection in happy and unhappy economies in relation to tuberculosis of animals.

Key words: disinfection, mycobacteria, concentration, exposure

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BAKTERICIDNO DEJSTVO DEZINFICIJENSA NA *MYCOBACTERIUM TUBERCULOSIS*

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Kratak sadržaj

Jedan od najvažnijih činilaca sistema profilakse i borbe protiv tuberkuloze životinja jeste dezinfekcija. Za svaki dezinficijens je važno da ima dobru rastvorljivost u vodi, širok spektar antimikrobne aktivnosti, da nema smanjenu antimikrobnu aktivnost u prisustvu organskih materija i tvrde vode, da nije toksičan za ljude i životinje, da nema neprijatan miris, da ne oštećuje objekte koji se dezinfikuju, da se lako čuva, prenosi i aplikuje i da je ekonomičan prilikom primene. Cilj istraživanja je bio izučavanje baktericidne aktivnosti novih dezinficijensa u odnosu na mikobakterijum mikroorganizam. Prvi deo istraživanja se odnosio na baktericidna svojstva dezinficijensa na atipične mikobaterije, tipa *M. fortuitum* № 122. Korišteni su preparati "Javelle-Kleyd" i "Geksadekon", pripremljeni prema uputstvu proizvođača. Zaključeno je da metode dezinfekcije koje su bile predmet istraživanja imaju baktericidnu aktivnost u odnosu na mikobakterijum uzročnika tuberkuloze, bovine tipa, sa dezinficijensima"Javelle-Kleyd" u koncentraciji 0,1 % i za vreme izlaganja 30 minuta i 1sat i sa preparatom "Geksadekon" u koncentraciji 3 % i vremenu izlaganja 5 sati. "Javelle-kleyd" i "Geksadekon" preparate u određenim razblaženjima i načinima upotrebe, je moguće primeniti u profilaksi na uročnika tuberkuloze kod životinja.

Ključne reči: dezinfekcija, mikobacterijum, koncentracija, izlaganje

INTRODUCTION

One of the main directions in the system of prophylaxis and fight against tuberculosis of animals belongs to the disinfection.

Disinfection is a complex of measures, aiming on elimination of causative agents of infectious diseases of man and animals in an environment, namely: disinfection of the second link of epizootic chain – factors of transmission. These measures enable the destruction of pathogenic microorganisms in the environment, in order to break the epizootic chain and stop development of epizootic process (1). Disinfection should be planned as a part of the prophylactic and epizootic measures (2).

For each disinfectant, it is important to have the following: good solubility in water, wide spectrum of antimicrobial action, not to reduce antimicrobial

activity in the presence of organic matters and hard water, not to be toxic or low-toxic for people and animals, not to have an unpleasant smell, not to damage the decontaminated objects, to be proof at storage, accessible for transporting and application, and also economic when exposed in the environment (3, 4).

In addition, during the preparation of a disinfectant, some hygienic terms are required. The disinfectant must not show a negative effect on animals and auxiliary personnel in the concentrations recommended for application. The preparation of the disinfectant must be without negative consequences and with no allergic or cumulative properties expressed (5).

For the elimination of causative agent for tuberculosis many facilities are offered in an external environment, but they do not answer to the requirements. Also, not enough of the bactericidal properties of the infectious agent for tuberculosis are studied so far (6). Therefore there was a requirement for the improvement of existing and for the development of more effective devices for disinfection (8).

MATERIAL AND METHODS.

The purpose of our researches was, to study a bactericidal action in new disinfectant, in relation to Mycobacterium. The study was done in SEC "Institute of experimental and clinical veterinary medicine", department of study on brucellosis and tuberculosis. Laboratory research was done according to the methodical recommendations "The order of testing of new disinfectants for veterinary practice" (7).

The first stage of study was on bactericidal properties in potential disinfected with atypical Mycobacteria, type of *M. fortuitum* № 122. For this purpose the preparations were done by the following "Javelle-Kleyd" and "Geksadekon" instructions.

The exposure of bactericidal properties to atypical Mycobacteria were done in the study with the analysis of bactericidal action of the disinfectant to the causative agent of tuberculosis, bovine kind. The test was carried out with the laboratory culture strain of Vallee. Biological research was performed according to the «Settings on diagnostics of tuberculosis of animals and birds» ratified on 26.05.1997 (9).

The estimation of the results gained during the study on bactericidal properties of disinfectant was done based on the results of crop and biological research.

RESULTS

Results which are presented in Table 1 verify that analysis with different modes of disinfectant "Javelle-Kleyd" are the following:

- For the concentration of 0,05 % and exposure time 1 hour and
- For the concentration of 0,1 % and exposure time 30 minutes, 1 hour, the preparation disinfects atypical Mycobacteria.
- With the disinfectant "Geksadekon", the results were the following:
- For the concentration of 3 % and exposure time 3 hours and 24 hours, the preparation disinfects atypical Mycobacteria.

Therefore, as next step, we conducted research with the test culture of bovine kind. The results of this exposure are given in Table 2.

Table 1. Results of research of bactericidal action of antimicrobials in crops, related to *M. Fortuitum* №122

Preparation (disinfectant)	Mode of application		Findings	
	Concentration	Exposure	Experimental group	Control
"Javelle-Kleyd"	0,03 %	15 minutes	+	+
		30 minutes	+	+
		1 hours	+	+
	0,05 %	15 minutes	+	+
		30 minutes	+	+
		1 hours	–	+
	0,1 %	15 minutes	+	+
		30 minutes	–	+
		1 hours	–	+
"Geksadekon"	2 %	1 hours	+	+
		5 hours	+	+
		24 hours	–	+
	3 %	1 hours	+	+
		5 hours	–	+
		24 hours	–	+
	5 %	1 hours	–	+
		5 hours	–	+
		24 hours	–	+

Legend: "+" – growth of Mycobacteria is present; "–" growth of Mycobacteria is not present.

Table 2. Crop method of determination of bactericidal properties in "Javelle-Kleyd" and "Geksadekon" disinfectants, in relation to the causative agent of tuberculosis, bovine type /strain of Vallee/

Preparation (disinfectant)	Test-culture	Concentration, %	Exposure	Experimental group	Control
"Javelle-Kleyd"	*M.bovis*	0,1	30 minutes	–	+
			1 hours	–	+
"Geksadekon"	*M.bovis*	3	5 hours	–	+
			24 hours	–	+

Legend: "+" – growth of Mycobacteria is present; "–" growth of Mycobacteria is not present.

Based on the information given in Table 2, it can be seen that the preparation "Javelle-Kleyd" in concentration 0,1 % and exposure of 30 minutes and 1 hour and also "Geksadekon" in concentration 3 % and exposure of 5 and 24 hours inactivate *Mycobacterium tuberculosis* of bovine kind, within the laboratory conditions. The growth of test culture *M.bovis* was noticed in control test tubes with nourishing environment provided at that time. For confirmation of positive results for crop method, the experiment on laboratory animals was performed. Research results on guinea-pigs are given in Table 3.

Table 3. Biological determination of bactericidal action of disinfectants "Javelle-Kleyd" and "Geksadekon" to the causative agent of tuberculosis, bovine kind

Preparation	Type of animals	Amount	Dose (cm^3)	Mode of application		Research findings	
				Concentration, %	Exposure	Experiment. group	Control
"Javelle-Kleyd"	Guinea-pigs	3	1	0,1	30 minutes	–	+
"Geksadekon"	Guinea-pigs	3	1	3	5 hours	–	+

Note: "+" tubercular changes in guinea-pigs, "–" no tubercular changes in guinea-pigs

Based on the information given in Table 3, it can be seen that in the guinea-pigs from experimental groups, in the internal organs there were no characteristic changes related to tuberculosis changes that could be found. On the other hand, in the control group, in lost (?) laboratory animals, pathological changes were discovered, that can be characterized as typical for tuberculosis. It should also be marked, that when allergic test for tuberculosis was performed in guinea-pigs, the intradermal reactions to tuberculin were discovered in the animals from the control group.

CONCLUSION

1. Crop and biological methods have bactericidal activity in relation to *Mycobacteria tuberculosis*, bovine kind with disinfectants "Javelle-Kleyd" in concentration 0,1 % and exposure of 30 minutes and 1 hour and "Geksadekon" in concentration 3 % and exposure time of 5 hours.
2. "Javelle-kleyd" and "Geksadekon" disinfectants in certain modes, are possible to apply for prophylactic and forced disinfection in happy and unhappy economies in relation to tuberculosis of animals.

LITERATURE

1. About the order of test new disinfection facilities for veterinary practice: Methodical pointing. *Gosagroprom of the USSR*. M.: 90, 1987. (7)
2. Babaykin V., Vasilenko P.: Disinfection with the use of aerozoley – an important link is in a prophylaxis but liquidations of diseases of zoons. *Veterinary medicine of Ukraine*. 2, 4, 2000. (5)
3. Leadthrough of veterinary disinfection objects of stock-raising: Instruction. M.: *Agropromizdat*, 1989. – 61 p. (2)
4. Rusenko I.: New index of efficiency of disinfected facilities for sanitation of stock-raising apartments. *Veterinary medicine of Ukraine*.7, 39 – 40, 2005. (3)
5. Selischeva N. V, M. V. Bogach, Y. V. Andrienko, V. P. Monastirli, O. A. Goncharuk: Monitoring of modern disinfectants in relation to the exciter of tuberculosis and atypical Mycobacteria *Veterinary medicine of Ukraine*. 2, 5 – 7, 2013. (8)
6. Setting is on diagnostics of tuberculosis of zoons and bird. – 1997. – 38 P. (9)
7. Volkov Y. P.: Prospects of development researches in an area of the development of disinfectants, Materials of scientific conference "Issues of the day disinfections, sterilizations, fumigations and deratizations ". M.; P. 13 – 14. 1992 (1)

8. Zarickiy A. M.: Disinfection: desinfection facilities and their applications. Zhytomyr: PP « Ruta »,. 1, 384, 2001. (6)
9. Yaschenko M. F.: Desinfection of stock-raising apartments bactericidal suds: *Scientific announcer NAU*. 36, 172 – 174, 2001 (4)

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ENTERIC BACTERIA IN FECAL SAMPLES OF EURASIAN GRIFFON VULTURES

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Abstract

Fecal samples originating from 15 Eurasian griffon vultures were collected during June 2012 in the territory of special nature reservation Uvac and examined for presence of enteric bacteria *Escherichia coli* and *Salmonella* spp. Salmonellas were isolated from five samples (33.3%) and serologically typed as *Salmonella enterica* subsp. enterica ser. Veneziana. *E. coli* was isolated from four samples (26.6%). Antimicrobial susceptibility testing revealed resistance to one and more antibiotics only in *E. coli* isolates.

Keywords: *Escherichia coli*, *Salmonella* Veneziana, Eurasian griffon vultures, antimicrobial resistance

ENTERIČNE BAKTERIJE IZOLOVANE IZ UZORAKA FECESA EVROAZIJSKIH BELOGLAVIH SUPOVA

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Kratak sadržaj

Uzorci fecesa od 15 beloglavih supova sakupljeni su u junu mesecu 2012 na teritoriji specijalnog rezervata Uvac i bakteriološki ispitani na prisustvo bakterija iz rodova *Salmonella* i *Echerichia coli.* Salmonele su izolovane iz pet uzoraka fecesa, a svi izolati serološki tipizirani kao *Salmonella* enterica subsp. enterica ser. Veneziana. *Escherichia coli* je izolovana iz 4

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uzorka. Izolovani sojevi enteričnih bakterija ispitani su na osetljivost prema antibioticima, a rezistencija na jedan ili više antibiotika je ustanovljena kod tri izolata *E. coli*.

Ključne reči: *Escherichia coli*, *Salmonella* Veneziana, evroazijski beloglavi sup, rezistencija na antibiotike

Introduction

Wild birds play an important role in the ecology and circulation of human and animal pathogens including viruses, bacteria, fungi and protozoa (Hubalek, 2004; Abulreesh et al., 2007; Benskin et al., 2009). In some European countries, wild birds (particularly gulls) are identified as reservoirs of enteric pathogens such as various serotypes of *Salmonella* (Kapperud et al., 1998; Hubalek, 2004; Čižek et al., 2007) and toxin-producing *Escherichia coli* strain (Abulreesh et al., 2007), which could be also multiple resistant (Palmgren et al., 2006; Čižek et al., 2007; Dolejska et al., 2007). Prevalence of salmonellosis among the wild-bird population has shown increasing tendency over the past 40 years as a result of artificial feeding by humans (Tizard, 2004). Wide range of *Salmonella* serotypes was isolated in wild birds, including multidrug-resistant strains of *S.* Typhimurium DT104 (Reche et al., 2003; Čižek et al., 2007). Enteric pathogens mainly reside in the intestinal tract of raptors or opportunistic carrion eating birds (such as vultures, crows and gulls), who feed on the ground, at sites where human waste is released or live by the fecally contaminated waters (Gerlach 1994; Tizard, 2004, Palmgren et al, 2006; Kocijan et al., 2009). Both *Salmonella* and *E. coli* can be pathogenic for birds; however, more frequently, the birds are potential healthy carriers and play a role in the dissemination of the agents in the environment by contaminating the natural waters and animal feed (Palmgren et al., 2006; Čižek et al., 2007). Though their role in the epidemiology of salmonellosis is still debated, several studies have confirmed the possibility that wild birds could serve as the source of human infection (Millan et al., 2004, Kruse et al., 2004).

Eurasian griffon vultures (*Gyps fulvus*) are at the *top* of the *food chain and play an essential role in the nature as they feed exclusively on carcasses of dead animals. The species inhabits the regions of Southern Europe, Southwestern Asia and North Africa. During the '90s of the last century, the survival of this species was critically endangered. Decrease in* Eurasian griffon vulture population is closely associated with abandoning of extensive livestock breeding in the area and thus food scarcity, poisoning (Sabočanec et al., 2005) and laying poisoned baits for large predators (Pavoković, 2005) as well as the EU Regulations on mandatory carcass disposal

aimed at prevention of Bovine Spongiform Encephalopathy. Limitation of natural habitat and reduced availability of their natural prey in our country resulted in changes of feeding habits of Eurasian griffon vultures. The diet of these wild birds is based mainly on slaughterhouse waste from intensive farming systems. Such diet could contribute to alterations of common microflora of the digestive tract. The available literature data on bacteriological examination Eurasian griffon vulture samples are still sparse both in the territory of Balkans and at a global level (Milan et al., 2004; Sabočanec et al., 2005; Kocijan et al., 2009). The aim of our study was to investigate presence of enteric bacteria in fecal samples (*Escherichia coli* and *Salmonella* spp.), which are not considered as a part of common intestinal microflora of Eurasian griffon vultures, and to examine the antibiotic susceptibility of these bacterial isolates.

Materials and methods

Sample collection

Location: Uvac River canyon valley with its three lakes (Uvačko, Zlatarsko and Radoinjsko Lake) as a Special Nature Reserve is protected natural asset of great importance according to the Decree of the Government of the Republic of Serbia („Official Gazette of the RS" No. 25/06 and 110/06). It occupies some 7 500 ha between mountain Zlatar massif in the Southwest and mountain Javor in the Northeast. The estimated population of griffon vultures in this reservation is currently around 100 nesting couples, and total population (including young birds) is some 300 birds. The adult nesting birds mostly do not leave their natural habitat, whereas young birds migrate during winter months to the north (via Slovenia and Italy to the Alps), to the south (Bulgaria, Greece, Israel and Africa) and to the west (France and Spain).

„The magnificent Uvac valley", natural habitat of Eurasian griffon vulture (http://www.politika.rs/rubrike/putovanja/U-carstvu-beloglavog-supa.lt.html)

Sampling was carried out at Uvac natural reservation in June 2012, during early morning hours. At the moment of sampling, around 50 Eurasian griffon vultures were present at the selected location. According to observation results, the birds were in good health condition. Fifteen fresh fecal samples were collected using sterile swabs. The samples were cooled and transported to the laboratory of clinical microbiology of the Scientific Veterinary Institute „Novi Sad".

Microbiology examination

Isolation of E. coli and Salmonella spp.:

Collected fecal swabs were inoculated into 2mL peptone water (Buffered peptone water, CM1049, Oxoid, Basingstoke, UK) and vortexed for 30 sec at maximum rpm. For the isolation of *E.coli*, 30 µL of suspension was inoculated onto the Columbia blood agar base (CM0331, Oxoid) with 5% sterile defibrinated sheep blood and MacConkey agar (CM0007, Oxoid). Inoculated plates were incubated at 37°C during 24-48h. Colonies of the characteristic appearance for the *Enterobacteriaceae*, were subcultivated on nutrient agar (CM0003, Oxoid). The colonies were examined using catalase and oxidase test and biochemical series test: "IMViC" (indole – methyl red-Voges Proskauer–citrate).

Isolates that resulted +/+/-/- on the "IMViC" test were further examined using BBL Crystal Enteric/Nonfermenter test (Becton Dickinson, Detroit, MI, USA). Isolation of *Salmonella* spp. was performed according to the protocol follow the ISO-6579: 2002 standard (Annex D), and biochemical confirmation of the isolates according to ISO 6579:2008 (9.5.3). Verification and serological typing of *Salmonella* isolates was performed in the National Reference Laboratory for *Salmonella, Shigella, Vibrio cholerae, Yersinia enterocolitica,* Institute of Public Health of Serbia "Dr Milan Jovanovic Batut", Beograd, Serbia.

Antibiotics susceptibility test:

Susceptibility of *Salmonella* and *Escherichia coli* isolates was examined by the disc diffusion method using Mueller-Hinton agar (CM337, Oxoid, Basingstoke, UK) according to Clinical and Laboratory Standards recommendation (document M100-S22, CLSI, 2012). The following antibiotics were tested: amoxicillin + clavulanic acid (20/10 µg), ampicillin (10 µg), cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg); ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulphonamides (300 µg), tetracycline (30 µg), trimethoprim (5 µg), trimethoprim + sulfamethoxazole (1,25/23,75 µg) (antimicrobial susceptibility discs Bio-Rad Laboratories, France). The plates were incubated for 18h at 37°C.

Results AND Discussion

In the '90s of the last century, the griffon vulture population in the Republic of Serbia was threatened by extinction. In the territory of Uvac-lakes, there were only seven individuals of this rare species from the *Accipitridae* family. The survival of this endangered species was enabled through continual delivery of slaughterhouse waste (some 50 tons per year). Nowadays, Uvac is the largest colony of this unique bird species in the Balkans, and among the large ones in Europe. Diet based on animal carcasses, i.e. sheep, bovine and horses from intensive farming systems, undoubtedly contributed to the survival of griffon vultures; however, feeding slaughterhouse waste could negatively affect the health of avian scavengers and promote transmission and spread of bacteria from these sources (Blanco et al., 2006).

In this research, bacteria of the genus *Salmonella* were isolated from five (33.3%) fecal samples originating from Eurasian griffon vultures. All isolates fermented glucose, produced H2S and L-lysine decarboxylase. Negative reactions were obtained for Voges Proskauer and indole tests, lactose fermentation, production of β-galactosidase (ONPG) and urease. In the national Reference Laboratory for *Salmonella, Shigella, Vibrio cholerae, Yersinia enterocolitica*, all isolates were serotyped as *Salmonella enterica* subsp. enterica ser. Veneziana

(11: i: e, n, x). This *Salmonella* serotype was first isolated from an apparently normal Italian civilian food handler in Venice, Italy, and described as a new serotype in 1945 (Bruner et al., 1945). *S.* Venezianae is *not listed among* the *15 most frequently isolated* serotypes in Serbia (www.who.int/gfn). According to the available literature, data on isolation of this serotype are scanty. In the period 1998-2008 in the U.S., only one case of laboratory-confirmed isolation of this serotype in humans was reported (CDC, 2008). S. Venezianae was isolated from mesenteric lymph nodes of one horse out of 500 examined samples taken from 100 slaughtered healthy horses (Bonardi et al., 1997). Disc diffusion test with S. Veneziana isolates did not reveal resistance against antibiotics used in the experiment.

The birds are generally considered as healthy carriers of *Salmonella* (Mikaelian et al., 1997; Milan et al., 2004), since their isolation is usually not associated with symptoms of the disease (Palmgren et al., 2006) nor macroscopic or microscopic lesions characteristic for salmonellosis (Milan et al., 2004). Epizootic outbreaks of salmonellosis in raptors are extremely rare as compared to other bird species (Milan et al., 2004). The incidence of one particular serotype indicates the existence of one same infection source or possibility of specific adaptation of *Salmonella* to the host, when it establishes itself as a part of the intestinal flora (Tizard, 2004). In free living raptors, examination of fecal samples revealed mainly low incidence of salmonellas, being 1.5% (Skov et al., 2008) , 1.9% (Mikaelian et al., 1997) and 4.19% (Reche et al. 2003). Within the framework of large-scale investigation of salmonellosis in wild animals, Milan et al. (2004) established a prevalence of 8.5% (7/82) in wild birds, while *S.* Typhimurium was isolated in one, out of three examined fecal sample originating from Eurasian griffon vultures.

Bacteriological examination of fecal samples in this research revealed presence of *Escherichia coli* in four (26.6%) samples. Kocijan et al. (2009) performed bacteriological, mycological and parasitological analysis of fecal samples (n=10) and vomit samples (n=5) of griffon vultures from the island Cres, Croatia. *Escherichia coli* was the most frequent bacterial isolate in both sample categories (feces 9/10, vomit samples 2/5). In raptors, *Escherichia coli* can be associated with diverse pathologic lesions (bumble foot, pericarditis, hepatic abscesses, salpingitis, and egg peritonitis) (Morishita et al., 1997). However, the virulence factors of particular *E.coli* strains, which are of importance in humans and mammals, do not accurately predict the relative importance of the strains for birds (Gerlach, 1994). In this research, one *E. coli* isolate manifested multiple-resistance to streptomycin, tetracycline, amoxicillin+clavulanic acid, ampicillin and t*rimethoprim/sulfamethoxazole, whereas two isolates were resistant to*

ampicillin. The reports on bacteriological examination of samples originating from Eurasian griffon vultures are extremely limited (Milan et al., 2004; Sabočanec et al., 2005; Kocijan et al., 2009), whilst data on antibiotic susceptibility of bacterial isolates are still lacking.

Undoubtedly, humans play an important role in spreading pathogens among the wild bird population through contamination of waters by sewage waste, inadequate waste management and by artificial feeding of birds. Predominance of enteric bacteria and antibiotic-resistant strains within the population of griffon vultures, as well as their role in survival and spread of such bacteria in the natural environment remains unclear. Transmission of bacteria from birds to mammals requires repeated exposure and large numbers of microorganisms (Gerlah, 1994), thus the importance of Eurasian griffon vultures as a source of human and animal infection is not significant, which is due to the „solitary lifestyle“ of these birds in inaccessible terrain. However, as feeding ecology is the principal factor that influences exposure of birds to intestinal bacteria (Benskin et al., 2009), the diet of griffon vultures based on slaughterhouse waste might have some negative impact upon health status of these birds and spreading of resistant strains of enteric bacteria over large distances by young migrating birds.

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LITERATURA

1. Albureesh H.H., Goulder R., Scott G.W.: Wild birds and human pathogens in the context of ringing and migration. *Ringing & Migration*, 23, 193–200, 2007.
2. Benskin C.M., Wilson K., Jones K., Hartley I.R.: Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biol Rev*, 84, 349–373, 2009.
3. Blanco G., Lemus J.A., Grande J.: Faecal bacteria associated with different diets of wintering red kites: influence of livestock carcass dumps in microflora alteration and pathogen acquisition. *J Appl Ecol*, 43, 990–998, 2006.
4. Bonardi S., Bottarelli A., Bentley S., Poeta A., Alberti C.A.: Isolation of Salmonella typhimurium and Salmonella veneziana from slaughtered horses. *Atti della Societa Italiana delle Scienze Veterinarie*, 51, 703-704, 1997.

5. Bruner D.W., Captain V.C., Joyce B.J.: Salmonella Veneziana – a new type. Veterinary Section of the 15th Medical General Laboratory, 1945.
6. Centers for Disease Control and Prevention, National Salmonella Surveillance System, Annual Summary, 2008.
7. Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement. Clinical and Laboratory Standards Institute document M100-S22. CLSI, Wayne, Pa, USA, 2012.
8. Dolejska M., Čižek A., Literak I.: High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from black-headed gulls in the Czech Republic. *J Appl Microbiol*, 103, 11-19, 2007.
9. Čižek A., Dolejska M., Karpiškova R., Dedičova D., Literak I.: Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella* strains resistant to antimicrobial drugs. *Eur J Wildlife Res*, 53, 55-60, 2007.
10. Gerlach H.: Disease etiologies: Bacteria, in: Avian medicine: principles and application, Section five, Chapter 33, Wingers Publishing, Inc., Lake Worth, Florida, pp. 949-965, 1994.
11. Hubalek Z.: An annotated checklist of pathogenic microorganisms associates with migratory birds. *J Wildlife Dis*, 40, 639–659, 2004.
12. International Organization for Standardization, Geneve, Switzerland, ISO 6579:2002, Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. - Amendment 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage (ISO 6579:2002/Amd 1:2007).
13. Kapperud G., Stenwig H., Lassen J.: Epidemiology of *Salmonella* Typhimurium O:4-12 infection in Norway: evidence of transmission from an avian wildlife reservoir. *Am J Epidemiol*, 147**,** 774-782, 1998.
14. Kocijan I., Prukner-Radovčić E., Beck R., Galov A., Marinculić A., Sušić G.: Microflora and internal parasites of the digestive tract of Eurasian griffon vultures (Gyps fulvus) in Croatia. *Eur J Wildlife Res*, 55, 71-74, 2009.
15. Kruse H., Kirkemo A.M., Handeland K.: Wildlife as source of zoonotic infections. Emerg Infect Dis, 10, 2067-2072, 2004.
16. Mikaelian I., Daignault D., Duval M.C., Martineau D.: *Salmonella* infection in wild birds from Quebec. *Can Vet J*, 38, 385, 1997.
17. Millán J., Aduriz G., Moreno B., Juste R.A., Barral M.: *Salmonella* isolates from wild birds and mammals in the Basque Country (Spain). *Rev sci tech Off int Epiz*, 23**,** 905-911, 2004.
18. Morishita T.Y., Aye P.P., Brooks D.L.: A survey of diseases of raptorial birds. *J Avian Med Surg*, 11, 77-92, 1997.

19. Palmgren H., Aspan A., Broman T., Bengtsson K., Blomquist L., Bergstrom S., Sellin M., Wollin R., Olsen B.: *Salmonella* in Black-headed gulls (*Larus ridibundus*); prevalence, genotypes and influence on *Salmonella* epidemiology. *Epidemiol Infect*, 134, 635–644, 2006.
20. Pavoković G., Sušić G.: Poisoning of 17 Eurasian Griffons by carbofuran on the Island of Rab, Croatia, in December 2004. *Vulture News*, 53, 24-25, 2005.
21. Reche M.P., Jimenéz P.A., Alvarez F., García de los Rios J.E., Rojas A.M., de Pedro P.: Incidence of salmonellae in captive and wild free-living raptorial birds in central Spain. *J Vet Med B Infect Dis Vet Public Health*, 50, 42-44, 2003.
22. Sabočanec R., Konjević D., Srebočan E., Petrinec Z.: Fatal poisoning of a Griffon vulture (*Gyps fulvus*) with methomyl. *Eur J Wildlife Res*, 51, 210–212, 2005.
23. Skov M.N., Madsen J.J., Rahbek C., Lodal J., Jespersen J.B., Jørgensen J.C., Dietz H.H., Chriél M., Baggesen D.L.: Transmission of *Salmonella* between wildlife and meat-production animals in Denmark. *J Appl Microbiol*, 105, 1558-1568, 2008.
24. Tizard I.: Salmonellosis in Wild Birds, Seminars in avian and exotic pet medicine. 13, 50-66, 2004.

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DETECTION OF AEROLYSIN (*aerA*) GENE IN *AEROMONAS HYDROPHILA* STRAINS ISOLATED FROM DISEASED CARP

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Abstract

Bacterial septicemia caused by motile aeromonads is common infection in the intensive fish production. *Aeromonas (A.) hydrophila* is often present in fish populations. Ubiquitous distribution of these bacteria in the aquatic environment, and the stress caused by intensive breeding are predisposing factors for the occurence of the disease. *A. hydrophila* is considered a major cause of septicaemia caused by motile aeromonads. Several *A. hydrophila* extracellular products (ECP) are considered as important factors in pathogenesis, primarily aerolysin (aerA), the extracellular lipase, cytolytic enterotoxin, hemolytic toxin and extracellular proteases. PCR detection of aerolysin (*aerA*) is considered a reliable method of identifying potentially pathogenic *Aeromonas* strains. In spring 2012, after a sudden increase in water temperature, disease occured in common carp population in one fish farm in Serbia. Five specimens of the one-year-old carp with clinical symptoms of motile aeromonas septicaemia were used for isolation of the bacteria. Identification of *A. hydrophila* was done on the basis of morphological, physiological, cultural and biochemical characteristics. PCR amplification of DNA from *A. hydrophila* isolates revealed presence of aerolysin (*aerA*) gene in all examined *A. hydrophila* isolates from carp with motile aeromonas septicaemia.

Key words: *Aeromonas hydrophila*, PCR, aerolysin, carp

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DOKAZIVANJE PRISUSTVA AEROLIZIN (*aerA*) GENA PATOGENIH SOJEVA *AEROMONAS HYDROPHILA* IZOLOVANIH IZ OBOLELIH ŠARANA

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Kratak sadržaj

Bakterijske septikemije izazvane pokretnim predstavnicima roda Aeromonas spadaju u česte infekcije riba u intezivnom gajenju, ali zahvataju i populacije riba otvorenih voda. Široka rasprostranjenost ovih bakterija u vodenoj sredini, i stres uslovljen intenzivnim gajenjem predstavljaju predisponirajuće faktore za nastanak oboljenja. *Aeromonas hydrophila* je primarni ili sekundarni uzročnik bolesti vodenih i kopnenih životinja i ljudi, i njegova patogenost je povezana s faktorima virulencije. *A. hydrophila* se smatra glavnim uzročnikom septikemije izazvane pokretnim aeromonadama. Nekoliko ekstracelularnih proizvoda (ECP) bakterije *A. hydrophila* se smatraju značajnim faktorima u patogenezi, pre svega aerolizin (aerA), ekstracelularne lipaze, citolitički enterotoksin, hemolitički toksin i ekstracelularne proteaze. Detekcija aerA pomoću PCR se smatra pouzdanim načinom identifikacije potencijalno patogenih sojeva *Aeromonas hydrophila*. Kod šarana u prolećnom periodu, prilikom naglog povišenja temperature vode, utvrđena je septikemična forma oboljenja koja je podsećala na prolećnu viremiju šarana. Pet primeraka jednogodišnje mlađi šarana sa kliničkim simptomima bakterijske infekcije su korišćeni za izolaciju bakterija. Identifikacija *A. hydrophila* izvršena je na osnovu morfoloških, fizioloških, kulturelnih i biohemijskih karakteristika. Za identifikaciju gena virulencije odabrane su kolonije *A. hydrophila* izrasle u čistoj kulturi na Rimler-Shotts medijumu. PCR amplifikacijom DNK iz izolata *A. hydrophila* dobijeni su PCR produkti veličine 462bp kod svih ispitivanih uzoraka. U našem istraživanju, pomoću PCR je dokazano prisustvo aerolizin gena kod patogenih sojeva *A. hydrophila* izolovanih iz obolelih šarana.

Ključne reči: *Aeromonas hydrophila*, PCR, aerolizin, šaran

INTRODUCTION

Aeromonas hydrophila, a Gram-negative, motile rod that is a member of the family *Aeromonadaceae* (Joseph & Carnahan 2000; Abbott et al. 2003), has been widely studied and is regarded as the most important bacterium causing "aeromonosis or haemorrhagic septicaemia or motile aeromonas septicaemia" in fish (Rhaman et al. 2001) and other aquatic animals (Hill et al. 2010; Pearson et al.2000). There have been a number of epidemiological studies indicating Aeromonas species as a cause of diarrheal disease in children, elderly people and immunocompromised patients (Figueras, 2005; von Gravaenitz, 2007).

The widespread of the bacteria in the aquatic environment and the stress caused by intensive breeding are predisposing factors for the disease. Stressful environmental factors, especially high water temperature, high levels of ammonia and nitrite, sudden changes in pH, and low concentrations of oxygen increases the possibility of disease occurence (Jeremic et al. 2005).

Several extracellular products (ECP) of *A. hydrophila* are considered important virulence factors, primarily aerolysin, extracellular lipase, cytolytic enterotoxin, hemolytic toxin and extracellular protease (John et al., 1997, Shome et al., 2005). Detection of virulence genes by PCR is very useful for the identification of pathogenic isolates of aeromonads (Uzbas et al., 2000).

Detection of aerolysin (*aerA*) using PCR and RFLP is considered a reliable for identification of virulent strains of *A. hydrophila* (Kingombe et al., 1999). The aim of this study was to determine the presence of aerA gene in *A. hydrophila* strains isolated from carp with motile aeromonad septicaemia.

MATERIAL AND METHODS

Five samples of the one-year-old carp with clinical symptoms of bacterial infection were used for isolation. From each fish, samples were collected from the kidney, liver, spleen and gills. The samples were streaked on tryptic soy agar (TSA, HiMedia), Mueller-Hinton agar (HiMedia) containing 5% defibrinated sheep blood erythrocytes (BA), selective Rimler-Shotts (RS) media (HiMedia), and incubated at 30^{o}C for 24 to 48^{h}. Following incubation, one typical colony (entire circular, convex, white to greyish, semitranslucent, size 2 to 3 mm, haemolytic) was selected from each plate with a pure culture and subcultivated in order to test the purity of isolates. The isolates were preliminary grouped according to colony morphology, haemolysis, and pigmentation before they were stored at –80^{o}C in 15% glycerol until further characterization. The type strain of Aeromonas hydrophila ATCC 7966 (American Type Culture Collection) was included in the phenotypic characterization.

Isolates were classified as *Aeromonas hydrophila* according to their reactions in the API 20E (Biomerieux) and following conventional tests, based on standard bacterial taxonomic procedures (Holt et al 1994; Austin and Austin, 2007).

Archives of Veterinary Medicine, Vol. 6, No. 2, 55 - 63, 2013.
Radosavljević V. i dr.: Detection of Aerolysin …

For identification of virulence genes, five colonies of *A. hydrophila* were selected and 24 hours old cultures were used for extraction of genomic DNA. DNA extraction was performed using commercial kit (QIAamp DNA Mini Kit, Qiagen) according to manufacturer's protocol. Detection of the aerolysin gene was performed using polymerase chain reaction (PCR) (Chu et al., 2005). Primers were used to detect 462bp aerolysin gene fragment (Aero1: 5'-CTCAGTCCGTGCGACCGACT-3' and Aero2: 5'-GATCTCCAGCCTCAGGCCTT-3'). Amplification was performed by 35 cycles of denaturation at 95° C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 2 min. After amplification, PCR products were characterized by 1.5% agarose gel electrophoresis in Tris–borate–EDTA buffer.

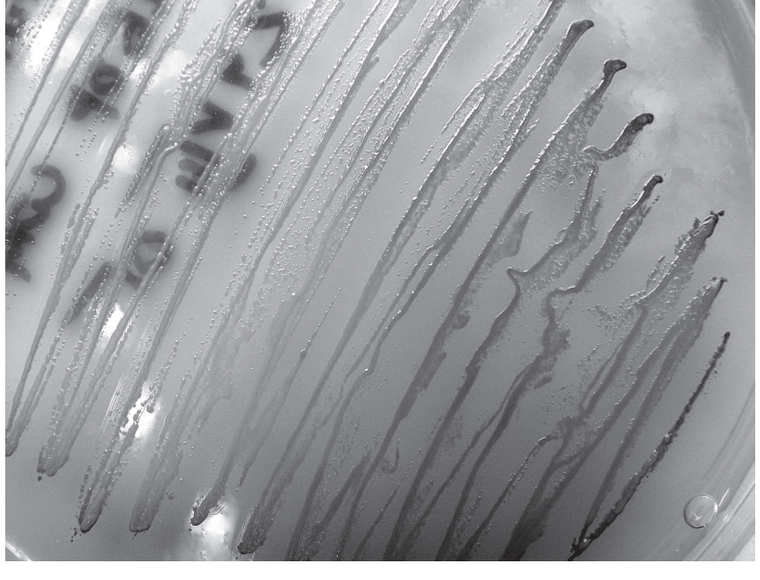
RESULTS

On a carp pond, localized in the northeastern part of the Republic of Serbia, in the spring, during a sudden increase in water temperature, increased mortality of young carp occured. The main signs of the diseased fish were anorexia, exophthalmus, redding due to haemorrhage of the skin and swimming at the surface of the pond, near fresh water supply. In scaled fish, scale pockets become edematous, causing lepidorthosis (Figure 1). Internal organs were edematous with hemorrhage and erythema on liver and kidney.

Figure 1. The pathological symptoms of the common carp suffering from motile aeromonad septicemia.

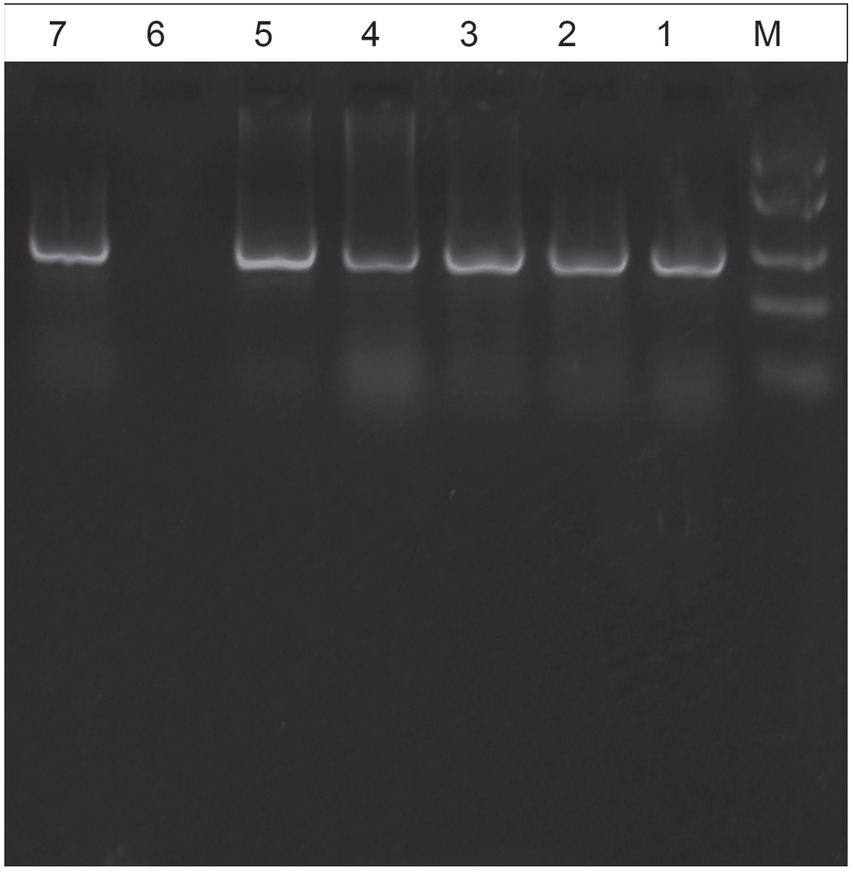
Pure cultures of *Aeromonas hydrophila* were obtained from all samples (Figure 2).

Figure 2. Growth of Aeromonas hydrophila on Rimler-Shotts medium.



A PCR amplification revealed that all *A. hydrophila* isolates were PCR positive for the *aer*A gene.

Figure 3: PCR amplification of *A.hydrophila* isolates for the 426-bp *aer*A gene. Lane M, 100-bp molecular weight marker; lane 1-5, 426-bp *aer*A amplified from the genomic DNA, lane 6-negative control, lane 7-positive control.



DISCUSSION

Motile aeromonads cause different pathologic conditions that include acute, chronic and latent infection. Severity depends on a number of factors including bacterial virulence, type and level of stress, resistance and physiological state of the host. In the acute phase, this condition is characterized by rapid fatal septicemia with little macroscopic evidence of disease. When present, the

most important symptoms are exophthalmos, skin redness and fluid collection in the scale pockets (Faktorovich, 1969).

There is evidence that the motile Aeromonas complex involves secondary and opportunistic pathogens, but ability of *A. hydrophila* to cause disease and death of fish should not be overlooked because occasionally highly virulent strains emerge. Regardless of whether or not the organism serves as a primary or secondary invader of stressed fish, it is often the final insult that leads to death (Plumb and Hanson 2011).

In attempt to explain the pathogenesis of infection caused by *A. hydrophila* several virulence factors were investigated. Toxins with haemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas spp*. (Chopra et al., 1990).

Although rare, *A. hydrophila* may cause high mortality among cultured fish without presence of severe external (stressful) influences. This inconsistency may result from the presence of A. hydrophila strains that possess specific virulent or pathogenic characteristics (Plumb and Hanson 2011).

In our study, PCR was performed to detect aerolysin (*aerA*) gene as a genetic marker for the determination of virulence. Role of aerolysin (*aerA*) gene in the pathogenicity of *Aeromonas* genus was previously demonstrated (Kozaki et al., 1989, Shaw, 2003). In present study, PCR amplification of *A.hydrophila* isolates for the 426-bp *aer*A gene, in samples from diseased fish, showed the presence aerolysin gene, which is an essential *A. hydrophila* virulence gene. Similar results were obtained in previous studies with *A. jandaei* (Chacón, 2003). It is well known that the screening of specific cytotoxin and hemolysin genes is the most effective way of detecting and characterizing *Aeromonas* virulence factors (Yousr et al., 2007).

CONCLUSIONS

Despite disagreement among scientists regarding significance of *A. hydrophila* infection, the frequency of its appearance in aquaculture environment, together with a high potential for stress, shows that this problem sholud not be ignored, because it is often what kills the fish. PCR test for the detection of aerolysin gene proved to be a useful tool for the detection of virulent strains of *Aeromonas hydrophila*.

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LITERATURE

1. Abbott S.L., Cheung W.K.W. & Janda J.M.: The genus Aeromonas: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *Journal of Clinical Microbiology* 41, 2348–2357, 2003.
2. Austin, B. and Austin, D.A.: Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish, 4th edn. Chichester, UK: Springer-Praxis, 2007.
3. Chacón, M.R., M.J. Figueras, G. Castro-Escarpulli, L. Soler, J. Guarro. Distribution of virulence genes in clinical and environmental isolates of *Aeromonas* spp. *Antonie van Leeuwenhoek*, 84 (4), 269-78, 2003.
4. Chopra, A.K., C.W. Houston and Kurosky A.: Genetic variation in related cytolytic toxins produced by different species of *Aeromonas*. *FEMS. Microbiol. Lett*., 78, 231-7, 1990.
5. Chu, W.H and Lu, C.P.. Multiplex PCR assay for the detection of pathogenic *Aeromonas hydrophila*. *Journal of Fish Diseases*, 28, 437–41, 2005.
6. Faktorovich, K. A.: Histological changes in the liver, kidneys, skin and brain of fish sick with red rot. Infectious diseases of fish and their control. Division of Fisheries Research Bureau of Sport Fisheries and Wildlife. Washington, D. C, 83-101, 1969.
7. Hill, W. A., Newman, S. J., Craig, L., Carter, C., Czarra, J. & Brown, P.: Diagnosis of *Aeromonas hydrophila*, Mycobacterium species, and *Batrachochytrium dendrobatidis* in an African Clawed Frog (*Xenopus laevis). J Am. Assoc. Lab. Anim. Sci*. 49, 2, 215-220, 2010.
8. Holt, J.G., Krieg, N.R., Sneathm, P.H.A., Staley, J.T. and Williams, S.T.: Bergey's Manual of Determinative Bacteriology, 9th edn. Baltimore, MD: Williams and Williams, 1994.
9. Figueras M.J.: Clinical revelance of Aeromonas spp. Rev. *Clin. Microbiol.* 16, 145-153, 2005.
10. Jeremić Svetlana, Radosavljević V., Jakić-Dimić, Dobrila.: Current bacterial diseases of freshwater fish. *Biotechniology and Animal Husbandry*.; 21, 3-4, 141-51, 2005.
11. John M.P., Stephen P.K. & Radomin S. Secreted enzymes of Aeromonas. *FEMS Microbiology Letters*. 15, 1–10, 1997.
12. Joseph S.W. & Carnahan A.M. Update on the genus Aeromonas. *American Society for Microbiology News* 66, 218– 223, 2000.
13. Kingombe, C.I., G. Huys, M. Tonolla, M.J. Albert, J. Swings, R. Peduzzi and T. Jemmi. PCR detection, characterization and distribution of virulence genes in *Aeromonas* spp. *Appl. Environ. Microbiol.*, 65, 5293-302, 1999.

14. Kozaki, S., A. Tsutomu, K. Yoichi and G. Sakaguchi.: Characterization of *Aeromonas sobria* hemolysin by use of monoclonal antibodies against *Aeromonas hydrophila* hemolysins. *J. Clin. Microbiol*., 27, 1782-86, 1989.
15. Pearson MD, Hirono I, Aoki T, Miranda R, Inglis V.: Virulence properties of motile aeromonads isolated from farmed frogs Rana tigerina and R. rugulosa. *Dis Aquat Organ*., 40, 3, 185-93, 2000.
16. Plumb JA, Hanson LA : Motile Aeromonas septicemia (MAS). In: Plumb JA, Hanson LA (Eds): Health maintenance and principal microbial diseases of cultured fshes, 3rd edition. Blackwell Publishing pp. 293-300, 2011.
17. Rhaman M.H., Suzuki S. & Kawai K.: The effect of temperature on Aeromonas hydrophila infection in goldfish, Carassius auratus. *Journal of Applied Ichthyology* 17, 282–285, 2001.
18. Shaw, D., H.J. Hodder,. Lipolysaccharides of the motile aeromonads; core oligosaccharides analysis as an aid to taxonomic classification. *Can. J. Microbiol*., 864-68, 2003.
19. Shome, R. , B., R. Shome., Y. Mazumder., A. Das., A. Kumar., H. Rahman, Bujarbaruah K. M. Abdominal dropsy disease in major carps of Meghalaya: Isolation and characterization of *Aeromonas hydrophila*. *Current Science*, 88, 12, 2005
20. Uzbas Z.Y., Lehner A. & Wagner M.: Development of a multiplex and semi-nested PCR assay for detection of *Yersinia enterocolitica* and *Aeromonas hydrophila* in raw milk. *Food Microbiology,* 17, 197 - 203, 2000.
21. Von Gravenitz A.: The role of *Aeromonas* in diarrhea: a review. *Infection* 35, 59-64, 2007.
22. Yousr, A.H., Napis, S., Rusul, G.R.A. and R. Son.: Detection of Aerolysin and Hemolysin genes in *Aeromonas spp*. isolated from environmental and shellfish sources by polymerase chain reaction. *ASEAN Food Journal*,14, 2, 115-22, 2007.

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SEROPREVALENCE OF *SALMONELLA* SPP. ON IMPORT BOARS FROM DENMARK TO SERBIA

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Abstract

The sera from 120 boars from 3 farrow-to-finish swine herds in Serbia were examined for *Salmonella* spp. antibodies in a cross-sectional study using an ELISA test. A total of 120 blood serum samples from boars imported from Denmark were examined. All boars were seronegative to *Salmonella* during the import, while after one year *Salmonella* spp. seroprevalence ranging from 0% to 45% was found in 3 herds using two tests in the time span of one year. Only on one farm during the import in 2011, after a year boars were still seronegative. A year later on the same farm, in boars imported in 2012 the seroprevalence was 30%. Seroprevalence of *Salmonella* spp. in boars tested on all farms was 24,17%.

Key words: boar, seroprevalence, *Salmonella,* import

Kratak sadržaj

Serumi od 120 nerastova iz tri farme u Srbiji sa zatvorenim ciklusom proizvodnje su ispitani na prisustvo antitela specifičnih za *Salmonella* spp. metodom ELISA testa. Svih 120 krvnih seruma je ispitivano od nerastova uveženih iz Danske. Svi nerastovi su bili seronegativni prilikom uvoza, dok godinu dana kasnije seroprevalenca *Salmonella* spp. se kretala od 0 do 45% u tri ispitivana zapata u dva navrata ispitivanja u period od godinu dana. Samo na jednoj farmi nerastovi uveženi 2011. godine su bili seronegativni i posle godinu dana. Na istoj farmi nerastovi uveženi 2012. godine posle godinu dana su imali seroprevalencu od 30%. Seroprevalenca *Salmonella* spp. kod svih uveženih nerastova na svim farmama je bila 24,17%.

Ključne reči: nerast, seroprevalenca, *Salmonella,* uvoz

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INTRODUCTION

Salmonella spp. does not normally cause clinical disease, but subclinical *Salmonella* spp. infections constitute an important food safety problem throughout the world (Kranker et. al., 2003). *Salmonella* spp. transmission in swine herds is a significant health and hygiene issue concerning public health and food safety. The major source of *Salmonella* contamination in pork is fattening the pigs from latently infected herds (Beloeil et al., 2004). In spite of the widely acknowledged value of controlling *Salmonella* in the live animal reservoir, and copious research endeavors, there is still much to learn about the control of *Salmonella* pre-harvest, as well as discerning the most cost-effective approaches to approaching control in the pork chain (Gorton et al., 2000). *Salmonella* IgG antibodies have been demonstrated in blood serum from boars using commercial kits, because these antibodies persist long after the infection (Šišak et al., 2011). The presence of antibodies indicates that the pigs were exposed to the enteric pathogen in a period of development, but on the other hand, the time needed for seroconversion suggests that pigs are carriers of *Salmonella* while still seronegative, and also different immune responses can affect the serological tests (Boyen et al., 2008).

Import of boars to Serbia occurs regularly in recent decades. When importing boars from breeding stock and/or countries free of certain diseases, there is a chance to confirm the presence of latent infections by certain diseases. The objectives of this study were to: examine *Salmonella* seroprevalence in boars from 3 farrow-to-finish herds, imported from Denmark. Boars were imported twice, in 2011 and 2012.

MATERIAL AND METHODS

Selection of herds. Three Serbian farrow-to-finish swine herds with a capacity of 2000 sows, with an intensive way of keeping the pigs were selected for the study. Each farm imported 20 boars from Denmark in 2011 and 2012. Boars spent 30 days in quarantine, and after that were forwarded for exploitation. All the boars had tags on their ears and a tattooed number. Artificial insemination has been performed on examined farms.

Sampling and laboratory analysis. Before leaving the quarantine (within 5 days from the due date), blood samples were taken from all boars for testing. After a year, blood samples were again taken from the same boars. Blood was taken by the puncture of the brachiocephalic plexus of the boars. A blood serum sample from each boar was frozen, and blood serum (harvested after

thawing) was examined for specific anti-bodies against *Salmonella* spp. using an indirect ELISA. Samples with an OD% > 10 were considered seropositive.

Data analysis. Data were entered into an Excel spreadsheet (Microsoft Excel 2010) and imported into Stata (Stata 8 Intercooled for Windows 9x) in which data were analyzed. Descriptive analysis was done in MiniTab version 14 (MiniTabR14b) and Excel (Microsoft Excel 2010).

RESULTS

Table 1. shows the prevalence estimates provided by blood serum sample collected from boars imported from Denmark. The sera from 120 boars, originating from 3 different farrow-to-finish herds, were examined by ELISA test for the presence of *Salmonella* antibodies.

Table 1. *Salmonella* spp. prevalence estimates provided by blood serum sample collected from boars imported from Denmark

Farm	Year	Number tested	Number positive (in quarantine)	Number positive (after year)	Prevalence estimates (%)
I	2011	20	0	4	20
	2012	20	0	3	15
II	2011	20	0	0	0
	2012	20	0	6	30
III	2011	20	0	7	35
	2012	20	0	9	45
Total		120	0	29	24.17

After the import, boars were placed in quarantine. After a period of resting from transport, within 5 days from the due date, blood samples were taken from boars for serological testing. All of the tested blood serum samples taken from boars during the quarantine in 2011 and 2012 were negative for the presence of antibodies specific for *Salmonella* (Table 1).

A year after the first blood sampling for serological testing, blood serum samples were taken again from the same boars.

In farm 1, *Salmonella* spp. seroprevalence in the boars was 20% (4/20) and 15% (3/20) in 2011 and 2012, respectively.

In farm 2, the blood serum sample from boars was negative in 2011. In 2012, the blood serum sample was positive in 30% (6/20).

In farm 3, Seroprevalence in the boars was 35% (7/20) and 45% (9/20) in 2011 and 2012, respectively.

DISCUSSION

This study provided a unique opportunity to compare serological status of boars prior to arrival and after spending a year in Serbia. All boars were seronegative upon arrival to Serbia. Boars were located on three different farms, where after a year their serological status should represent an indicator of *Salmonella* spp. presence on monitored farms. After one year from import of 60 boars in 2011, 11 boars were found seropositive, whereas on one farm they were all seronegative. The farm where all boars were seronegative after one year, conducted high-level biosecurity measures during that year, boars were kept in a separate building, had no contact with other animals, as well as people who worked with them. After one year from import of boars in 2012, on the same farm there was 6/20 seropositive boars. On the farm during this period isolated *Salmonella* from feed and as a result of the findings of six seropositive boars. Feed as a source of infection for pigs and the prevalence of *Salmonella* in feed have been investigated by Molla et al., 2010. While the other two investigated farms had not high levels of biosecurity measures. According to the literature data, herd seroprevalence rates for *Salmonella* are 93% in Germany, 59% in Denmark, 79% in Greece and 72% in Sweden, evaluated at test cut-off of OD%>10, and herd cut-off of 1 or more seropositive animals. The average rate within herd seroprevalence was 24% for Germany, 9% for Denmark, 14% for Greece and 10% for Sweden (Lo Fo Wong, 2001). Our study found higher seroprevalence in 5/6 herds; interestingly, the same herd that was seronegative became positive in the next year. The average seroprevalence within herd was 24.17, which is also a higher level compared to research results of other authors (Nowak et. al., 2007; Bonde and Sørensen 2012).

CONCLUSION

Based on the results the survey to be concluded that in Serbia there is a high seroprevalence of *Salmonella*. This suggests the necessity of more intensive implementation of biosecurity measures on farms, as well as control of feed for pigs, which can be a potential source of various infections. The high seroprevalence of *Salmonella* in pigs Serbia is a potential risk of contamination of the meat, and therefore infection in human.

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REFERENCES

1. Beloeil P.A., Chauvin C., Proux K., Madec F., Fravalo P., Alioum A.: Impact of the *Salmonella* status of market-age pigs and the pre-slaughter process on *Salmonella* caecal contamination at slaughter. *Veterinary Research*, 35, 513–530. 2004
2. Bonde M., Sørensen J.T.: Faecal *Salmonella* shedding in fattening pigs in relation to the presence of *Salmonella* antibodies in three pig production systems. Livest. Sci. 150, 236–239, 2012
3. Boyen F., Haesebrouck F., Maes D., Immerseel F., van Ducatelle R., Pasmans F.: Non-typhoidal *Salmonella* infections in pigs: a closer look at epidemiology, pathogenesis and control. *Vet. Microbiol*. 130, 1–19. 2008
4. Gorton S.J., Kliebenstein J.B., Beran G.W., Baum D.: Economic analysis of *Salmonella* impacts on swine herds. *Swine Research Report*, Paper 47, 2000
5. Kranker S., Alban L., Boes J., Dahl J.: Longitudinal study of *Salmonella* enterica serotype Typhimurium infection in three Danish farrow-to-finish swine herds. *Journal of Clinical Microbiology*, 41, 2282–2288, 2003
6. Lo Fo Wong D.M.A.: Epidemiology and control options of *Salmonella* in European pig herds. PhD, University Copenhagen, 2001
7. Molla B., Sterman A., Mathews J., Artuso-Ponte V., Abley M., Farmer W., Rajala-Schultz P., Morgan Morrow W.E., Gebreyes W.A.: *Salmonella* enterica in commercial swine feed and subsequent isolation of phenotypically and genotypically related strains from fecal samples. *Applied and environmental microbiology*, 76, 21, 7188-7193, 2010
8. Nowak B., von Muffling T., Chaunchom S., Hartung J.: *Salmonella* contamination in pigs at slaughter and on the farm: A field study using an antibody ELISA test and a PCR technique. *International Journal of Food Microbiology*, 115, 259–267, 2007
9. Šišak F., Havličkova H., Matiašović J., Karpiškova R.: Serological and Bacteriological Evaluation of *Salmonella* status in swine herds. *Czech J. Food Sci*. 29, 102-108, 2011.

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COMPARATIVE INVESTIGATION OF DIFFERENT METHODS FOR THE DETECTION OF INFECTION IN RABBITS CHALLENGED WITH *L. interrogans* SEROTYPE *hardjo*

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Abstract

Keeping up-to-date with modern diagnostic techniques for leptospirosis as well as continuous improvement of laboratory diagnostic methods resulted in abundant knowledge on the nature and consequences of this infection and its importance in both human and veterinary medicine. In that respect, development and introduction of novel diagnostic tests and procedures have become the paramount issue in the diagnostics of leptospirosis and related infectious diseases. Thus, the goal of this research was to investigate the application of diverse laboratory methods and to evaluate their validity in the diagnostics of leptospirosis. Eleven rabbits were artificially infected with live cultures of *L. interrogans* serovar *hardjo* by the method of skin scarification. Blood and blood serum samples of challenged animals were collected every other day throughout the 3-week period (i.e. until day 21), and then once weekly during following five weeks. Blood sera were tested for the presence of *L. interrogans* serovar *hardjo* specific antibodies applying the methods of microscopic agglutination (MA) test and ELISA. Blood samples were examined using the method of cultivation in liquid medium by *Johnson* supplemented with 200*μg*/1*ml* 5- fluorouracil (5-*FU*). Presence/absence of *L.interrogans* serovar *hardjo* was confirmed by polymerase chain reaction (*PCR*) method. In this reaction, a pair of primers separated from the basic structure of the *Leptospira interrogans rrs* (16S) gene. In MA test, the presence of specific antibodies against *L. hardjo* in rabbits was confirmed in 67 (36.61%) of 183 investigated sera. Initial positive specific antibody finding was recorded on day 9 post challenge, and it persisted until day 17. In ELISA test, positive and suspect findings

were confirmed in 67 and 18 samples, respectively. Initial ELISA-positive finding was observed on day 15, showing increasing tendency throughout the monitoring period and reaching its maximum value on day 42. Method of blood sample cultivation resulted in isolation of *L. interrogans* serovar *hardjo* in 33 (18.03%) on day 3 at the earliest, whilst highest isolation rate was observed on day 17 post challenge. Applying polymerase chain reaction (PCR) method, genome or genome sequences of *L. interrogans* serovar *hardjo* were detected in 67 (56.30%) out of 119 blood serum samples. PCR method revealed positive finding as early as on day 1 post challenge, whereas the highest rate of positive findings was recorded on day 19. Comparison of the results obtained by methods of cultivation and PCR during the period from experimental day 1 to 21, i.e. period prior to administration of chemotherapeutic agents, demonstrated high level of linear correlation of $r = 0.8105$ at the 0.01 significance level. After dihydrostreptomycin therapy administered from day 21 post infection, *L. interrogans* serovar *hardjo* could not be isolated using the method of blood sample cultivation. Contrary to that, PCR method revealed the presence of *L. interrogans* serovar *hardjo* genome in 23 samples.

Key words: Leptospirosis, diagnostic methods, *L. interrogans* serovar *hardjo*

UPOREDNO ISPITIVANJE RAZLIČITIH METODA ZA DOKAZIVANJE INFEKCIJE VEŠTAČKI INFICIRANIH KUNIĆA SA *L. interrogans* SEROTIP *hardjo*

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Kratak sadržaj

Praćenjem savremenih metoda dijagnostike leptospiroze, a posebno usavršavanjem laboratorijskih dijagnostičkih metoda, došlo se do brojnih saznanja o prirodi i posledicama ove infekcije, o njenom zdravstvenom značaju kako u veterinarskoj tako i humanoj medicini. Sve ovo ima za posledicu nastojanje da se u dijagnostici zaraznih bolesti kakva je i leptospiroza, razvijaju i uvedu novi dijagnostički testovi i procedure. Imajući u vidu sve navedeno, kao cilj ispitivanja postavljena je potreba da se ispita primena ra-

zličitih laboratorijskih metoda i oceni njihova valjanosti u dijagnostikovanju leptospiroza. U ogledu je veštački inficirano postupkom skarifikacijom kože, 11 kunića živom kulturom *L. interrogans* serovar *hardjo*. Kunićima su uzeti uzorci krvi i krvnog seruma, svakog drugog dana do 21. dan, a zatim 1 nedeljno narednih 5 nedelja. Krvni serumi su ispitani na prisustvo specifičnih antitela protiv *L. interrogans* serovar *hardjo* metodima mikroskopske aglutinacije (MA) i *ELISA*. Uzorci krvi su ispitani metodom kultivacije na tečnoj hranljivoj podlozi po *Johnson*-u sa dodatkom 200*μg*/1*ml* 5- fluorouracila (5-*FU*), a prisustvo/odsustvo genoma *L.interrogans* serovar *hardjo* metodom lančane rakcije polimeraze (*PCR*). U reakciji je korišćen par prajmera izdvojen iz osnovne strukture *Leptospira interrogans rrs* (16S) gena. Specifičnih antitela protiv *L. hardjo* kod kunića primenom MA utvrđena su kod 67 uzoraka seruma ili 36,61% od ukupno ispitanih183, pozitivan nalaz specifičnih antitela najranije je zabeležen 9. dana od inficiranja i održavao se do 17. dana. Primenom *ELISA* testa pozitivni nalazi utvrđeni su kod 67, a sumnjivi kod 18 uzoraka. Najraniji pozitivan nalaz *ELISA* testom zabeležen je petnaestog dana, a zatim broj pozitivnih nalaza raste, dostiže maksimum 42. dana. Metodom kultivacije uzoraka krvi, *L. interrogans* serovar *hardjo* je izolovana kod 33 (18,03%) uzorka, najranije trećeg dana, a najveći procenat izolata zabeležen je 17. dana od inficiranja. Primenom metoda lančane reakcije polimeraze (*PCR*) genom ili delovi genoma *L. interrogans* serovar *hardjo* dokazan je u krvnom serumu kod 67 (56,30%) od 119 uzoraka. Primenom metoda lančane reakcije polimeraze (*PCR*) pozitivan nalaz zabeležen je već prvog dana, a najveći procenat pozitivnih nalaza zabeležen 19. dana od inficiranja. Poredeći rezultate dobijene metodom kultivacije i *PCR* počev od 1. do 21. dana eksperimenta odnosno za period pre prmene hemioterapeutika, ustanovljen je visok nivo linearne korelacije od $r = 0,8105$ na nivou značajnosti od 0,01. Nakon primene dihidrostreptomycine od 21. dana nakon inficiranja, u uzorcima krvi kunića metodom kultivacije nije izolovana *L. interrogans* serovar *hardjo.* Za razliku od metoda kultivacije, primenom *PCR* metoda, dokazana je prisustvo genoma *L. interrogans* serovar *hardjo* kod 23 uzorka.

Ključne reči: Leptospiroza, metodi dijagnostike, *L. interrogans* serovar *hardjo*

INTRODUCTION

Most cases of *L. interrogans* serotype *hardjo* infection in animals are asymptomatic, yet associated with shedding of leptospires in the environment via the urine. Identification of infected animals, particularly carriers, is a multifaceted problem in the view of validity of applied serological tests as well as highly complex and time-consuming isolation procedure. Keeping up-to-date with modern diagnostic techniques for leptospirosis as well as continuous improvement of laboratory diagnostic methods resulted in abundant knowledge on the nature and consequences of this infection and its importance in both human and veterinary medicine. In that respect, development and introduction of novel diagnostic tests and procedures have become the paramount issue in the diagnostics of leptospirosis and related infectious diseases. Thus, the goal of this research was to investigate the application of diverse laboratory methods and to evaluate their validity in the diagnostics of leptospirosis. Immunological assays are highly applicable in the diagnostics of leptospirosis and thus increasingly used in laboratory practice. Reaction of microscopic agglutination (MA), being an eligible diagnostic method, has been the most widely used serological test so far. In the recent years, ELISA has become widely used. However, all the aforementioned tests demonstrated some limitations and drawbacks regarding sensitivity and specificity in the detection of subclinical infections in animals. While the method of isolation is the most accurate and reliable diagnostic tool yet highly demanding, time-consuming and expensive, the polymerase chain reaction (PCR) is sufficiently sensitive, specific and rapid method enabling detection of even small number of leptospires. Capacity of polymerase chain reaction (PCR) to detect presence of 5-10 bacteria per *ml* of the sample indicates its high sensitivity, which makes it more appropriate and applicable as compared to the method of cultivation *Smith et al.* (1994).

MATERIAL AND METHODS

Population of 11 rabbits were challenged with live culture of *L. interrogans* serovar *hardjo* by skin scarification procedure. Blood and blood serum samples of challenged animals were collected every other day throughout the 3-week period (i.e. until day 21), and then once weekly during following five weeks. Blood sera were tested for the presence of *L. interrogans* serovar *hardjo* specific antibodies applying the method of microscopic agglutination (MA) test with live seven-day-old cultures of reference leptospiral serotypes (*Royal Tropical Institute Amsterdam*, *The Nederland*) as well as modified *ELISA* for

detection of *L. hardjo*-specific antibodies in blood serum samples of infected rabbits. In this assay, basic components of the commercial bovine kit were used (*IgG* and *IgM*, producer: *Central Diagnostic, Lelystad*, *the Netherlands A*). The modification has implicated replacement of the conjugate with anti-rabbit immunoglobulins (*IgG*, *IgA, IgM*) produced in pig and conjugated with peroxidase (*MP Biomedicals, Inc; Aurora, Ohio* SAD, *cat. No 1135)*. The titre of used immunoglobulins was determined by chessboard titration. The working titre of conjugated immunoglobulins was 1:200. Detection of *L. hardjo* in blood serum was performed by the method of cultivation in liquid medium (by *Johnson*) supplemented with bovine albumin and 200 *μg/ml* 5 fluorouracil (*5FU*) and by polymerase chain reaction (*PCR*). The reaction was done according to adapted protocol for isolation and diagnostics of leptospiral DNA described by *Merien et al.* (1992). Isolation of DNA from urine samples was performed according to *QIamp® DNA Mini and Blood Mini Kit* (*Quiagen®*, Germany) protocol for DNA purification from blood and body fluids (spin-protocol). PCR reaction was accomplished using two oligonucleotide primers described by *Mrerien et al.* (1992), which correspond to nucleotides 38-57 *A Lepto (5'-GGC GGC GCG TCT TAA ACA TG-3')* and *B Lepto (5'-TTC CCC CCA TTG AGC AAG ATT-3')* from the primary structure of *L. interrogans* serotype *canicola* strain *Moulton* 16S *rRNA* gene. The resulting specific product was 331 *bp* in size. DNA amplification was performed in a 25 μl reaction, using a thermal cycler "*MultiGene Thermal Cycler, TC9600-G*" manufactured by *LabnetInternational*, *Inc (NY., USA*). Temperature regimen encompassed 15-minute *Taq* polymerase activation period at 95 ^{0}C with simultaneous phase of initial denaturation of DNA template from the sample, followed by 35 cycles encompassing 1.5-minute denaturation at 94 ^{o}C, 1-minute hybridization (*annealing*) at 63 ^{o}C, 2-minute elongation at 72 ^{o}C and final elongation (extension) at 72 ^{o}C during 10 minutes. The resulting amplification product was applied onto the 1.5% gel supplemented with *ethidiumbromid* and subjected to electrophoresis. The results were captured and interpreted using the illuminator.

RESULTS AND DISCUSSION

The results of the detection of specific antibodies against *L. hardjo* in rabbits using dark field microscopic agglutination (MA) are displayed in Table 1 and Chart 1. The results pertain to the period from the start of the experiment until day 56, i.e. the experimental period is divided into two phases. The first phase encompassed sampling from day 1 to day 21, whereas second phase encompasses weakly sampling throughout 5-week period after administration of chemotherapeutic agents.

Table 1. Finding of antibodies against *L. hardjo* in blood serum of rabbits challenged by skin scarification (microscopic agglutination method, MA)

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	-	-	-	-	1:100	1:100	1:100	1:100	1:250	1:100	1:50	1:25	-	-	-	-	-
2	-	-	-	1:25	1:50	1:100	1:100	1:100	1:.250	1:100	1:25	1:25	1:25	-	-	-	-
3	-	-	-	-	1:25	1:50	1:50	1:100	1:100	1:100	1:25	1:25	-	-	1:50	1:25	1:25
4	-	-	1:50	1:100	1:250	1:500	1:250	1:500	1:500	1:100	1:100	1:100	1:100	1:75	1:75	1:50	1:25
5	-	-	-	1:100	1:500	1:100	1:250	1:100	1:250	1:100	1:50	1:50	1:50	1:25	1:25	1:25	1:25
21	-	-	-	-	1:50	1:100	1:250	1:250	1:250	1:250	1:100	1:100	1:100	1:250	1:250	1:100	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø
23	-	-	-	-	1:50	1:100	1:100	1:100	1:50	-	-	-	-	-	-	-	-
24	-	-	-	1:100	1:100	1:100	1:100	1:100	1:50	-	1:25	1:25	1:25	1:50	1:100	-	-
25	-	-	-	1:50	1:100	1:250	1:250	1:250	1:100	1:100	1:50	1:25	1:25	1:100	1:25	-	-
26	-	-	-	1:100	1:250	1:500	1:250	1:250	1:100	1:100	1:100	1:100	1:50	1:50	1:25	1:25	-

Legend: (-) negative (+) positive Ø – died rabbits

The results of examination of 183 blood sera of rabbits infected via skin scarification and rubbing of live cultures of *L. hardjo* (Table 1) revealed seropositive finding in 67 (36.61%) samples. Monitoring of the increase of geometric mean titre (GMT) of *L. hardjo* specific antibodies, positive titre result was diagnosed on day 9 post infection (Chart 1). Monitoring of antibody titre values of rabbits during the further course of infection revealed that they persisted at the weakly-positive level (1:100) until day 17 post challenge, when the titre values dropped below the lower limits of positivity of MA method (titre <1:100).

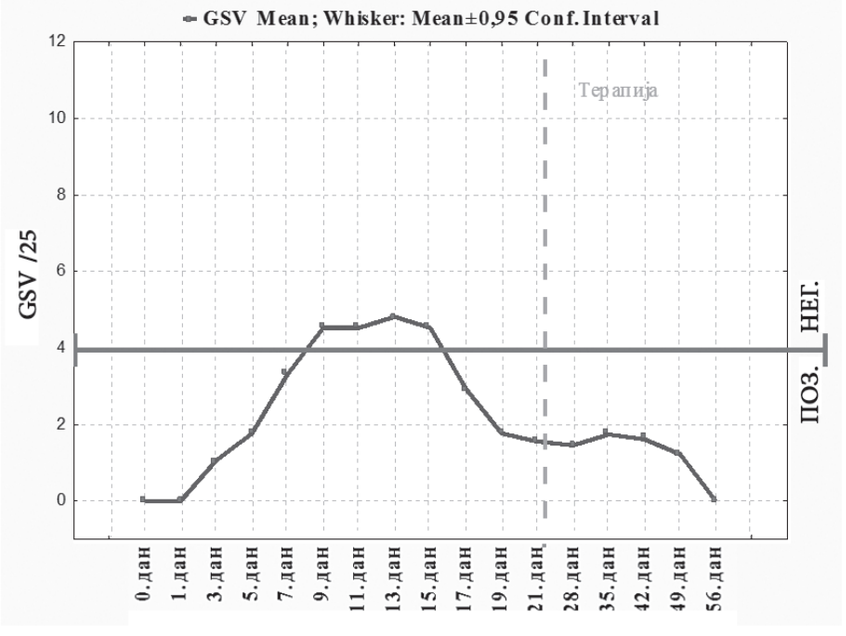


Chart 1. Distribution of *GMT* antibodies against *L. hardjo* in blood sera of rabbits challenged by skin scarification (microscopic agglutination method, MA).

Collected rabbit serum samples were also examined in an immunoenzyme assay, *ELISA*. *L. hardjo* antibody finding obtained by ELISA revealed positive result in 67 (36.61%) samples (rate > 45%), and suspect result (rate 20-45%) in 18 (9.84%) samples. In this test, the earliest diagnosis of seropositive result was accomplished on day 7 (Table 2). Monitoring of distribution of geometric mean titre values revealed that titre of L. *hardjo* specific antibodies exceeds the boundary positivity level on day 15 post challenge. The highest GMT value was recorded on 42nd day of investigation period, showing decreasing tendency in the following days (Chart 2).

Table 2. Finding of antibodies against *L. hardjo* in blood serum of rabbits challenged by skin scarification – ELISA (expressed in %)

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	13%	5%	10%	5%	16%	13%	24%	16%	21%	2%	13%	19%	25%	63%	124%	40%	129%
2	2%	4%	6%	7%	4%	2%	2%	3%	7%	0%	13%	4%	22%	47%	94%	77%	33%
3	12%	3%	13%	4%	4%	-2%	1%	0%	5%	0%	6%	3%	29%	66%	128%	72%	98%
4	4%	6%	4%	13%	19%	8%	8%	9%	13%	13%	25%	28%	31%	57%	145%	124%	129%
5	1%	4%	1%	13%	3%	9%	9%	15%	17%	-3%	36%	39%	44%	69%	103%	90%	89%
21	1%	-6%	-5%	3%	5%	6%	8%	18%	94%	116%	114%	127%	110%	93%	112%	91%	85%
22	-3%	-2%	-7%	25%	4%	6%	3%	1%	48%	19%	39%	27%	31%	W	W	W	W
23	0%	-1%	-1%	-1%	3%	2%	1%	3%	92%	65%	81%	90%	106%	130%	118%	123%	91%
24	-4%	7%	5%	3%	5%	21%	29%	32%	72%	46%	127%	107%	115%	124%	149%	125%	135%
25	14%	-2%	13%	16%	76%	52%	43%	50%	124%	101%	109%	77%	99%	114%	108%	102%	97%
26	1%	-3%	-5%	3%	0%	5%	4%	11%	54%	88%	109%	98%	82%	113%	174%	120%	109%

Legend: (-) negative (+) positive Ø – died rabbits

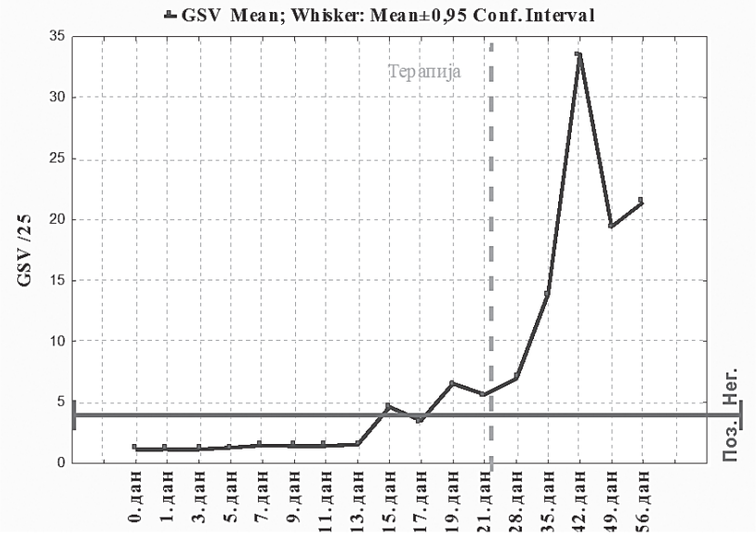
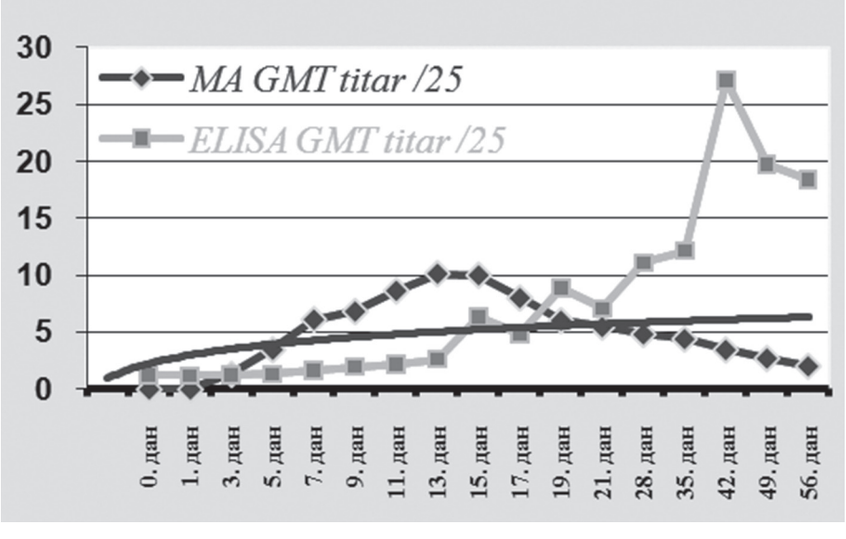


Chart 2. Distribution of *GMT* antibodies against *L. hardjo* in blood sera of rabbits challenged by skin scarification – diagnosed by ELISA

Comparison of GMT values obtained by MT and ELISA tests revealed the following: samples examined by MA test manifested increased levels of specific antibodies, which exceeded the boundary level of 1:100 (*GMT* titre 4 $log2/_{25}$) as early as on day 7. The level of *L. hardjo* specific antibodies diagnosed by MA method increases until reaching the maximum level on days 13 and 15, and then shows decreasing tendency and drops below the boundary positivity level on day 42 (*Cole J.R.Jr,et al.1973*). Contrary to MA method, *ELISA* demonstrated considerably longer period (15 days) between the infection and exceeding the boundary positivity levels being 45% (*PP*) or (*GMT* titre 4 $log2/_{25}$). During the further course of the experiment, GMT values manifested permanent increasing tendency until day 42, when they reached the maximum levels, and then started to drop down gradually. It is to be emphasized that MA method enables seropositivity diagnosis from day 7 to day 42 post-infection. In ELISA, the time span of diagnosis extends from day 15 to day 56, i.e. to the end of the investigation period (Chart 3).

Chart 3. Distribution of *GMT* values for antibodies against *L. hardjo* in blood sera of rabbits challenged by skin scarification – diagnosed by MA test and ELISA



Testing of correlation levels of GMT values for the applied methods (i.e. MA and ELISA) in the population of rabbits challenged by skin scarification revealed weak inverse linear correlation of r = -0.3277 with 0.05 level of

significance. Furthermore, the parity of arithmetic means of GMT values for MA test and ELISA was tested and *t*-value (t = -2.288) and *P* value 0.029 (P <0.05) were calculated, indicating mutual difference between the arithmetic means of tested sets with a risk of 0.05 (*Merien F.et al.1995;Márcia Costa Ooteman et al.2006;* Grgić Ž *et al.* 2007).

Analysis of the results obtained by the method of isolation on culture media, *L. hardjo* was isolated from 33 (18.03%) out of 183 examined blood sera of rabbits challenged via skin scarification. Using the isolation method, *L. hardjo* was detected as early as the day 3 post-challenge, and the highest rate of positive findings was recorded on day 17 post-infection. The results are presented in Table 3 (*Schonberg A.,et al. 199.;Johnson R. C. Et al.1973*).

Table 3. Results of reisolation of *L. hardjo* from blood of rabbits challenged via skin scarification using cultivation in liquid and semisolid medium acc. to *Johnson* supplemented with 200*μg*/1*ml* 5 *FU.*

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
4	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
5	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
21	-	-	+	-	-	-	+	+	-	+	-	+	-	-	-	-	-
22	-	-	-	-	-	-	-	+	+	+	-	-	-	W	W	W	W
23	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-
24	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
Σ	0	0	1 9,09%	0	0	0	2 18,18%	6 54,54%	8 72,73%	9 81,82%	4 36,36%	3 27,27%	0	0	0	0	0

Legend: liquid medium o Semisolid o, liquid + semisolid o dead rabbit W positive (+) negative (-)

The presence of *L. hardjo* in blood of infected rabbits was also examined using the method of polymerase chain reaction (PCR). *L. hardjo* was detected in 67 (56.30%) out of 119 examined samples. By the use of PCR, *L. hardjo* was detected as early as on day 1, and the highest rate of positive findings was observed on day 19 post-challenge (Table 4). The method of polymerase chain reaction enabled detection of *L. hardjo* also in the second stage of the experiment, i.e. after administration of antimicrobial therapy, which is an important point for the interpretation of the results obtained in this assay. Polymerase chain reaction method demonstrated high sensitivity, that is, ability of detecting 5-10 bacteria per 1ml of the sample as well as short time of examination, which makes it more appropriate than the method of cultivation (*Gerritsen M. J. et al.1991; Grgić Ž. et al. 2012*).

Table 4. Finding of *L. hardjo* in the sera of rabbits infected via skin scarification using the *PCR* method

Anim No.	Days post infection												Days post therapy				
	0.	1.	3.	5.	7.	9.	11.	13.	15.	17.	19.	21.	28.	35.	42.	49.	56.
1	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+
2	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+
3	-	-	-	-	+	+	-	+	+	+	+	+	-	+	+	+	+
4	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	-	-
5	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-
21	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
24	-	-	-	-	-	+	-	+	-	+	+	+	-	+	+	-	-
Σ	0	2 28,57%	1 14,29%	2 28,57%	3 42,85%	3 42,85%	3 42,85%	6 85,71%	5 71,43%	6 85,71%	7 100%	6 85,71%	4 57,14%	6 85,71%	5 71,43%	4 57,14%	4 57,14%

Legend: *PCR* positive (+) *PCR* negative (-).

Numerous authors (*Maria Rosa et al. 2005; Cousins D.V., 1991*) reported on the capacities of ELISA and its advantages over the MA method (*Brown P.D, 1995* and *Cousins D.V., et al., 1991*). In that respect, our results should contribute to better understanding of this diseases as well as evaluation of the validity of the applied diagnostic methods. In our research, microscopic agglutination method (*agglutination-lysis test*) was the basic assay, which in recent

period underwent certain modifications aimed at improving the reliability of ELISA method (*Cole et al., 197;, Márcia Costa Ooteman et al.,2006*). The articles of *Bercovich Z. et al.* (1990) and *Levett P.N.* (2002) confirmed the ability of this method to detect specific antibodies in the serum of infected animals at the earliest between the day 5 and day 10 post-challenge, which corresponds with our results. The method of cultivation of leptospires is highly reliable; however, it manifests some considerable limitations such as poor stability of leptospires and their rapid decay in the environment, time-consuming isolation procedure as well as high level of biohazard for the personnel. The method of polymerase chain reaction (PCR) enables detection of leptospiral infection at a very early stage thus increasing the chance for prompt and successful therapy. This is of particular importance in latent human and animal infections and their suppression and prevention. Data from the available literature pertaining to comparison of the results obtained by PCR and other methods strongly indicated its high sensitivity as compared to other methods used so far in the diagnostics of leptospirosis (*Gravekamp et al., 1993; Grgić Ž., 2011, Doctoral Dissertation*).

LITERATURE

1. Bercovich Z., Taaijke R. and Bokhout B.A.; Evaluation of an ELISA for the Diagnosis of Experimentally Induced and Naturally Occurring Leptospira hardjo Infection in Cattle; Vet. Microbiol.; 21(3); 255-262, Jan. 1990.
2. Brown P.D., Gravekamp C., Carrington D.G., Van-De-Kemp H.; Hartskeerl R.A., Edwards C.N., Everard C.O.R., Terpstra W.J., Levett P.N.*; Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis; J.-MED.-MICROBIOL. vol. 43, no. 2, pp. 110 - 114; 1995.
3. C. Gravekamp, H. van De Kemp, D. Franzen, D. Carrington, G.J. Schoone, G.J.J.M. Van Eys, C.O.R. Everard, R.A. Hartskeerl and W.J. Terpstra, Detection of seven species of pathogenic leptospires by PCR using two sets of primers, Journal of General Microbiology 139, pp. 1691–1700, 1993.
4. Cole J.R.Jr, Sulzer C.R. and Purchell A.R.; Improved microtechnique for the leptospiral microscopic agglutination test. Appe. Microbiol. 25; 976-1980, 1973.
5. Cousins D.V., Robertson G.M., Parkinson J. and Richards R.B.; Use of the Enzyme Link Immunosorbent Assay (ELISA) to Detect the IgM and IgG Antibody Response to Leptospira interrogans Serovar hardjo in Pregnant Ewes;l Zbl. Bakt. 275 (3), 335-342, 1991.

Archives of Veterinary Medicine, Vol. 6, No. 2, 71 - 85, 2013.
Grgić Ž. i dr.: Comparative investigation ...

6. Gerritsen M. J., Olyhoek T., Smits M. A. and bokhhout B. A.; Sample preparation for polymerase chain reaction-based semiquantitative detection of Leptospira interrogans serovar hardjo subtype hardjobovis in bovine urine, Journal of Clinical Microbiology, Vol. 29, No 12, p 2805-2808, 1991.
7. Grgić Ž., Đuričić B., Vidić B., Savić S., Pušić I.: Application of polymerase chain reaction (PCR)in detection of L.interrogans serotype hardjo in cattle = Primene lančane reakcije polimeraze (PCR)u dokazivanju L.interrogans serotype hardjo kod goveda.Proceedings, Second International epizootiology Symposium [i]XIV Serbian epizootiology days, April 18-21, 2012, Belgrade, glavni i odgovorni urednik Tamaš Petrović, Beograd, SVD, Sekcija za zoonoze, 2012, Str.108-116, ISBN 978-86-83115-20-4 (eng), M33 1,0
8. Grgić Ž., Đuričić B., Vidić B., Savić-Jevđenić S.: Uporedna ispitivanja komercijalnog i modifikovanih elisa testova u dijagnostici L.hardjo kod goveda = A commparatfve examination of comercial and modified elisa test for a diagnostic of L.hardjo in cattle.Zbornik radova i kratkih sadržaja, IX epizootiološki dani sa međunarodnim učešćem, Požarevac 28-31.mart 2007.godine, urednik Bosiljka Đuričić, Beograd, SVD, Sekcija za zoonoze, 2007, str.208-211, ISBN 978-86-83115-07-5 (srp), M64 0,2
9. Grgić Ž.: Uporedno ispitivanje bakterioloških i seroloških metoda i polimeraza lančane reakcije u dijagnostici leptospiroze goveda = Comparative Investigation of Bacteriological and Serological Methods and Polymerase Chain Reaction in the Diagnostics of Leptospirosis in Cattle : doktorska disertacija.Beograd, Fakultet veterinarske medicine, Katedra za zarazne bolesti životinja i bolesti pčela, 2011 M71 6,0
10. Johnson R. C., Walby J., Henry R. A. and Auran N. F.: Cultivation of parasitic leptospires: effect of pyruvate. Applied Microbiology. 26: 118-11, 1973.
11. Levett P.N., Branch S. L.; Evaluation of two enzume-linked immunosorbent assay methods for detection of immunoglobulin M amtibodies in acute leptospirosis; Am. J. Trop. Med. Hyg., Vol 66, 6, p 745-748, 2002.
12. Márcia Costa Ooteman, Annamaria Ravara Vago and Matilde Cota Koury; Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis; Journal of Microbiological methods Vol. 65, 2, p 247-257, may 2006.
13. Merien F., Baranton G., Perolat P.; Comparison of polymerase chain reaction with microagglutination test and culture for diagnosis of leptospirosis; J.-INFECT.-DIS. vol. 172, no. 1, pp. 281-285, 1995.
14. Schonberg A., Hahn Hey B., Kampe U., Schmidt K., Ellis W.A.; The isolation and identification of Leptospira interrogans serovar bratislava from a pig in Germany; Zentralbl-. Veterinarmed-B, Vol. 39, No 5, p 362-368, 1992.

15. Smith, C.R., Ketterer, P.J., McGowan, M.R. and Corney, B.G.: Areview of laboratory techniques and their use in the diagnosis ofLeptospira interrogans serovar hardjo infection in cattle. Aust. Vet.J.; 71, (9): 290-294., 1994.

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

3. Poglavlje u knjizi:
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Qinn P.: Clinical Veterinary Microbiology. London, Mosby, 1998

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

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