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SEROSURVEILLANCE OF EQUINE INFECTIOUS ANAEMIA IN A REGION OF VOJVODINA

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Abstract

Equine infectious anemia is a consequence of a persistant infection of the horses with Lentivirus. Pathogenesis of the disease is very variable, what can bee seen through a wide range of clinical forms of the disease – from inaparrent infection to death. Diagnostics of EIA is based on clinical symptoms, detection of antibodies and virus. Antibodies can be identified with Hi, VN, CFIT, cELISA, SA-ELISA and AGID test. RT-PCR technique enables the detection of and/or quantification of viral RNA level in blood of infected animal. First reliable serological test for EIA was AGID test. Modified AGID test is considered today as aknowledged, international standard for the detection of antibodies against EIA virus and it enables detection of more then 95% of ll positive animals. Horses with positive findings with this test are considered infected and should be euthanized or placed in strict isolation. Further measures to control the spread of this disease are insect-vector control and disinfection of surgical and other equipment in use on successive animals. The results of a study during a twenty year period, in the region of AP Vojvodina show that from the total of 11.972 horses blood samples, with te use of AGID test, positive results were found in 21 or 0,17% of horses.

Key words: Equine infectious anemia, horses, seroprevalence, AGID test

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ISPITIVANJE RAŠIRENOSTI INFEKTIVNE ANEMIJE KOPITARA NA PODRUČJU VOJVODINE

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Kratak sadržaj

Infektivna anemija kopitara je posledica perzistentne infekcije konja lentivirusom. Patogeneza IAK je vrlo promenljiva, što se reflekturje kroz širok spektar kliničkih formi bolesti – od inaparentne infekcije do uginuća. Dijagnostikovanje IAK bazira na kliničkim znacima, detekciji antitela i virusa. Antitela se mogu utvrditi pomoću HI, VN, CFIT, CELISA, SA-ELISA i AGID testa. RT-PCR tehnika omogućuju detekciju i/ili kvantitafikaciju nivoa virusnih RNK u krvi inficirane životinje. Prvi pouzdani serološki test za IAK bio je AGID test, a modifikovani AGID test se danas smatra priznatim internacionalnim standardom za detekciju antitela za EIAV i moguća je detekcija > 95% svih pozitivnih životinja. Konji sa pozitivnim serološkim testom smatraju se zaraženim, moraju se neškodljivo ukloniti i držati strogo izolovano. Kontrola vektora i dezinfekcija hiruških instrumenata i druge opreme su mere koje se primenjuju u sprečavanju širenja ovog oboljenja. Rezultati ispitivanja tokom dvadesetogodišnjeg perioda na području AP Vojvodine, ukazuju da su od ukupno 11972 uzorka krvnih serum konja, primenom AGID testa, pozitivni nalazi utvrđeni kod 21, ili 0,17% konja.

INTRODUCTION

Equine infectious anaemia (EIA) is a persistent viral infection of equidas. The causative agent, EIA virus (EIAV) belongs to *Lentiviruses* from family *Retroviridae*, subfamily *Orthoretrovirinae*. Equine infectious anaemia occurs in cases of persistent *Lentivirus* infection of horses. The disease is spread out in the whole world and occurs in horses, ponies, mules and donkeys. Clinical cases of the disease was described for the first time in France in the middle of 19th century and viral etiology was identified in 1904 (Timoney et al.,1988). During the following years there was no progress in the introduction of pathogenesis of the disease, only in epidemiology, clinical manifestation of the disease and pathology. All attempts for the virus to be transfered to different experimental

animals, except horses, were unsuccsesfull (Cook et al. 1996). A reliable diagnostic method was not introduced until 1970 (Coggins and Norcross, 1970). On the contrary to that, during the last few years a break through was done in analysing biological and biochemical characteristics of EIA virus and its pathogenesis.

EPIZOOTIOLOGY

EIA virs can be found in many countries in the world, at all continents. The disease can be found in horses in Europe and the level of incidence depends of the density of the equine population, presence of the vectors and also different programs for control of this disease (Sellon, 1993; Toma, 1980). Equine infectious anaemia is found also in USA, Canada and Latin America, where a high level of incidence was found (Cook et al. 1996; Hall et al.,1988; Timoney et al., 1988). The most important way of infection with EIA in nature is with contaminated blood, mostly transfered by blood sucking artropods (Kemen and Coggins, 1972). The virus is spread via interrupted feeding of bloodsucking horseflies on a clinically ill horse and then on susceptible horses. Transmission can also occur by the iatrogenic transfer of blood through the use of contaminated needles, because the virus can remain vital up to 4 days (Cook et al. 1996).

Epidemiological proof, with a fact that EIA virus cannot replicate in mamals other then equides, (Kemen and Coggins, 1972) shows that persistently infected horses, ponies, donkies and mules represent the only reservoir of the virus in the nature. Besides, according to the known data so far, wild type of EIA virus will not replicate in any kind of insect or insect cell lines (Foil and Issel, 1991). That it why it is considered that artropod transimission of the virus is only mechanical (Kemen i Coggins, 1972). Succsessful machanica transmission of the virus depends on everal factors such as level of the viru in blood or tissues of the host (titar), characteristics of the vector and its behaviour, behaviour of the host, climate, close presence of the woods, shelters (most of the Tabanides do not enter indoor spaces).

PATHOGENESIS AND CLINICAL SYMPTOMS

Pathogenesis of EIA virus is very variable, caused by the characteristics of the host and the virus, which reflects in a wide range of clinical forms if the disease, from inaparen infection to death. Two important parametrs in pathogenesis of EIA are a lifetime persistence of EIA virus in infected host with a spo-

radic occurence of the disease. Life time persistence of the virus comes from a capability of the virus to intergrete itself into a hromosome DNA of the host. Proviral DNA in integrated and non intergrated state can easily be detected during the period of acute infection. Yet, specific sequences of the virus cannot be detected in asymptomatic horses which indictes that the number of cells which contain inergreted DNA viruses is accually low (Rice et al., 1989). In special conditions such as immunosupression (Dreguss and Lombard, 1954), the reapperiance of clinical symptoms is conditioned with the characteristics of EIA virus – it can mutate and make new demands for the immune system of the host.

The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight and oedema of the lower parts of the body. If death does not result from one of the acute clinical attacks, a chronic stage develops and the infection tends to become inapparent. The incubation period is normally 1– 3 weeks, but may be as long as 3 months. The severity of the disease varies and it can be in a asymptomatic form or in a form with a high morbidity rate, even with fatal ending. Factors of the host and virus which influence this variability are still not completely clear.

Clinical manifestation of EIA disease can be acute or chronic and there is also the inapparent state of the carrier. Acute form of the disease is often connected with the primary infection and clinical symptoms include pyrexia, anorexia, depression and petechial bleeding of mucosa. Anaemia is not characteristic in acute infections, except in very severe cases when there can also be seen epistaxys and ventral oedema. In chronic infections cycles of healing and return of the disease can be seen with classic symptoms of anemia, edema and weight loss. Death of the animal can occur 4 week later at the earliest after the infection. If the animal lives through the acute phase, the frequency and the severity of the clinical episodes progressively drops (90% can be seen during the first zear after the infection), until the animal becomes an inaparent reservoir of pathogens (Timoney et al., 1988.) Nevertheless, some animals serologicaly positive to EIA virus, never had any clinical symptoms, or they were in uch a mild form that they could not b noticed by the owner (Issel and Coggins, 1979).

DIAGNOSTICS

Diagnostics of EIA is based on clinical symptoms , analysis of fagocytic blood cells which contain ingested erythrocites (sideroleucocytes / which are not pathognomonic for EIA, but often are present in acute infection), detecti-

on of antibodies against viral components and detection of the virus. Diagnostics based on clinical symptoms is complex because of the variability of the symptoms and the exsistence of inaparent carriers.For field strains of EIA virus which replicte only in monocitic/macrofage cells, detection of the virus can be done with a transfusion of 250ml of whole blood (collected in acid-cytrate solution of dextrosis) from a horse suspected for EIA to a seronegative horse as a receiver. RT-PCR technique enables detection and/or quantification of the viral RNA level in blood of infected animal.

Antibodies can be detected by the following tests: Hi, VN, CFIT, cELISA, SA-ELISA and AGID test. The first reliable serological test for EIA was the AGID test (Coggins and Norcross, 1970). However, there are reports that in infected horses can be gained negative findings and what is even more important, some of them can spread the virus (Toma, 1980). Detection in these animals requires more sensitive serological tests. Noumerous modifications of ELISA method have been described so far with a p26 as antigen. Competitive ELISA method (cELISA) uses mAb for p26 and ELISA with synthetic antigen (SA-ELISA)can also be used. When using ELISA methods, false positive findings can be expected. Although cELISA and SA-ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs have to be confirmed using the AGID test (Lew et al.,1993) The AGID test also has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions by lines of identity. Agar gel immunodiffusion (AGID) tests (Coggins et al., 1972) and enzyme-linked immunosorbent assays (ELISAs) (Suzuki et al., 1982) are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams. In rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never rise high enough to be detectable (Toma, 1980).

More advanced dignostics is possible with the use of RT-PCR technique, which enables detection and/or quantification of the viral RNA level in blod (Nagarajan and Simard 2001).

OUR INVESTIGATIO

During a period of twenty year, blood serum semples of horses were analysed, from two different epizootical regions – from southern Backa and Srem region. Horses originated from two stables, several horse clubs, private owners and one collection point where horses were in qurantine before tran-

sportation for exportation.

Agar-gel immunodifussion test was used (Coggins test-VMRD Inc.) and in total 11.972 horse serum samples were analysed.

Findings after the analysis of blood serum samples from horses, for equine infecious anemia are shown in Table 1. In the perid of study from the total of 11.972 horse serum samples, positive results were found in 21 animal, which is 0,17%.

Table 1. Finding of blood serum samples from horses nalysed for equine infectious anemia during the period 1994- 2013

Year	No of horses analysed	No of horses positive for EIA
1994	475	3
1995	312	0
1996	508	1
1997	421	1
1998	214	0
1999	184	2
2000.	593	0
2001.	1359	0
2002.	446	0
2003.	593	1
2004.	381	2
2005.	1065	1
2006.	417	0
2007.	874	0
2008.	425	0
2009.	522	1
2010.	611	0
2011.	826	0
2012.	957	6
2013.	789	3
TOTAL	**11.972**	**21 (0,17%)**

DISCUSSION

Equine infectious anaemia can cause damage and it can be a deadly disease. Until today, treatment for horses that have EIA is not known. Also, there is no vaccine on the market against EIA virus for the protection of horses. But with control measures and management techniques that are strict and appropriate for implementation in horse breeding, the chances of infection with EIA virus can be pretty much reduced.

Horses infected with EIA virus represent a danger and threat to the community. The consequences can be of different levels and risk from EIA is still to be estimated. In order to prevent the spreading of EIA virus, strict control measures should be applied (Cook et al. 1996). In some rural areas horses still participate in everyday life, taking part in agriculture and transportation. A decrease of the potential risk of great economical losses resulting from infectious diseases occurring in horses is very important.

A significantly higher number of infected animals has been found in Greece. During the period 2001-2008, a total of 7.872 horse serum samples were tested at the Centre of Veterinary Institutes of Athens. Antibodies against equine infectious anaemia (EIA), were found in 4.5% of the samples and seropositivity for EIA was determined (Mangana-Vougiouka et.al. 2013).

To the contrary of this, there are data from several regions in Turkey. A study was done where the material consisted of 8.947 horse serum samples, including 8.769 horses and 178 donkeys, from Ardahan, province in north east part of Turkey(Albayrak and Ozan, 2010). Blood was collected from all horses and donkeys and the sera were analysed for the presence of antibodies for equine infectious anaemia virus (EIAV) using an enzyme-linked immunosorbent assay. All animals were negative for antibodies against EIA virus. EIA infections are also reported in different countries (Pearson and Knowles 1984; Lew et al. 1993; Nagarajan and Simard 2001). In Armenia and Georgia EIA has not been reported so far, but the presence of haematophagous vectors as important risk factors of EIA for equines has been found (Erdem, 2007). Since 1981, in Serbia there is a program for prevention and, eradication of EIA in horses(Vidić et. al. 1998.). EIA is a disease that is mandatory for reporting to the OIE and disease which is under annual program of monitoring, administered by the Ministry of Agriculture and environment protection of Serbia.

Gained results show that EIA is present in horses from the region included in the study and there is a very low prevalence. There is no tendency of the virus to spread and we can assume that the gained results are a consequence of a horse trade market, because EIA was found in horses from other regions of Serbia, intended for slaughter.

PROPHYLAXIS AND CONTROL

An attenuated live vaccine, developed in the early 1970s, was extensively used in China (OIE Terr. Manuel, 2008) between 1975 and 1990. Until today, there is no available vaccine against EIA virus, except in China, because of the complex development of the vaccine which is conditioned by the life cycle and

antigen performances of *Lentivirus*. Control measures for EIA have the aim to reduce the probability of the appearance of infection. This is achieved by the good management on the farm, strict laws and regulations and the capability of detecting and separation of infected animals. Good practice on farms involves separation of infected animals, grazing animals far from the edge of the woods, shelter from the tabanides attack, usage of spray and repellents for reduction of the vectors and also the usage of the proper procedures of vaccination or blood sampling can help in the prevention of the spreading of EIA. Besides all this, most of the countries have their own regulations for EIA with the measures "test and remove", which limit the breeding and trade of the infected animals. All of the horses with positive finding are considered infected and should be euthanized or placed in strict isolation. Further measures to control spread of this disease are insect-vector control and disinfection of surgical and other equipment between use on successive animals. These regulations include the obligation of permanent identifikation of infected animals (lip tatoo, stamps, electronic implants).

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PREVENTION OF CLASSICAL SWINE FEVER SPREADING IN CROSS-BORDER REGION THROUGH IMPROVEMENTS OF SANITARY STANDARDS AND EDUCATION OF FARMERS

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Abstract

This paper describes the implementation of the project "Prevention of CSF spreading in the cross-border region through improvements of sanitary standards and education of farmers - STOP CSF" implemented and funded under the IPA Cross-Border Programme Croatia-Serbia 2007-2013. The project had been implemented in Serbia by Scientific Veterinary Institute "Novi Sad" from Novi Sad and the project partners in the Republic of Croatia were Osjek-Baranja County and the Regional Developmental Agency of Slavonia and Baranja Ltd from Osijek. The project was implemented in the period 17 January 2011 - 17 July 2012 (18 months). All planned project activities have been fully implemented. A promotional leaflet was designed, edited and printed in 10000 copies (5000 copies in Serbian and 5000 copies in the Croatian language). On the territory in Serbia where the project was carried out (Southern Backa and Srem district) all of the copies of leaflets printed in Serbian language were distributed. The Manual "The prevention of classic swine fever (CSF) in rural farms" of authors, Sava Lazić, Tamaš Petrović, Jasna-Prodanov Radulović and Radoslav Došen, was also edited and printed in 4000 copies (2000 on Serbian and 2000 on the Croatian language). On the territory in Serbia where the project was carried out all the copies of the manual printed in Serbian language were distributed. On the topic of CSF, 10 workshops have been held, visited by 237 participants in Serbia and 4 joint workshops have been held (two in Serbia and two in Croatia) for farmers both from Serbia and Croatia with the total of 84 farmers attended (43 from Croatia and 41 from Serbia). Therefore the workshops in

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Serbia were attended by 278 participants. For better education and training for implementation of biosecurity measures in the prevention of CSF, in the period from the 21st to the 26th of November 2011 there was organized a study tour to Vehta (Bremen) in Germany. On the study tour, there were 15 farmers and 4 members of the Project team from Serbia, and 15 farmers with 3 members of the Project team and an interpreter from Croatia. In the area of project implementation in Serbia, on family farms, there were 18 disinfectant barriers built. A cost-benefit analysis was made that scientifically and professionally determined that the invested funds are multi functional and instrumental in the prevention of CSF. The film „Preventing the CSF in rural households" (30 min. long) and a TV spot (60 seconds long) were made. They have been broadcasted on over 10 local TV stations. The peak point of project activities was the International Conference: "Preventing the spread of CSF in the border region of Croatia-Serbia (STOP-CSF)" which was held on the 7th and 8th of June 2012 in Novi Sad. At the conference 25 scientific and professional papers were presented by the speakers from Germany (EU Reference Laboratory for CSF), by high scored professionals from the neighbouring countries (Italy, Slovenia, Austria, Romania, Hungary, Bulgaria, Macedonia, and Bosnia and Herzegovina) and the countries where the project was implemented (Croatia and Serbia). The first day of the Conference there were 152 participants, and 158 on the second day, mainly veterinarians. The general impression was that the Conference was successful, the lectures and discussions provided explanations on many issues from epizootiological surveillance, prevention and diagnostics of Classical Swine Fever (CSF).

Key words: IPA Project, Cross-Border Programe Croatia-Serbia, Classical swine fever

SPREČAVANJE ŠIRENJA KLASIČNE KUGE SVINJA U POGRANIČNOM REGIONU KROZ POBOLJŠANJE SANITARNIH STANDARDA I OBRAZOVANJE FARMERA

Sava Lazić[1], Jovan Kvaščev[1], Radoslav Došen[1], Jasna Prodanov-Radulović[1], Ivan Pušić[1], Gordana Stojanović[2], Tamaš Petrović[1]

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Kratak sadržaj

U ovom radu je dat opis realizacije projekta: "Sprečavanje širenja klasične kuge svinja u pograničnom regionu kroz poboljšanje sanitarnih standarda i obrazovanje farmera (STOP–KKS)", koji se sprovodio i finansirao u okviru IPA Međugraničnog programa Hrvatska-Srbija 2007-2013. Projekat u Srbiji je realizovan od strane Naučnog instituta za veterinarstvo "Novi Sad" iz Novog Sada i sa partnerima iz Hrvatske, Osječko-Baranjska županija i Regionalna razvojna agencija Slavonije i Baranje iz Osjeka. Projekat je sproveden u period od 17. januara 2011. do 17. jula 2012. godine (18 meseci). Sve planirane projektne aktivnosti su u potpunosti realizovane. Edukativni letak je uređen i odštampan u 10000 primeraka (5000 primeraka na srpskom jeziku i 5000 primeraka na hrvatskom jeziku. Kopije na srpskom jeziku podeljene su na teritoriji realizacije projekta u Srbiji, a to su Južnobački i Sremski okrug. Priručnik "Sprečavanje pojave klasične kuge svinja u seoskim domaćinstvima", autora: Lazić Save, Petrović Tamaša, Prodanov-Radulović Jasne i Došen Radoslava, je takođe uređen i odštampan u 4000 primeraka (2000 primeraka na srpskom jeziku i 2000 primeraka na hrvatskom jeziku). Svi primerci priručnika na srpskom jeziku su podeljeni na teritoriji realizacije projekta u Srbiji (Južnobački i Sremski okrug). Na temu klasične kuge svinja održano je 10 radionica, kojima je prisustvovalo 237 učesnika i 4 zajedničke radionice (dve u Srbiji i dve u Hrvatskoj) za farmere iz Srbije i Hrvatske, na kojima je učestvovalo 84 farmera (41 iz Srbije i 43 iz Hrvatske). Radi boljeg obrazovanja i obuke u primeni bio-sigurnosnih mera za sprečavanje pojave klasične kuge svinja u periodu od 21-26. novembra 2011. organizovano je studijsko putovanje u Vehtu (Bremen) u Nemačku. Na studijsko putovanje išlo je 15 farmera i 4 člana projektnog tima iz Srbije i 15 farmera, 3 člana projektnog tima i prevodilac iz Hrvatske. U oblasti realizacije projekta u Srbiji na 18 porodičnih farmi izgrađene su dezinfekcione barijere. Izrađena je "*Cost-Benefit*" analiza, kojom je na naučnoj i stručnoj osnovi utvrđeno da su uložena sredstva multifunkcionalna i imaju značajnu ulogu u sprečavanju pojave klasične kuge svinja. Izrađen je film "Sprečavanje pojave klasične kuge svinja u seoskim domaćinstvima" u trajanju od 30 minuta i TV spot od 60 sekundi, koji su emitovani na više od 10 lokalnih TV stanica. Vrhunac projektnih aktivnosti bila je međunarodna konferencija pod nazivom "Sprečavanje širenja klasične kuge svinja u pograničnom regionu Hrvatska-Srbija (STOP-KKS)", koja je održana 7. i 8. juna 2012. u Novom Sadu. Na konferenciji je prezentovano 25 referata, a predavači su bili iz Nemačke (EU referentne laboratorije za KKS), zatim ze-

malja u okruženju (Italija, Slovenija, Austrija, Rumunija, Mađarska, Bugarska, Makedonija i Bosna i Hercegovina) i zemalja gde se projekat realizuje (Hrvatska i Srbija). Na konferenciji je prisustvovalo prvog dana 152, a drugog dana 158 učesnika, uglavnom veterinara. Konferencija je bila uspešna, a predavanjima i diskisijom data su objašnjenja na više pitanja o epizootiološkom nadzoru, sprečavanju i dijagnostikovanju klasične kuge svinja.

Ključne reči: IPA projekat, Među-granični program Hrvatska-Srbija, klasična kuga svinja

INTRODUCTION

Classical swine fever (CSF) still presents a major threat to health and welfare of pigs and the economy of any country. The incidences of classical swine fever result in massive morbidity and mortality of pigs. The compulsory safe disposal of infected animals, suspicious animals and all animals that could have been in direct or indirect contact with the infected pigs causes great economic losses. Therefore, this diseases still influences the sustainability of pig farming in many EU countries, especially the countries of the Balkan Peninsula.

Direct loses occurring by death of diseased pigs and by safe disposal of all pigs that were or may have been in contact with infected and / or diseased pigs, while indirect losses are often unpredictable, and they are usually larger than the direct losses (Saatkamp H.W. et al. 2002). The last, but in the same time one of the biggest outbreaks of CSF was in The Netherland and Germany during 1997 and 1998. During that epidemic, CSF was registered in 420 pig farms in The Netherlands, about 11 million of pigs were killed and safely disposed, and the total damage is estimated at 243-466 million euros (Mangen J.J.M. et al. 2002). The feral pigs still represents the risk for CSF virus transmission and for the new outbreaks in the EU countries. Populations of feral pigs are increasing, so a lot of efforts have been made in the development and implementation of different strategies to prevent the occurrence of this disease in feral pigs (Guberti V. et al. 2012).

In Serbia, the CSF existing continuously, with occasional CSF free periods, for more than 20 years (the disease was detected for the first time on May 30th 1990, on the territory of Mačva district), and economic losses that are occurred during this period were extremely high (Djuričić B. et al. 2001, Djuričić B. et al. 2002,). The total number of infected pigs in the former Yugoslavia in the period 1994 - 1999 was 8,460, 21,979 animals were killed and safely disposed during the outbreaks, and a total of 41,941,516 animals have been vaccinated during that period (Djuričić B. et al. 2012.). From 2000 to 2007, the CSF has often appeared in small rural farms throughout the territory of the Republic

of Serbia, and often in the form of an epidemic. Areas, such as: Srem, Mačva, Northern Bačka, Smederevo-pomoravski County and Southwest parts of Serbia are sites where the CSF usually appeared during this period. The CSF was registered in 1,381 farms during this period and the number of infected pigs was 17,887. There are no exact data for the number of succumbed and safely destroyed pigs for that period, but there are data for the period from 2005 to 2007, when a total of 4,750 died, and 15,483 pigs have been safely destroyed (Milićević V. et al. 2009, Milošević B. 2010). The last epidemic of CSF in the Republic of Serbia was in late 2010, on one industrial pig farm and in a few rural backyards. During this epidemic 202 pigs have been infected, 155 animals died, and 9,063 pigs have been safely destroyed (Pušić I. et al. 2012). The total losses have been estimated on about 2 million euros.

Strategy for prevention, control and eradication of CSF in the Republic of Serbia is based on a systematic and planned vaccination of animals, on the control of animals immune status and on implementation of biosecurity measures on pig farms. By monitoring of the immune status of the vaccinated pigs, which was conducted in late 2010, was found that the percentage of protected pigs in the whole territory of Serbia is around 80%. However, in some areas the percentage of protected pigs was significantly lower - about 60% (Mićović Z. et al. 2012). Experience of many countries that had eradicated KKS suggests that the vaccination can be stopped when the protection is achieved in 90% of the total pig population, but with the strict application of biosecurity measures. Possibilities for implementation of biosecurity measures on pig farms is a key factor in preventing the occurrence and spread of CSF, but also the limiting factor for the termination of vaccination of pigs against CSF in Serbia (Hristov S. et al. 2012, Došen R. et al. 2012, Lazić S. et al. 2012.).

In Croatia, the CSF has often occurred on small rural farms in the period from 1996-1997 to 2003, although a constant vaccination of pigs against classical swine fever have been conducted at that time (Pavlak M. et al. 2011, Labrovic A. et al. 2012). No vaccination policy in CSF control in Croatia was introduced from 1st of January 2005. However, in 2006 and 2007 until the beginning of 2008 classical swine fever was registered in 295 rural households in Croatia and 19,320 pigs was killed and safely disposed (Croatian Ministry of Agriculture Fishers and Rural Development, Veterinary Directorate, 2012). By termination of vaccination, and then by removing of all pigs that have been vaccinated against CSF from the herds, the pig population in Croatia become completely naive and unprotected from the potential occurrence of this disease. Therefore, the only way to protect the pig's is by implementation of biosecurity measures, that are prescribed in a number of laws and regulations.

Implementation of biosecurity measures is conditioned by the commitment of all involved in the rearing of pigs, especially the owner, no matter how many pigs the owner has on the farm. Experiences from previous years still suggest that the smaller, so-called family farms are often a source of infection and highly contribute to the spread of CSF. However, in several cases have been proved that a good awareness of the owner, as well as other family members, about the disease such as the CSF, has contributed to the early detection of the disease and prevent further virus transmission. This significantly contributed to the lower economic losses. The family pig farms owners also need to know that by concealing of the disease and by negligent behavior they cause enormous damage (Lazić S. et al. 2011). Therefore by raising the awareness, and goodly informed pigs breeders about CSF in small rural farms, can be a key milestone in the implementation of biosecurity measures, especially in pigs breeding without vaccination against classical swine fever.

In the whole world, and especially in the EU, the great efforts are made in establishing and implementing of effective measures to prevent the occurrence and spread of CSF, for rapid diagnosis, for early detection, and especially for building of fast early warning systems, efficient education of pigs owners and breeders and capacity building for the implementation of biosecurity measures to reduce the risk of introduction and spread of CSF virus in rural areas. The project "Prevention of CSF spreading in the cross-border region through improvements of sanitary standards and education of farmers - STOP CSF" was proposed in October 2009 and accepted to be co-financed by the EU in December 2010. The European Union delegation in Zagreb and Belgrade are recognized the importance of this project, not only for the region between the two states, but also for much broader area. Thus, the "STOP-CSF" project aims at improving hygiene standards and education of farmers in order to reduce the risk of classical swine fever in the border region of Croatia and Serbia.

The project "Prevention of CSF spreading in the cross-border region through improvements of sanitary standards and education of farmers - STOP CSF" has been implemented and funded under the IPA Cross-Border Programme Croatia-Serbia 2007-2013. The project had been implemented in Serbia by Scientific Veterinary Institute "Novi Sad" from Novi Sad and the project partners in the Republic of Croatia were Osjek-Baranja County and the Regional Developmental Agency of Slavonia and Baranja Ltd from Osijek. The project budget was € 456,642, and the implementation had started 17.01.2011 and lasted 18 months.

The main objective of the project was to reduce the risk for occurrence and prevention of the spread of classical swine fever (CSF) by improving the sani-

tary standards and education of farmers in the border region of Osijek-Baranja County in Croatia and the South Backa and Srem County in Serbia.

The project main goals are to inform, educate and raise awareness, on the prevention of the spread of CSF, of farmers and peoples in rural areas, owners of small slaughterhouses, peoples involved in animal transportation, hunters and veterinarians through educational workshops, through preparation of manuals about the CSF disease, leaflets, educational movie and TV commercials, through study visit tour of Serbian and Croatian farmers to intensive pig farming facilities in Germany, by organization of the International Scientific conference on CSF and by implementation of biosecurity measures in small pig production systems in Serbia and Croatia.

Disinfection barriers for vehicles and personnel at the entrance in the pig production facilities (small family farms) could be an example of improving sanitary standards in the Serbia – Croatia border region. As one of the key project activity, these disinfection barriers have been built on family farms in the Osijek-Baranja County (22 disinfection barriers) and on family farms in South Backa and Srem County (18 disinfection barriers).

PROJECT DESCRIPTION

Main Objectives:

The main objective of the project was to reduce the risk of occurrence and prevention of the spread of classical swine fever (CSF) by improving the sanitary standards and education of farmers in the border region of Osijek-Baranja County in Croatia and the South Backa and Srem County in Serbia.

Overall objective of the project is: **Reducing economic damages in the region caused by CSF due to non-compliance with EU standards**

Specific objective of the project is: **Improving sanitary standards in order to reduce the risk of CSF transmission in the cross-border region**.

Target Group:

Members of family farms dealing with pig production; representatives of pig farmers associations, and producers in the sector of pig breeding.

Tasks (Activities):

1. Project management
2. Education and informing of family farms' members and members of hunting associations

- Joint Workshops (joint workshops with farmers from Croatia and from Serbia)
- Workshops (national workshops)

3. Creation of data base – preparation for comparative analysis
4. Creation of conditions for installation of disinfectant barriers on 40 family farms
5. Preparation and printing of educational-informative materials
   - Leaflet
   - Manual
   - Movie and TV spot
6. Organization of international conference on regional level
7. Organization of a study tour for visiting intensive pig production systems (in Germany)

Territorial Coverage:

Republic of Croatia: Osijek-Baranja County, municipalities of Erdut, Belje, Draž, Čeminac, Magadenovac and Semeljci

Republic of Serbia – municipalities of Novi Sad, Bač, Bačka Palanka, Ruma, Šid and Sremska Mitrovica.

RESULTS

Task Nr: **1.**

Task title: **Project management:**

Six Kick off meetings of the Steering Committee, 3 in Croatia and 3 in Serbia had been conducted. In Croatia the meetings were held on 24.01.2011 in Osijek, on 14.09.2011 in Dalj and on 03.05.2012 in Osjek. In Serbia the meetings were held on 16.05.2011 in Novi Sad, on 26.10.2011 in Zasavica, and on 05.07.2012 in Novi Sad. On the Steering Committee meetings, conducted activities were analyzed and further plans of implementation were determined for the upcoming period. Logs and attendance sheets were produced and sorted. The project team from the Scientific Veterinary Institute held 73 team meetings. These team meetings were held once a week, and on the meetings the previous work was analyzed and assignments and tasks were distributed for the following week. All meetings have been logged.

Task Nr: **2.**

Task title: **Education and information of family farms members (OPG) and members of hunting associations:**

For the aim of raising awareness on CSF in cases of outbreak, and informing on implementation of biosequrity measures, hygiene standards, EU regulations and national laws and acts, there were 10 workshops held in the following Counties: Sremska Mitrovica (2), Ruma (1), Šid (1), Bač (2), Bačka Palanka (1), Novi Sad (3). In total 237 participants attended on workshops and from that number: 139 farmers, 42 hunters, one butcher, 29 veterinarians and 26 people from other professions. There were also 4 joint workshops held, 2 were held in Serbia, one in Zasavica (Sremska Mitrovica County) on 26.10.2011 where the participants were: 10 farmers from Croatia and 12 from Serbia and one in Novi Sad on 14.12.2011 where the participants were 10 farmers from Croatia and 10 from Serbia. In Croatia the joint workshops were held in Dalj on 14.09.2011 where the participants were 10 farmers from Serbia and 10 from Croatia, also in Donji Miholjac where the participants were 9 farmers from Serbia and 10 from Croatia. It was planned that 240 people from Serbia should be educated through national workshops and joint cross border workshops and by the end of this activity there are 278 participants educated in total. Attendance sheets and reports from each workshop have been collected. For the purposes of the workshops, two lap tops, one video been, a screen and a camera, were procured.

Task Nr: **3.**

Task title: **Creation of a data base – preparation for comparative analysis:**

The Cost - Benefit Analysis (CBA) had been elaborated on 68 pages with 4 graphs, 5 geographical maps and 34 tables, through many aspects and by *"Monte Carlo"* simulation of epidemiological situation and economic losses in outbreak situation of CSF, it was determined that the intended funds of the project would pay off during the first year. Minimal advantages of the raised biosecurity measures and the building of disinfectant barriers are estimated to be 7 to 6 times greater than the amount invested.

Task Nr: **4.**

Task title: **Creation of conditions and installation of disinfectant barriers:**

Disinfectant barriers represent one of the key factors in hygiene standards in farms in prevention of CSF and other infectious disease. In the project activities it was planned that 18 barriers should be built in the southern Bačka and Srem County. First of all polls and research were conducted in order to determine which farms and family lands were appropriate for building the barriers. The key factor in determination was the number of farms with pigs and the amount of the pig population on the space they were grown, in the area of project

implementation. From 47 farms where research was conducted, 18 were picked for the building of barriers. In this stage there are 18 disinfectant barriers built. Geographically the count would be: in Sremska Mitrovica County there were 9 barriers built, in Bačka Palanka there are 4 barriers built, in Šid County there are 2 barriers built, in Ruma County there are 2 barriers built and in Bač County there is one disinfectant barrier built. All of the barriers were built according to the rule book "Rules of the veterinary and sanitary conditions for the farming and breeding of cattle, live stock and small live stock (chickens and rabbits), (the Official gazette of the Republic of Serbia number 81/2006). Owners of farms had to provide an owners document of the parcel on which the barrier is being built and sign a contract with the Scientific Veterinary Institute "Novi Sad" in which the building and usage of the barrier is regulated.

Task Nr: **5.**

Task title: **Preparation and printing of educational-informative materials:**

One of the key factors in raising awareness, in the goal of preventing CSF, is informing and educating the farmers. In order to educate and inform the farmers the project foresees that on the topic of CSF: 10000 leaflets be prepared, designed, edited, printed and distributed (5000 in Serbian and 5000 in the Croatian language), 4000 Manuals (2000 in Serbian and 2000 in the Croatian language), a 30 minutes long film is to be produced and distributed and from it a one minute TV spot is to be broadcasted on local television, as well as promoting this topic through local newspapers and radio. The project team of the Scientific Veterinary Institute "Novi Sad", during the implementation, has prepared the planned number of leaflets, 5000 were delivered to the partners in Croatia, and 5000 leaflets were distributed on the project implementation area in Vojvodina. A Manual titled **"The prevention of CSF in rural farms"** (dr Sava Lazić, dr Tamaš Petrović, Jasna-Prodanov Radulović and Radoslav Došen) was edited and printed in the planned number of 4000 copies. On the territory where the project was carried out (Southern Backa and Srem district) all the copies of the manual, printed in the Serbian language, were distributed and 2000 copies in the Croatian language were sent to partners in Croatia. The Croatian partners provided the texts in Croatian that were implemented in the leaflets and Manual.

Task Nr: **6.**

Task title: **Organization of international conference on regional level:**

According to the project activities, a international scientific conference entitled **"Preventing the spread of CSF fever in the border region of Croatia-Serbia (STOP - KKS)"** was organized by the Scientific Veterinary Institute "Novi

Sad". The conference was held in Novi Sad, on the 7th and 8th of June 2012, in Hotel "Park". The Conference was held according to a defined program, and 25 papers were reported. The speeches were given by the head of the EU Reference Laboratory for CSF from Hanover, the heads of National Reference Laboratories for CSF in the neighbouring countries, and the heads of the Reference Laboratories for CSF in Croatia and Serbia, representatives of the Veterinary Directorate of Croatia and Serbia and several experts from Serbia. Analyzing the papers of the conference it can be said that they gave a significant contribution to a better understanding, control and surveillance of CSF. In the light of latest scientific findings 16 papers presented the measures of epizootiological surveillance, control and diagnostics of CSF. The presented papers (4) on the topic „Biosecurity measures" provided answers to key moments of the risk of disease introduction into a pig herd, as well as implementation of measures that prevent these risks. The major contribution to better understanding of the occurrence and spread of CSF from feral to domestic pigs was presented in five reports. All the papers have significant scientific and professional basis, and can be applied on the entire Balkan region. Therefore, all the papers and abstracts presented at the Conference and printed in the Proceedings **"Preventing the spread of CSF in the border region of Croatia - Serbia"** fully correspond to the given topics, all were concise and comprehensive. On the first day 152 participants attended the Conference, and on the second day 158 participants. In addition to the lectures, an intensive discussion was held. Every participant received material consisting of a handbag (with printed signs of: EU, IPA Cross-border program Croatia-Serbia, Scientific Veterinary Institute "Novi Sad" and the sign of Osijek-Baranja County, printed name of the conference, place and date), the Proceedings, the manual "Preventing the emergence of CSF in rural households", three copies of a leaflet "Prevention of spread of CSF in the border region through the improvement of sanitary standards and education of farmers (STOP-CSF)", a DVD with the educational movie and TV spot on CSF, a notebook, a pencil and an identification card. The conference has been accredited by the Veterinary Chamber of Serbia, according to the Rulebook on Education of veterinarians. The general impression is that the conference was successful and that the lectures and discussions gave the explanations to many issues on epizootiological surveillance, prevention and diagnosis of CSF.

Task Nr: **7.**

Task title: **Organization of a study tour:**

For the goal of introducing and implementing biosecurity measures for prevention of infective diseases of pigs, a study tour in Germany was realized,

including farmers and project team members from Croatia and Serbia. The tour was conducted in Vechta (Bremen) from the 20th to the 26th of November 2011. The study tour was organized by the Croatian partners in cooperation with the "Big Dutchman" company. The study tour participants were: 15 farmers and 4 project team members from Serbia and 15 farmers, 3 project team members and an interpreter from Croatia. During the stay in Germany, the participants visited the headquarters of the "Big Dutchman" Company in Vechta, that deals in production and distribution of farm equipment for swine breeding, as well as complete technological lines for food distribution, energy, maintenance and ventilation that is based on the principals of the newest technical and scientific standards and is in sync with all biosecurity measures for the well being of the animals. During the Study tour the "Big Dutchman" Company organized two workshops with the topic of swine breeding and diseases prevention. One of the workshops was held by the representative of the Interest Group of swine breeding, Urlich Polschner, who represented his group, their goals and tasks. This group functions in the Vechta area, where they represent many farmers with the total breed of 7.5 million pigs. During the workshop, advantages the Interest Group provides were highlighted as was the need for such group to be formed in Serbia and Croatia. The other workshop was held by a veterinary doctor Horst Gaumann that presented the most important biosecurity measures on farms, and also presented his experiences in biosecurity measures and their role in the prevention of many infective diseases. Through a lecture and discussion, Mr. Gaumann presented actual measures of disease prevention in Germany and the roles of veterinary doctors in Germany. It can be concluded that the study tour in Germany, was successful and productive, especially if it is analyzed by the theoretical and practical aspects that were demonstrated along with the presentation of proper swine breeding and proper biosecurity measures that could certainly be implemented in Serbia and Croatia. One of the important aspects of this tour is that farmers from Serbia and Croatia had the chance to spend time together, exchange experiences and ideas, and form contacts for future cooperation.

DISCUSSION AND CONCLUSIONS

The grade for realized tasks results of the project is very high. Results that should be highlighted are the ones achieved through activities regarding the education of farmers through workshops, a study tour to Germany, the construction of disinfectant barriers and the International Conference. The Project aimed to educate, through workshops, 200 participants (farmers, veterinarians and a like) on the topics of CSF and biosecurity measures. However,

through 10 workshops in Serbia, 237 participants were educated and informed on these topics, which is 18.5 % more than the planned number. If the interest of farmers, for these workshops were to be graded, the interest of farmers from Sremska Mitrovica County should be highlighted. In the two workshops held in Sremska Mitrovica (the villages of Kuzmin on 03.09.2011 and Zasavica on 27.10.2011) there were 61 participants, which is 52.5% more than the planned number. It should be noted, as well, that Sremska Mitrovica has the largest number of family swine farms in the entire area of project implementation. The high grade for the workshops implementation is supported by the discussions between the participants and the lecturers. All of the workshops included a discussion on various topics, but the vaccination of swine in the Republic of Serbia, the risks of stopping the vaccination, the repercussions and advantages of this measure, as well as implementation of biosecurity measures and the competitiveness of Serbian swine meat in the European market, were the most common topics. When we include the joint workshops, held in Croatia (Dalj and Donji Miholjac) and in Serbia (Zasavica and Novi Sad), from the educational point of view, it can be concluded that in Serbia there were 278 participants, which is 15.83 % more than anticipated. A large contribution, to the more than successful implementation of workshops, goes to the lecturers (Dr. Sava Lazić, Dr. Tamaš Petrović, Mrs. Jasna Prodanov-Radulović and Mr. Radoslav Došen, the Project Team of the Scientific Veterinary Institute „Novi Sad"), they represent the leading experts, in this filed, in the Republic of Serbia.

The Study tours to Germany meet the expectations of all the participants, especially the farmers, so this activity will also be graded very highly. During the stay in Germany, the farmers had the chance to get accounted with the latest in technical advancements in swine breading, as well as the latest biosecurity measures that insure maximum profit in swine farming.

The making of the education and informational material (leaflets, Manual, TV spot and film) are also graded with the highest marks. The educational and informative material was made in the goal of raising awareness of the public on CSF and the necessary of biosecurity measures that need to be implemented in order to prevent this infectious disease in family farms. Rich illustration and the explanatory text, made the material easy to understand and quick to the point.

The construction of the disinfectant barriers represents a great contribution to the sustainability of the project in the period of the next 10 years. Also it is a significant improvement of sanitary standards on family swine farms. This is why this activity must be ranked the highest.

The International Conference held on the 7th and 8th of June, 2012, is graded very highly, and this is confirmed by a large number of attendees and lecturers.

This event represents the crown of the project and the successful realization of all projects activates. Through the presented papers, relevant facts were presented which further inspired the participants to debate and discuss the topics. The fact that the Conference was a success is supported by the conclusion that was reached by the participants, that in the goal of exchanging information and good experiences such a conference should be held periodically, every second year.

The main outcome of the project is the raised awareness of the border region population on CSF. The education of family farmers (swine breeders) and the construction of disinfectant barriers on family farms, in the goal of bettering the sanitary conditions and preventing CSF, are the most advantageous point of the project for the border region between Croatia and Serbia. Education of family farmers was conducted through 10 workshops in Serbia, 10 workshops in Croatia and 4 joint workshops, where family farmers from both Croatia and Serbia participated together. Throughout the workshops there were 278 participants educated. The population in the boarder region was introduced with dangers of CSF spreading, through leaflets (5000 copies in Croatian and 5000 copies in the Serbian language), Manuals (2000 copies in Croatian and 2000 copies in the Serbian language) and through broadcasting of a TV spot. In the border region of Serbia, 18 disinfectant barriers were built and 22 barriers in the border region of Croatia.

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WEST NILE VIRUS SURVEILLANCE PROGRAM IN SERBIA

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Abstract

Serological and virological examination of the presence of human and animal infection caused by West Nile Virus (WNV) as well as the presence of the virus in vectors, which has been conducted during the past few years, confirmed an active virus circulation in the territory of the Republic of Serbia. Based on the obtained results and anticipated intense circulation of WNV, which poses substantial risks for both public and animal health in Serbia, and having in mind its crucial role in the protection of public health, Veterinary Directorate of the Ministry of Agriculture and Environmental Protection infront the Veterinary Service launched and funded the national WNV monitoring program starting from April 2014. The Program encompassed the entire territory of the Republic of Serbia and was conducted by scientific and specialized veterinary institutes and field veterinary service in close collaboration with qualified entomologists and ornithologists. The principal objective of the monitoring – surveillance program is early detection of WNV in monitored regions, timely reporting of the virus presence and activation of human health service institutions and local authorities aimed at establishing the control measures - eradication of mosquitoes, informing the local community and taking all relevant preventive measures for human health protection. The surveillance program of the WNV occurrence and spread is based on direct and indirect surveillance of WNV in natural environment. Indirect surveillance encompasses serological testing of seronegative sentinel horses and poultry for the presence of WNV infection, and it is performing continuously and periodically during the most

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intensive mosquito activity (May – September). The number of sentinel animals that should be tested was defined at the district level, according to the rate of anticipated risk of WNV infection. Direct surveillance was performed through periodical and continuous testing of pooled mosquitoes samples collected at two-week intervals during peak mosquito season (May – September) and samples of wild birds (tissues of dead birds and throat swabs of captured live susceptible bird species). The number of samples was stipulated according to the anticipated risk rate in particular regions.

Key words: West Nile virus, surveillance program, Serbia

PROGRAM NADZORA PRISUSTVA VIRUSA ZAPADNOG NILA U SRBIJI

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Kratak sadržaj

Sprovedena serološka i virusološka ispitivanja prisustva infekcije uzrokovane virusom Zapadnog Nila (WNV) kod različitih vrsta životinja i ljudi, kao i vektora virusa u proteklih nekoliko godina su potvrdila aktivnu cirkulaciju ovog virusa na području Republike Srbije. Na osnovu ovih rezultata i realne predpostavke o nastavku intenzivne cirkulacije WNV i opasnosti za, pre svega, javno zdravlje ali i zdravlje životinja na području zemlje, veterinarska služba na čelu sa Upravom za veterinu Ministarstva poljoprivrede i zaštite životne sredine je prepoznala svoju značajnu ulogu u zaštiti javnog zdravlja i od aprila 2014. godine je pokrenula i finansirala nacionalni program monitoringa WNV. Pomenuti program nadzora na celokupnom području Republike Srbije sprovode veterinarski naučni i specijalistički instituti i nadležne veterinarske stanice u saradnji sa entomolozima i ornitolozima. Osnovni cilj programa monitoringa je rana detekcija prisustva WNV na nekom području i pravovremeno alarmiranje humane zdravstvene službe i lokalnih samouprava radi kontrole – suzbijanja koma-

raca, informisanja stanovništva i preduzimanja svih mogućih preventivnih mera zaštite ljudi. Program monitoringa – nadzora pojave i širenja WNV se zasniva na indirektnom i direktnom praćenju prisustva WNV u prirodi. Indirektno praćenje virusa se vrši serološkim testiranjem na WNV seronegativnih – sentinel konja i živine, koja se vrše kontinuirano i periodično u periodu najveće aktivnosti komaraca (maj – septembar). Broj sentinel životinja koje se prate je definisan na nivou svakoga okruga Republike Srbije i to u odnosu na visinu rizika od pojave infekcije WNV. Direktno praćenje prisustva WNV se vrši periodičnim i kontinuiranim ispitivanjima zbirnih uzoraka komaraca uzorkovanih svake dve nedelje u periodu njihove najveće aktivnosti (maj - septembar) i divljih ptica (tkiva uginulih i briseva živih prijemčivih vrsta divljih ptica) na prisustvo ovoga virusa. Broj uzoraka za ispitivanje je takođe određen po okruzima na osnovu visine rizika.

Ključne reči: virus Zapadnog Nila, program nadzora, Srbija

INTRODUCTION

West Nile virus (WNV) is a neurovirulent mosquito-borne *Flavivirus* with zoonotic potential, which is maintained in nature in an enzootic transmission cycle between avian hosts and ornithophilic mosquito vectors. The virus occasionally infects other vertebrates, including humans and horses, in which it may cause sporadic disease outbreaks that may result fatal. West Nile virus (WNV) was first isolated from a febrile woman in the West Nile district of Uganda in 1937 (Smithburn et al., 1940) and today is considered as the most widespread flavivirus in the world, endemic in Africa, Asia, Europe, Middle East, Australia and Americas (Trevejo and Eidson, 2008; Calistri et al., 2010; Weissenböck et al., 2010; Papa et al., 2011).

WNV infections have been described in a wide variety of vertebrates (Komar et al., 2003). The virus is maintained in an enzootic cycle between ornithophilic mosquitoes, mainly of the *Culex* genus (Hayes et al., 2005; Ziegler et al., 2012), but also *Aedes* and *Ochlerotatus* genus, and certain wild bird species (Savini et al., 2012; Ziegler et al., 2012). WNV was found in more than 150 species of wild and domestic birds (van der Meulen et al., 2005). Wild birds are important to public health because birds migrating across national and intercontinental borders and becoming a long-range virus vectors (Linke et al., 2007). Following infection, many bird species produce levels of viraemia that are sufficient for transmitting the virus to mosquitoes (Komar et al., 2003). Human and mammals, especially horses, are occasional, dead end hosts and play limited roles in the natural cycle because viraemia is generally too low to

infect mosquitoes (Dauphin et al., 2004; Valiakos et al., 2011), however severe neuroinvasive disease and occasionally with fatal outcomes can occur.

In Europe, until the 1990´s WNV had caused sporadic outbreaks with rare reports of encephalitis but its epidemiological behaviour changed when it re-emerged in Romania, Russia and the Mediterranean basin causing dozens of humans and horses deaths (Castillo-Olivares and Wood, 2004; Blitvich, 2008; Calistri et al., 2010). Also, only recently the strains of WNV lineage 2 were identified in Europe: in 2004 and 2005 in goshawks and birds of prey in Hungary, in 2007 in Volgograd, Russia, and in 2008 and 2009 in goshawks and a falcon in Austria (Bakonyi et al., 2006; Erdélyi et al., 2007; Platonov et al., 2008; Wodak et al., 2011). Since 2008, WNV has been heavily spreading throughout central and southeastern Europe, constituting a serious veterinary and public health problem for Europe (Barbic et al., 2012; Ziegler et al., 2012).

In Serbia, WNV situation was mostly unknown until 2009. Serological testing of horses sampled during 2009-2010 by ELISA based on WNV recombinant envelope E (rE) protein and PRNT showed for the first time in Serbia that 12% of 349 horses from northern part of country presented specific neutralizing WNV antibodies. Positive horses were found in 14 of the 28 municipalities studied, which are up to 200 km distant (Lupulović et al., 2011). In another study, presence of WNV specific antibodies was found in 28.6% (72) of 252 examined horse sera samples collected from 7 different stables and locations in Vojvodina province and Belgrade area, during 2007-2011. WNV seroprevalence ranged per stable from 13.3% up to 40% seropositive animals (Medić et al., 2014). In addition, just one year later, to asses WNV presence in the environment immediately after the human WNV outbreak in 2012, during November and December of 2012, presence of anti-WNV IgG antibodies were examined by commercial ELISA test in blood sera samples of 130 horses from 6 stables and 1 settlement in Vojvodina province, northern Serbia. Positive results were obtained in 49.23% (64/130) samples. Per stable, percent of seropositive animals was from 35% to 64% (Petrović et al., 2014). This prevalence (49.23%) obtained in horses during 2012 was much higher than that found in horses during 2009 and 2010 (12%), including the confirmed seroconversion in at least 8 horses tested negative in 2010, thus confirming an intensive WNV circulation in 2012 on the territory of Serbia (Petrovic et al., 2014). Similarly, 96 horses from 5 tested stables during 2012 were tested again during 2013 with the same methodology. High prevalence of 46.88% (ranged between stables from 23.53-75.0%) with new cases of seroconversion were detected also indicating an intensive WNV circulation in 2013 (unpublished data).

WNV circulation in Serbia was also confirmed in wild birds as virus natural host. In total, 92 blood sera and 81 pooled tissue samples were collected

from 133 dead and live captured wild resident and migratory birds (45 species within 27 families) from January until September 2012 in Vojvodina Province - northern part of Serbia. WNV antibodies were detected by ELISA and PRNT in 7.6% (7/92) blood sera and virus presence was confirmed in tissue of 8 out of 81 (9.87%) and blood of one bird. Most of the antibody or virus positive birds were strictly resident, suggesting endemic presence of WNV in Serbia (Petrovic et al., 2013). By phylogenetic analysis of genomic sequences, all WNV isolates were classified as a lineage 2 strains that clusters with the viruses responsible for the most recent human and animal outbreaks reported in neighbouring countries (Petrovic et al., 2013).

The first studies on the presence of WNV in mosquitoes as virus vectors date back to the period 2005-2010. A total of 56757 mosquitoes (841 pools of 50 individual insects) originating from 66 localities in 29 settlements in Vojvodina were examined. The presence of WNV genome was established in only three pooled-samples of mosquitoes collected during 2010 in the territory of Detelinara (part of the city of Novi Sad). The isolate was typed as lineage 2 WNV (Petric et al., 2012). This study was furher done during 2012 and 2013, when significantly increased prevalence of WNV in mosquitoes was established. Even more than 9% of the mosquito pools examined during 2012-2013, mainly of species *Culex pipiens* was tested positive for WNV presence (unpublished data).

The history of WNV infection among human population in Serbia is mostly unknown, and only scarce historical data exists. First serological investigation of WNV infection presence in human population in Serbia was conducted in 1972 and antibodies against WNV were found in 2.6% - 4.7% of human sera (Bordjoški et al., 1972). In another study, antibodies against WNV were detected, depending on location, in 1 to 8% of tested human sera in Serbia (Vesenjak-Hirjan et al., 1991). After a gap of many years, more recent serological examinations show presence of anti-WNV IgG antibodies in 18 out of 451 (3.99%) human collected from 2005 to 2010 in Vojvodina province with yearly rates varying between 1.97% and 6.04% (Petrić et al., 2012). Except this data, as to our knowledge, no clinical manifestation of disease was ever reported in Serbia until 2012. In August 2012, an outbreak of WNV infection in humans was reported for the first time ever in Serbia (EpiSouth Weekly Epi Bulletin - N°232 and N°240, 2012; ECDC, 2012), being the first time that WNV infections in the country have been associated with clinical symptoms. As of November 30, 2012, a total of 71 West Nile fever cases were reported, among which 42 were clinically and laboratory confirmed, and in 9 cases resulted fatal (lethality of 12.7%). All the cases were detected in central

and northern part of the country, 72% of them in the Beograd district (ECDC, 2012; Obrenovic et al., 2013; Popovic et al., 2013). This epidemic continued, and became even more severe during 2013. As of November 2nd, 2013, a total of 303 West Nile fever cases were reported, among which 202 were clinically and laboratory confirmed, and 103 were classified as probable cases. Infection in 35 cases resulted fatal (lethality of 11.6%). Almost all of the cases were also detected in central and northern part of the country (Institute of Public Health of Serbia, 2014).

The aforementioned serological and virological examinations confirmed active circulation and endemic presence of WNV in the territory of the Republic of Serbia. Based on the obtained results and anticipated intense circulation of WNV that poses substantial risks for both public and animal health in Serbia, and having in mind its crucial role in the protection of public health, Veterinary Directorate of the Ministry of Agriculture and Environmental Protection infront the Veterinary Service launched and funded the national WNV surveillance program starting from April 2014. The methodology of implementation and management of this surveillance program is presented in this article.

METHODOLOGY OF WNV SURVEILLANCE PROGRAM IN SERBIA

The surveillance program encompassed sentinel species (poultry and horses), mosquitoes (particularly species *Culex pipiens,* which were confirmed as most prevalent WNV vectors in our region) and wild bird species, which are natural virus reservoirs and populate the natural habitats in Serbia, either temporarily or permanently. The surveillance program was conducted throughout the year according to the provided guidelines. Active surveillance was performed by serological examination of sentinel poultry and horses and by testing of virus presence in samples of mosquito vectors (sampled by dry-ice baited traps in the period of most prominent vector activity using special traps), as well as in the samples of all collected dead wild birds belonging to the species susceptible to WNV (tested throughout the year). Passive surveillance encompassed serological (testing of paired serum samples) and virological examination of clinically ill horses manifesting signs of CNS dysfunction.

The active and passive surveillance encompassed all municipalities in the Republic of Serbia. The selection and distribution of sampling localities in each county-region is defined by epizootiological services of scientific and specialized veterinary institutes according to the assessment of the risk of exposure to WNV. By assessing the exposure risk, the following is taken into consideration:

1. already available results of serological examination of horses;
2. existence of areas suitable for mosquitos such as standing waters, rivers, water flows, canals etc.;
3. settlements with recorded human infections (according to the data obtained from the Institute of Public Health of Serbia – "Batut" and regional Institutes of Public Health in the relevant territory)

Based on the available data on the presence and circulation of WNV in the Republic of Serbia, the districts, i.e. Counties, were categorized according to risk of WNV infection outbreak (Table 1).

Table 1. Categorization of districts - Counties in the Republic of Serbia according to risk of WNV outbreak based on available results of laboratory examination of horses and vector mosquitoes, as well as of human cases

Regions – Counties with particularly high risk	**Regions – Counties with moderate risk**
North Bačka County	North Banat County
South Bačka County	West Bačka County
Middle Banat County	Šumadija County
South Banat County	Pomoravlje County
Srem County	Bor County
City of Belgrade	Zaječar County
Mačva County	Zlatibor County
Kolubara County	Moravica County
Podunavlje County	Raška County
Braničevo County	Rasina County
	Nišava County
	Toplica County
	Pirot County
	Jablanica County
	Pčinj County

ACTIVE SURVEILLANCE

1. Serological surveillance

Serological surveillance implicated sampling and examination of blood sera of sentinel horses and poultry for the presence of WNV specific antibodies (sentinel animals are individuals that have not been in contact with WNV, that is, do not possess specific antibodies against WNV in the blood). Serological examination is performed using ELISA technique. The testing is performed in authorized scientific and specialized veterinary institutes.

1.1. Serological surveillance of sentinel poultry

Serological testing of sentinel poultry encompassed blood samples of poultry kept in extensive breeding system (backyard poultry), where only poultry hatched during the year of testing shall be tested on anti-WNV antibody presence. The tested population should be mainly located in the suburban areas (predominantly rural settlements). In high-risk regions (10 Counties – Table 1), the sampling is performing in ten settlements with highest risk, i.e. five poultry blood samples were collected per one settlement, from at least one husbandry with extensive poultry keeping system (backyard poultry). The sampling and examination thereof is performing during the period May-September, i.e., in 6 sampling ocassions: one in May (by the end of the month), one in June, two in July, one in August (middle of the month) and one in September (until 15th September), meaning ones or twice monthly, depending on the risk of infection in the relevant period of the year. In regions/Counties with lower risk of WNV infection outbreak (15 Counties – Table 1), the sampling is performing in 6 high-risk settlements by collecting up to 5 blood sera per one settlement from at least one husbandry with extensive (backyard) keeping system. The sampling and examination thereof is performing during the period June-September, i.e., in 4 sampling ocassions: one in June, one in July, one in August (middle of the month) and one in September (until 15th September). Throughout entire surveillance period (from May/June until September), the obtaining of blood samples had to be done at the same locations for all sampling ocassions. The term "location" in this Program considered the area of selected settlements. Sampling plan is presented in Table 2.

1.2. Serological surveillance of sentinel horses

During the preparatory period of WNV surveillance in horses and with an aim of selecting appropriate sentinel animals, mandatory serological surveillance of horses was conducted from March to May 2014 to identify WNV-

seronegative animals, which are to be used as sentinel animals in the WNV surveillance program.

Serological testing of sentinel horses' blood sera implicated collecting of up to 50 samples from as many as possible locations (minimum 3) in each high-risk County (10 Counties – Tables 1 and 2) and up to 30 samples from as many as possible locations (minimum 3) in each lower-risk region (15 Counties – Tables 1 and 2). The collected samples are testing for the presence of anti-WNV antibodies by applying ELISA test. The sampling should be performed successively from the same sentinel animals, three times (first sampling – during June; second sampling – during July; third sampling – during August 2014).

2. *Virological surveillance*

Virological surveillance encompassed sampling and examination of organs and tissues of dead birds or throat swabs of captured live wild birds (susceptible species), as well as examination of pooled samples of vector mosquitoes (species *Culex pipiens*) for the presence of West Nile Virus – WNV. The virus presence was also tested in samples of brain and cerebrospinal fluids from dead horses with clinically manifested neurological dysfunction (passive surveillance). Virological testing is performing by molecular methods (*real-time RT-PCR* or *RT-PCR*) in the National reference laboratory for WNV in Specialized Veterinary Institute „Kraljevo" (VSI Kraljevo), as well as in virology laboratories of Scientific Veterinary Institute „Novi Sad" (NIV-NS) and Scientific Veterinary Institute of Serbia (NIVS).

2.1. Virological surveillance of wild birds

Dead wild birds found in the natural environment, particularly the resident species most susceptible to infection, e.g. *Corvidae* (magpie, crow, raven, rook. etc.), raptors (northern goshawk, falcon and eagle) and singing birds as well as birds died in rehabilitation centres, zoos or bird breeding farms (mostly raptors such as falcons, eagles, hawks…) were submitted to the aforementioned laboratories for testing for the presence of WNV. If dead birds were unavailable for laboratory examination, the sampling in high-risk regions (May – October) could be performed from captured live WNV-susceptible birds species by obtaining throat swabs or by hunting of certain bird population (*Corvidae*) for examination purposes (in cooperation with the hunting associations). Storage and transportation of samples to the authorized laboratory strongly requires maintenance of cold-chain conditions (refrigeration or freezing (swabs)). Samples obtained from dead birds (brain and parenchymatous

organs) and throat swabs are testing for the WNV presence using molecular methods (*real-time RT-PCR* or *RT-PCR*). Collection of dead-bird samples and testing od virus presence is performing throughout the year in high-risk regions (10 Counties – Tables 1 and 2) and in the period May-October in lower-risk regions (15 Counties – Tables 1 and 2)

2.2 Virological surveillance of mosquitoes – WNV vectors

Vector mosquitoes (*Culex pipiens*) are testing for the WNV presence by molecular methods (*real-time RT-PCR* or *RT-PCR*). The mosquitoes were examined as pooled sample (50-100 individual insects per pool) per one sampling point. Mosquitoes are collecting by dry-ice baited traps in the period of their most intensive activity (May-September) at the semi-urban and urban localities suitable for their survival and reproduction (standing waters, rivers, water flows, canals etc.) in the vicinity of susceptible animals (i.e., close to horse stables and poultry farms). The collection of mosquitoes should be performed at two-week intervals from 10 localities throughout the high-risk Counties (7 samplings, starting by end May, and then by mid and end of following months until mid September). In lower-risk Counties, the sampling should be performed monthly at 5 localities throughout the County (5 samplings, once a month, starting by end May, and then in the second half of the following months until mid September) (Tables 1 and 2). Native mosquito samples (without liquid) collected by dry-ice baited or other traps require rapid cooling (freezing) and immediate transportation to the laboratory (VSI Kraljevo, NIV-NS, or NIVS) for examination while still frozen. To the purpose of sampling and entomological examination, establishing of close cooperation with entomologists is highly recomended.

PASSIVE SURVEILLANCE

All horses with clinically manifested neurological dysfunction had to be subjected to testing for WNV infection in the framework of passive surveillance. The testing encompassed serological examination of paired samples of blood sera collected at 3-4 week intervals. The presence of WNV-specific antibodies is done in the corespondent scientific or specialized veterinary institute. In cases of lethal outcome in horses, samples of brain tissue and cerebrospinal fluid need to be submitted for laboratory examination for the presence of WNV (laboratories of VSI Kraljevo, NIVS or NIV-NS).

Table 2. WNV surveillance plan (sampling and testing)

	High-risk regions/Counties	**Lower-risk regions/Counties**
1. Testing of sentinel animals (domestic poultry and horses) aimed at early detection of WNV circulation		
Surveillance of sentinel poultry on rural households – poultry hatched in current year (backyard poultry)	Serological testing at the authorized institute in the period May-September from 10 settlements / County; 5 samples / settlement from et least one household according to described schedule. 6 samplings (1 in May; 1 in June; 2 in July; 1 in August – by middle; 1 in September (until 15 Sept)	Serological testing at the authorized institute in the period June-September from 6 settlements / County; 5 samples / settlement from et least one household according to described schedule. 4 samplings (1 in June; 1 in July; 1 in August – by middle; 1 in September (until 15 Sept)
Surveillance of sentinel horses	Serological testing of 50 sentinel horses in the authorized institute, sampling from minimum 3 localities per County. Sampling and blood testing of same horses to be performed three times (in three occasions) (June-July-August)	Serological testing of 30 sentinel horses in the authorized institute, sampling from min 3 localities per County. Sampling and blood testing of same horses to be performed three times (in three occasions) (June-July-August)

	High-risk regions/Counties	Lower-risk regions/Counties
2. Testing aimed at early detection of WNV in natural reservoirs and vectors		
Virus surveillance in wild birds	Application of *RT-PCR* or *real time RT-PCR* methodology for testing samples of dead susceptible bird species throughout the year, or up to 100 samples of purposely hunted birds or live captured susceptible bird species per County during the period May - October	*RT-PCR* or *real time RT-PCR* methodology for samples of up to 50 dead birds (WNV-susceptible species) per County during the period May - October
Virus surveillance in vectors - mosquitoes (*Culex pipiens*)	Collecting mosquitoes at 2-week intervals in the period May-September at 10 localities within the County and testing the virus presence by *RT-PCR* or *real time RT-PCR* methodology (7 samplings in the period from end May to the first half of September)	Collecting mosquitoes at monthly intervals in the period May-September at 5 localities per County and testing the virus presence by *RT-PCR* or *real time RT-PCR* methodology (5 samplings once a month in the period from second half May to the first half of September)

SAMPLING PROCEDURE, SAMPLE DISTRIBUTION AND REPORTING

According to the provisions of the Surveillance program, sampling of calculated amount of blood samples from sentinel horses and poultry from settlements, households and stables, as well as obtaining of basic epizootiological and anamnestic data is done by authorized veterinary service and epizootiolo-

gists in scientific or specialized veterinary institutes responsible for serological testing of blood samples of sentinel horses and poultry for the presence of WNV-specific antibodies.

Responsible epizootiologists of scientific and specialized veterinary institutes collected basic epizootiological and anamnestic data, as well as the samples of wild birds and mosquitoes in their regions. The samples were submitted to laboratories for testing for the presence of WNV, that is, national reference laboratory fro WNV in the VSI Kraljevo or Virology laboratories of NIV-NS and NIVS. The carcasses of susceptible species of wild birds and throat swabs of captured susceptible live wild birds, as well as mosquito samples (pooled samples consisting of 50-100 mosquitoes per one sampling ocassion and sampling locality) collected in the territories of North Bačka, West Bačka, South Bačka, Srem, North Banat, Middle Banat and South Banat Counties were submitted to the virology laboratory of the NIV-NS. The carcasses of susceptible species of wild birds and throat swabs of live susceptible wild birds, as well as mosquito samples (pooled samples consisting of 50-100 mosquitoes per one sampling ocassion and sampling locality) collected in the epizootic regions controlled by Specialized Veterinary Institutes VSI Niš, VSI Kraljevo, VSI Zaječar and VSI Jagodina were submitted to the national reference laboratory for aviary influenza, Newcastle disease and WNV in VSI Kraljevo. The samples collected in the epizootical region controlled by NIVS, that is, epizootical regions of NIVS, VSI Požarevac and VSI Šabac were submitted to the virology laboratory of the NIVS.

The Institutes communicated the regular monthly testing reports to the Veterinary Directorate by the 10th of the following month. In cases of positive seroconversion finding (positive serological finding in previously seronegative sentinel animals) during testing or establishment of virus presence in wild birds, vectors (mosquitoes) or horses (during active or passive surveillance), SUSPECTED CASE on presence of infectious disease is set up and reporting immediately, without delay, to the responsible veterinary inspector, Veterinary Directorate and to the National reference laboratory of the VSI Kraljevo. Positive and suspect samples were immediately submitted to the National reference laboratory of the VSI Kraljevo for final confirmation and diagnosis. In case of positive finding, the National reference laboratory sends the confirmatory official report to the sender and to the Veterinary Directorate. Based on positive seroconversion finding, or positive finding for virus presence, the Veterinary Directorate declares the presence of WNV, i.e., POSITIVE CASE of WNV infection in the relevant region. Veterinary Directorate communicates the information on Suspected and Positive Cases of WNV infection (confirmation of suspect infection – positive case) to the Ministry of Health.

Blood serum samples obtained from horses and poultry confirmed positive for the presence of WNV-specific antibodies, as well as the virus-positive samples of wild birds and mosquitoes must be stored in frozen state to the purpose of further examination (including potentially virus-positive samples of diseased/dead horses tested during passive surveillance process).

To provide the unremitting and timely WNV surveillance, particularly its stages that require technically complex procedures (surveillance of wild birds and vector mosquitoes), timely planning and implementation of required resources (personnel, equipment, reagents) by all participating parties is of vital importance. Surveillance of wild birds and mosquitoes, as a technically complex segment of the procedure, requires the following preceding actions and prerequisites:

- official approval for capturing and sampling of wild birds;
- participation of qualified ornithologists or at least certified ringers (binders) for performing field activities and accurate identification of wild bird species used for obtaining samples
- cooperation and working together with hunting societies and organizations;
- legally registered, i.e., reported nets for capturing wild birds;
- participation of qualified entomologists to perform accurate identification of trapped mosquitoes (at species level) to be examined for the presence of WNV and for field activities aimed at trapping and collecting of mosquitoes;
- sufficient number of adequate traps for collecting the mosquitoes (*Culex pipiens*);
- dry ice for storing and transportation of samples to the laboratory

The WNV surveillance program has been conducted during 2014 in the territory of Serbia. The program is still ongoing, and its evaluation will be performed by the beginning of 2015. Based on the data obtained in this evaluation, the effects of the Program will be assessed. We believe that the obtained data will enable potential corrections and amendments to the Program, thus making it highly effective instrument for further surveillance of this vector-borne zoonotic viral infection in the territory of Serbia.

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A METHOD FOR DETECTING AND TYPING OF SALMONELLA BY MULTIPLEX PCR

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Abstract

Today in Ukraine's market is increasing the volume of trade with livestock products. Also the number of catering services and grocery shops selling ready-made food is growing throughout the country. The veterinary service should have time to check the quality of all of these products. Only traditional bacteriological methods of isolation and identification of pathogens of toxicoinfection, which is not enough in terms of increasing turnover of products, are used today. The one of the most dangerous toxicoinfections is salmonellosis. Typing different *Salmonella* species gives an answer about the source of infection. The aim of our work was to develop a system of identification of *Salmonella* and typing among them five serovars based on the polymerase chain reaction (multiplex PCR). We performed analysis of the nucleotide sequences of the five members of the genus *Salmonella*, on the basis of which a primer designed for the identification of any member of the genus Salmonella with simultaneous typing - *Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Gallinarum-Pullorum by multiplex PCR. The protocol of multiplex PCR was optimization with simples positive DNA matrix.

Key words: salmonella, multiplex PCR, typing DNA.

MULTIPLEX PCR METOD ZA DETEKCIJU I TIPIZACIJU SALMONELA

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Kratak sadržaj

Danas na ukrajinskom tržištu postoji povećanje obima trgovine animalnim proizvodima. Takođe, broj ketering agencija i trgovina mešovite robe koje prodaju gotovu hranu raste u celoj zemlji. Veterinarskoj službi treba dosta vremena za proveru kvaliteta svih tih proizvoda, jer danas na raspolaganju imamo samo tradicionalne bakteriološke metode izolacije i identifikacije uzročnika toksoinfekcija, što nije dovoljno s obzirom na sve veći promet proizvoda. Jedna od najopasnijih toksoinfekcija je salmoneloza. Tipizacija različitih *Salmonella* vrsta daje odgovor o izvoru zaraze. Cilj našeg rada bio je razvijenanje sistema zasnovanog na lančanoj reakciji polimeraze za identifikaciju bakterija iz roda *Salmonella* i tipizaciju pet serotipova (multiplex PCR). Izvršili smo analizu nukleotidnih sekvenci za pet članova roda *Salmonella* i dizajniranje prajmera za identifikaciju bilo kog člana roda *Salmonella* sa istovremenom tipizacijom - *Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Gallinarum-Pullorum metodom multiplex PCR. Optimizacija protokola metode multiplex PCR je izvršena sa jednostavnim pozitivnim matricama DNK.

Ključne reči: *Salmonella*, multiplex PCR , DNK tipizacija

INTRODUCTION

Salmonellosis - one of the most dangerous diseases that is caused by serotypes of bacteria of the genus *Salmonella*, which have mechanisms for habitat and parasitism in the gastrointestinal tract (Althouse et al., 2003; Chiu et al., 2010).

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According to the current classification, *S. enterica* is divided into six subspecies: *Salmonella enterica* subspecies enterica, *Salmonella enterica* subspecies salamae, *Salmonella enterica* subspecies arizonae, *Salmonella enterica* subspecies diarizonae, *Salmonella enterica* subspecies houtenae and *Salmonella enterica* subspecies indica, which differentiate by the biochemical activity and represent the number of subtypes I, II, IIIa, IIIb, IV , and VI , respectively. *Salmonella enterica* subspecies enterica is mostly isolated in the majority of cases of *Salmonella* infection from animal and human (Althouse et al., 2003; Battistuzzi et al., 2004).

Salmonella contamination occurs through the consumption of contaminated food: eggs and egg products, milk and dairy products, meat birds and other animals. Another way of infection is the transfer of infections through tap water, in addition, the sources of infection can be the open water (Bailey, 1998). According to the FAO, 20% of poultry products in the world are contaminated with *Salmonella*, and they can persist for a long time in the animal facilities because they can form a surface film (Vestby et al., 2009; http://www.fao.org/docrep/012/i1133e/i1133e00.htm). Annually on the planet are registered 21 million cases of typhoid fever, and about 216 thousand cases (Zhou and Pollard, 2010).

Worldwide, the monitoring of the incidence of salmonellosis in which tracked various options for its manifestation. As well as a comparison of *Salmonella* strains isolated from humans and animals (Chiu et al., 2009; Chiu et al, 2010; Laupland et al, 2010).

The system of quality control of food, raw materials, based on the use of bacteriological methods of investigation (D'Aoustlnt, 1991).

As an alternative to traditional bacteriological methods for the identification and typing of *Salmonella* proposes the use of polymerase chain reaction (dos Santos, 2001; Zahraei Salehi et al., 2005; Eyigör et al, 2007; Cao et al., 2008; Mirmomeni et al., 2008; Zhou et al., 2010).

Analysis of antigen alleles H1 (i, g, m, r or z10) allowed fast typed serological variants enteritidis, hadar, heidelberg and typhimurium (Hong et al., 2008).

To date, Ukraine has not yet widespread methods of rapid diagnosis of salmonellosis. Typing of the pathogen is an essential component of diagnosis, because it can give an answer about the alleged source of infection. For this reason, the aim of our work was the development of the national test system based on the polymerase chain reaction, which would like to identify and typed some key members of the genus *Salmonella* (Gerylovich, 2011).

METHODS AND MATERIALS

The objects of our study were *Salmonella spp*., *Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. *Gallinarum*-Pullorum. For the construction of genus-and species-specific primers electronic databases of sequences of essential genes in *Salmonella* contained in the international database GenBank (http://www.ncbi.nlm.nih.gov/genbank/) were analyzed.

Multiple alignment of selected sequences, and their subsequent analysis to select PCR primers was performed using the computer program Bio Edit (v.7.2.4).

The protocol of polymerase chain reaction has been developed on the basis of the primer systems with a certain temperature, the selection of components for the formulation of the multiplex PCR and the identification of the genus *Salmonella spp*. and typing of the five listed above serotypes (Elnifro, 2000; Kaderali, 2007).

One-day-old cultures of *Salmonella* from the museum sector study mycoplasmoses and salmonellosis are grown for meat - peptone medium were used as the source of positive DNA-matrix.

Extraction of total nucleic acid was carried out using micro columns. To 450 μl of Extraction buffer was added 100 μl of *Salmonella* culture. After lysis of the containments from the tubes were transferred to microcolumns and centrifuged. This was followed by washing with ethanol followed by extraction of total nucleic acid of TE-buffer.

DNA concentration was calculated by spectrophotometery at 260 nm.

RESULTS AND DISCUSSIONS

The nucleotide sequences of the major genes were analyzed. The greatest breadth of sample homogeneity and sequenced portions of the gene was detected in *invA* for all members of the genus *Salmonella*. In the computer analysis of the gene sequences *invA* was selected 22 pairs of oligonucleotides - potential pairs of primers for PCR. The PCR product limited by size of 387 bp in length, and olygonucleotides were called Salm3_4.

For *Salmonella enterica* ser. Enteritidis specific motifs were found in the gene *SefA*. Sequence analysis of this gene allowed to establish the potential 6 primer pairs. The primers flanking portion length 299 bp were selected.

The gene *fliC* demonstrated specificity for *Salmonella enterica* ser. Typhimurium. The primers flanking region 420 bp were choosed.

Gene *viaB* contained specific motives for *Salmonella enterica* ser. Typhi. Accordingly, on this basis was chosen area, which limited the targeted gene fragment length 738 bp.

For the genome of *Salmonella enterica* ser. Dublin serospecific motifs were found in SeD_A1104 gene. When bioinformatics studies were identified primers flanking the product of 203 bp.

Finally, gene SG0266 was elected by containing specific motifs for *Salmonella enterica* ser. Gallinarum-Pullorum. Specific primers flanking length of 97 bp region were selected in analyzed area.

Table 1. Nucleotide sequence and PCR product.

Salmonella	Primer	5*-3*	PCR product, bp.
Salmonella spp.	Salm 3	GCTGCGCGCGAACGGCGAAG	387
	Salm4	TCCCGCCAGAGTTCCCATT	
Salmonella enterica ser. Enteritidis	Sent F	AAATGTGTTTTATCTGATGCAAGAGG'	299
	Sent R	GTTCGTTCTTCTGGTACTTACGATGAC	
Salmonella enterica ser. Typhimurium	Styp F	CCCCGCTTACAGGTCGACTAC	433
	Styp R	AGCGGGTTTTCGGTGGTTGT	
Salmonella enterica ser. Typhi	Styphi_F	CACGCACCATCATTTCACCG	738
	Styphi_R	AACAGGCTGTAGCGATTTAGG	
Salmonella enterica ser. Dublin	Sdub_F	ACGCGAAATCTGATGGTCTT	203
	Sdub_R	GCCCACCAGTTGTGAAAGGC	
Salmonella enterica ser. Gallinarum-Pullorum	Sgal_F	CCGCACAACACATCAGAAAG	97
	Sgal_R	AGCTGCCAGAGGTTACGCTG	

After synthesis of primers, we performed optimization of the PCR protocol. As the positive control for PCR we used DNA extracted from the one-day-old culture of *Salmonella* which have been stored in the museum NSC " IECVM ".

The obtained DNA matrix concentration after measuring with a spectrophotometer, we have led to the same concentration and then put PCR.

The first stage was carried out testing each primer pair using the standard composition of the reaction mixture at different temperatures.

To determine optimal temperature parameters PCR was performed with various primer annealing temperature: 58° C, 60° C, 63° C and 65° C. As a result, established the following optimal amplification:

- Initial denaturation - 94° C – 2 min;
- Denaturation- 94° C – 30s;
- Annealing - 63° C – 30s;
- Extension - 72° C – 40s;

} 40 cycles

- Final extension - 72° C – 5min

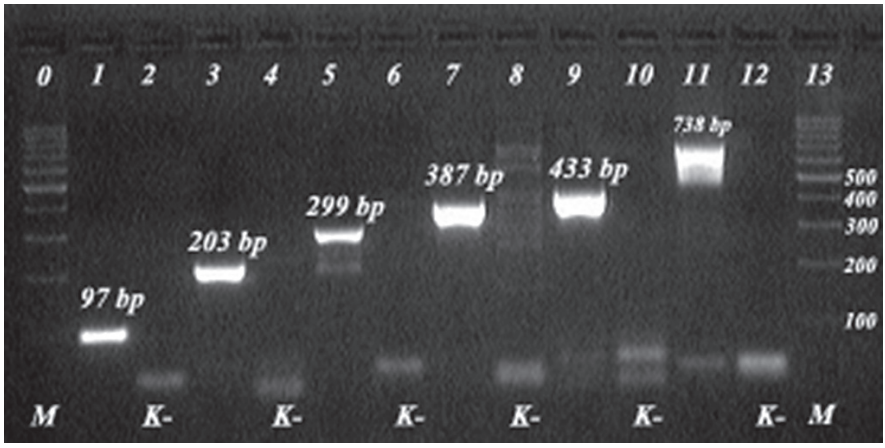

Figure 1. Electropherogram of results of initial testing of primers with positive DNA template: Track number 1 - corresponds the *Salmonella enterica* ser. Gallinarum-Pullorum aplicon (97 bp) lane number 3 - corresponds to the amplicon for *Salmonella enterica* ser. Dublin – (203 bp), lane number 5 - corresponds to the amplification *Salmonella enterica ser.* Enteritidis – (299 bp), lane number 7 - corresponds to the amplicon for *Salmonella* spp. – (387 bp), track number 9 - corresponds to the amplicon for *Salmonella enterica ser.* Typhimurium – (433 bp), track number 11 - corresponds to the amplicon for *Salmonella enterica* ser. Typhi – (738 bp.)

To set up the multiplex PCR, the optimization of reaction was performed using the basic sets for the amplification produced by Thermo Scientific (Lithuania).

Table 2. The composition of the reaction mixture for multiplex PCR

№	Components	
1	10×DreamTaq Buffer	2,5 µl
2	dNTP Mix, 2 mM each	2,5 µl
3	25 mM MgCl2	0,5 µl
4	Primer Styphi_Forward, (conc. 40 pM)	40,0 pM
5	Primer Styphi_Reverse, (conc. 40 pM)	40,0 pM
6	Primer Styp _ Forward, (conc. 40 pM)	20,0 pM
7	Primer Styp _ Reverse, (conc. 40 pM)	20,0 pM
8	Primer Salm_3 Forward, (conc. 40 pM)	20,0 pM
9	Primer Salm_4 Reverse, (conc. 40 pM)	20,0 pM
10	Primer Sent_ Forward, (conc. 40 pM)	10,0 pM
11	Primer Sent_ Reverse, (conc. 20 pM)	10,0 pM
12	Primer Sdub_ Forward, (conc. 20 pM)	10,0 pM
13	Primer Sdub_ Reverse, (conc. 20 pM)	10,0 pM
14	Primer Sgal_ Forward, (conc. 20 pM)	10,0 pM
15	Primer Sgal_ Reverse, (conc. 20 pM)	10,0 pM
16	Template DNA	10 pg – 1 µg
17	DremTaq DNA Polymerase	10,0 U
18	Water, nuclease-free	to 25,0 µl
Total volume		25, 0 µl

We have increased the time of denaturation of DNA to 45s for multiplex PCR-protocol establishment. The annealing of primers was also prolonged to the 45s, elongation time was increased to 1 minute. Final elongation was 10 min :

- Initial denaturation - 94° C – 2 min;
- Denaturation- 94° C – 45s;
- Annealing - 63° C – 45s;
- Extension - 72° C – 1min;

} 40 cycles

- Final extension - 72° C – 10 min

This mode is enabled to carry out the amplification of the expected fragments (Fig. 2) in a single reaction.

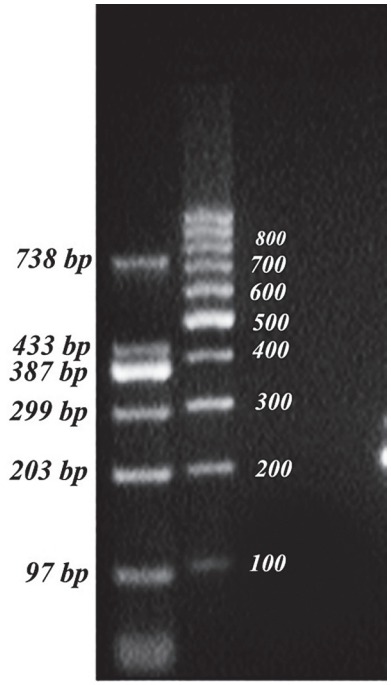

Figure 2. Electropherogram results Multiplex - PCR

CONCLUSIONS

The primer system, which allows simultaneous identification of a multiplex - PCR its five core members (*Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Gallinarum-Pullorum) has been developed.

Multiplex PCR protocol could be applied in the laboratories for identification and typing of *Salmonella* in the shortest possible time. Also, the system can be convenient for monitoring *Salmonella* contamination of various objects, while typing their main representatives.

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SUGGESSTED MODEL FOR MONITORING AND CONTROL OF FOODBORNE PATHOGENS IN WILD BOAR´S MEAT

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Abstract

Wild animal meat harvesting and processing is significantly different from classical livestock meat production and represents a challenge by itself. Implementation of concept "from forest to fork" encompasses influence of hunting ground ecology, type of hunting, field evisceration of hunted game, meat inspection after shooting or transport etc. The objective of this paper was to propose a model for monitoring and control of food born pathogens in wild boar´s meat. Hazard analysis emphasized the importance of several pathogens: *Mycobacterium spp., Salmonella spp., Trichinella spp.,* and a five-step control program were proposed. The program includes management of microbial contamination of wild boar meat, control measures for live animals, control measures during hunting and after shooting, guidelines for official meat inspection with specific details for control of identified hazardous pathogens and control measures for wild boar carcasses processing. The research on presence of food born pathogens in wild boar meat is still scarce, while the *Trichinella spp.* live cycle is well described and there are relevant data about epidemiology and natural reservoirs of the parasite in this part of Europe, little is known about tuberculosis and salmonellosis prevalence in wild boar population. Thus, implementation

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of control program, with strictly defined control measures cannot be overemphasized, and should include veterinary officials as well as hunters and others involved in game meat chain.

Key words: meat, wild boar, control program, food borne pathogens

PREDLOG MODELA PRAĆENJA I KONTROLE ALIMENTARNIH PATOGENA U MESU DIVLJIH SVINJA

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Kratak sadržaj

Lanac proizvodnje mesa divljači se umnogome razlikuje od klasične proizvodnje mesa domaćih životinja i predstavlja jedinstven izazov. Primena koncepta „od šume do trpeze“ obuhvata uticaj ekosistema lovišta, lova, evisceracije na zemlji, pregleda nakon klanja, transporta itd. Osnovni cilj ovog rada je predlog programa praćenja i kontrole anlimentarnih patogena u mesu divljih svinja. Identifikacijom hazarda izdvojeni su sledeći patogeni: *Mycobacterium spp., Salmonella spp., Trichinella spp* i predložen je program kontrole koji obuhvata: opcije za kontrolu mikrobiološke kontaminacije mesa divlje svinje, kontrolne mere kod živih divljih svinja, kontrolne mere tokom lova i odstrela, opis službene kontrola odstreljene divljači sa specifičnostima za kontrolu identifikovanih hazarda i kontrolne mere tokom obrade trupova divljih svinja. Prisustvo alimentarnih patogena u mesu divljih svinja još uvek je relativno neistražena oblast. Dok o *Trichinella spp* postoje relevantni podaci i poznat je način održavanja životnog ciklusa ovog parazita na našim prostorima o prevalenci tuberkuloze i salmoneloze malo se zna. Stoga je primena ovakvog programa sa jasno definisanim merama kontrole više nego svrsishodna. Dobro je poznata činjenica da lica koja rukuju sa izlovljenom divljači imaju malo ili nikakvo znanje o osnovnim higijenskim principima. Primena programa za kontrolu alimentarnih patogena treba da uključi kako lica u lovištima tako i veterinare koji vrše službenu kontrolu.

Ključne reči: meso, divlje svinje, program kontrole, alimentarni patogeni

INTRODUCTION

Game disease control includes the control of disease spread from wildlife reservoirs to domestic animals, as well as control of zoonotic emerging infectious diseases that represent a significant threat to public health. The possibility of disrupting ecological balance in wildlife population is of special concern in the implementation of wild animal disease control measures. It is widely accepted that complete eradication of shared infectious agents is almost impossible in wildlife hosts serving as natural reservoirs of human and livestock infections.

Harvesting and processing of wild game meat is substantially different from classical livestock meat production and represents a challenge by itself. Implementation of the concept "from forest to fork" encompasses the influence of hunting ground ecology, type of hunting, field evisceration of hunted game, meat inspection after shooting or transport etc. The risk assessment of food borne pathogens in wild boar addresses the following two main items: defining epidemiological differences between wild animals and livestock and the criteria for food safety assessment to be followed for game meat.

Domestic animals raised for food production in farm conditions undergo regular veterinary health control as well as *ante* and *post* mortem inspection at slaughterhouses, while in game species; only *post mortem* examination is widely practiced.

Official criteria for game meat risk assessment are still lacking, and are hard to be established because average daily game meat varies significantly in different regions. Consumption of game meat is limited in general population, but some specific groups consume significant amounts. Game meat consumption in hunter families in EU is estimated at 4 kg/year (Ramanzin et al., 2010).

The objective of this paper was to propose a model for monitoring and control of food borne pathogens in wild boar meat. The program includes management of microbial contamination of wild boar meat, control measures for live animals, control measures during hunting and after shooting, guidelines for official meat inspection with specific details for control of three selected hazardous pathogens and control measures in the processing of wild boar carcasses.

HAZARD IDENTIFICATION

Two criteria were established in wild boar meat hazard identification: evidence of spread of hazardous pathogens during handling, processing and con-

sumption of wild boar meat and evidence of presence of shared pathogens in wild boar population in specific geographical region. Hazard analysis emphasized the importance of three pathogens:

- *Mycobacterium spp*. (bacterial agent that infects animals; sometimes without clinical manifestations, transmitted to humans via contact with infected carcass or raw meat and products)
- *Salmonella spp*. (zoonotic bacterial agent, naturally occurs in animal´s digestive organs, contaminates carcasses in abdominal shots or during inadequately performed evisceration);
- *Trichinella spp*. (parasitic agent, humans typically acquire the infection by consuming raw or undercooked meat infested with infectious *Trichinella* larva)

Mycobacterium bovis

Mycobacterium spp. represents the major biohazard in large game. In the South Bačka region (SBR), three hotspots of endemic bovine tuberculosis (BTB) were identified, in the municipalities of Žabalj, Titel and Novi Sad. The prevalence of infected cattle in affected herds varied from 11.10% to 59.18% (Pusic et al., 2009). Data on BTB presence in wild boar population in the same area are lacking, and a field research is in progress.

Infection of humans with *M. bovis* may occur by inhalation of aerosols or through consumption of milk or at some extent meat contaminated with the bacilli.

There is evidence for human infection through wild boar meat consumption (Ashford et al., 2001).

The most important natural reservoir species for maintaining BTB in Europe´s wildlife population are badger (*Meles meles*), wild boar (*Sus scrofa*) and deer belonging to subfamily *Cervinae*. Wild boars can serve as a reservoir of infection for domestic animals but may also be of public health significance as a direct source of human infection (Gortazari et al, 2008). Epizootical investigations considering BTB in Vojvodina province revealed that there are tuberculous districts in South Backa region with a high incidence of BTB positive animals and herds (Pusic et al., 2007; Pusic et al ., 2013).

Serbian BTB eradication program is based on regular annual bovine intradermal tuberculin testing, compulsory slaughter of positive animals coupled with frequent skin testing in infected and incontact herds, movement restrictions, meat inspection at abattoirs and computer based evidence (Pusic et al., 2008; Pusic et al., 2009a,b,c). Implementation of similar surveillance and eradication programs in wild animal populations is quite impossible to achieve.

Trichinella spp

Trichinellosis is widespread in most of Europe. In Serbia, the endemic regions for trichinellosis are Srem, the valleys of Danube, Drina and Kolubara (Petrovic et al., 2012a). Investigation of trichinella outbreaks in Vojvodina province during the period 2002-2011, revealed that eating of raw, poorly processed or undercooked meat and products from backyard domestic pigs was a main source of human infection (Urosevic et al., 2013). Wild boar to human transmission of live trichinella larvae through meat consumption are well documented in Serbia (Urosevic et al., 2013). In Europe, wildlife represents the most important reservoir of *Trichinella*, which makes eradication impossible and explains why the parasite continue to circulate, even though the prevalence in wildlife can be very low for many years (Rafter et al., 2005).

Research on trichinellosis carried out by Petrovic et al. (2012a,b,c) in Vojvodina region revealed high prevalence of infection in jackals (8.33%), foxes (5%), and at less extent wild boars (1%). In some European countries, where eradication of trichinellosis in domestic animals is accomplished (like Denmark), the prevalence of sylvatic trichinellosis is extremely low (0.001%) (Enemark et al., 2000). The parasitic burden in omnivorous and carnivorous game in Vojvodina region is higher (3 larvae/10g) if compared to countries with no trichinellosis registered in domestic animals (e.g. Denmark 1 larva/10 g). The infestation is particularly high in wild boars in Vojvodina, reaching even 1.100 larvae/g (Petrovic et al., 2013a). High incidence of sylvatic trichinellosis in a particular geographic area poses substantial risk of infection spread to domestic pigs, especially in the grazing habitat (Petrović et al., 2013a,b). In pigs, trichinellosis is usually contracted through cannibalism, ingestion of synantropic and sylvatic animals, or feces of animals in early stages of infection (1-2 post infection) (Petrovic et al., 2014).

Salmonella spp.

Salmonella spp infection in wild boar population is traditionally associated with S. *typhimurium*, but in recent decades, the range of serotypes isolated from carcasses, tonsils, feces and lymph nodes is much broader. Large differences in the prevalence of different *Salmonella spp*. serotypes are not only reported among species (for example, it is higher in wild boar than in ruminants), but also between particular regions (i.e., higher prevalence was established in the southern states of the EU) (Table 1).

Table 1. The prevalence of Salmonella spp. in wild boar in different European countries (*Paulsen et al.*, *2011*)

Animal species/sample	Country	No. samples	Positive samples
Wild boar/feces	Italy	2365	441 (18.7%)
	Portugal	77	17 (22.1%)
	Switzerland	73	4 (5.5%)

In Serbia, the researches on game pathogens conducted so far were predominantly focused on wild birds and enteropathogens other than *Salmonella spp*. (Stojanov et al., 2012; Velhner et al., 2012). The prevalence of *Salmonella spp.* in carcasses of wild boar is relatively low, less than 10% (unpublished data), but the infection in wild boar is much more common as compared to wild ruminants. Therefore, inadequate evisceration and/or direct bullet shot through abdominal cavity increase the risk of *Salmonella spp.* contamination of the meat of wild boar (Wisniewski, 2001).

CONTROL OPTIONS FOR MICROBIOLOGICAL CONTAMINATION OF WILD BOAR MEAT

According to recommendations of Codex Alimentarius Commission (CAC, 2005), the control is focused on hygiene and game meat inspection in the primary stages of the meat chain (including transport), as the important measures for the control of the meat of hunted game. The recommendations have been implemented in national legislation of countries of Central Europe (Regulations (EC) No 853/2004 and 854/2004).

The control measures are divided into two groups:

(1) visual inspection for the presence of disease and assessment of all major changes (clearly visible pathological changes, severe contamination from the environment or in case of suspected specific biohazard, additional laboratory testing is recommended)

(2) implementation of practical skills and knowledge aiming to prevent dissemination and multiplication of food borne pathogens (i.e. *Salmonella spp*.) on/in edible tissues.

CONTROL MEASURES

1. Control measures in wild boar population

Control of zoonotic agents in wild animal population could be attempted through vaccination campaigns, by keeping animals in isolated geographical areas or fenced hunting grounds to prevent contact with domestic animals and wild animal additional feed ban (Zanella et al. 2008).

Wild animal population size regulation (managing overabundance) contributes in infectious disease control by decreasing transmission rate. When field evisceration is performed, offals and other remains should be removed from hunting ground to disrupt transmission chain of food born pathogens, especially tuberculosis and trichinellosis (Gortazar et al., 2006; Petrović et al., 2012a). Wild boars are at a greater risk of contracting tuberculosis compared to deer, as a result of a hunting management policy (Vincente et al., 2006). The main risk factor is artificial additional nutrition of wild pigs to preserve the high density of population, which enhances hunting opportunities but also facilitates the transmission of infectious diseases (Vincent et al., 2007). Scavenging habits of wild boars and eating offals of deer that have succumbed to the disease or were killed sanitary increases the risk for tuberculosis, salmonellosis and trichinellosis (Gortazer et al., 2008).

According to Petrović et al. (2014), life cycle of *T. spiralis* in Vojvodina region includes circulation from domestic pigs to wild boars and *vice versa,* which is associated with characteristic behavior of this animal species. Wild boars are very tolerant to the presence of humans, often commingling with domestic pigs on common pastures, and have access to laystall and food waste. Improper disposal of pig carcasses and offals in the field represents the greatest risk factor for trichinellosis maintenance and spread in pig population.

In actual scientific literature, there are no published recommendations (at least to our knowledge) on *Salmonella spp*. control measures in live wild boars. Therefore, it is important to control the hygiene during hunting, evisceration, bleeding and cleaning of carcasses, cooling and transportation, coupled with sampling of processed carcasses and testing for the presence of Salmonella *spp*.

2. Control measures during hunting and shooting

Good hunting practice should be implemented as observation prior shooting "*ante mortem*" inspection, that includes observation of the behavior of the living animal, constitution and presence of suspect alterations that may be indicative for infective or parasitic disease (coughing, diarrhea, abnormal movements or position of limbs, rough hair etc.). The stress that animal suffers

prior killing may be acute or protracted, and is largely dependent to a hunting method. Prolonged stress and the number, position and caliber of the entry wounds have a great influence on the microbiological condition of the carcass, causing invasion of bacteria from digestive tract or insertion of bacteria and dirt with the bullet itself. During hunting, a risk of animal´s muscle glycogen storage depletion is increased, resulting in an increase in pH levels in the meat, which negatively affects the quality and microbiological status of meat (Wiklund and Smulders, 2011). Hunter's skill and experience, as well as the used bullet and its caliber greatly influence the game meat quality (Atanassova et al., 2008).

3. Official game meat inspection

Official meat inspection, immediately after shooting, is carried out by an authorized veterinarian or veterinary inspector in a depot for cooling and temporary storage of game carcasses (Urošević et al., 2012a).

Hunting manager provides information about hunting process and all relevant details to veterinary official. These include data of carcass marking, information on abnormalities in behavior or health status of animals prior shooting, and potential alterations in organs and carcass after evisceration (Urosevic et al., 2012b).

The evisceration in large game has to take place as soon as possible after killing, especially the stomach and bowels. All the eviscerated organs must be kept until the veterinary inspection is completed. Skinning and cutting up game is not allowed in field conditions.

Veterinary inspection of meat and viscera of hunted wild boars must identify:

- any abnormalities or alterations that may indicate that the meat presents a health risk for humans or other animals;
- presence of any sign of disease or condition that makes meat unsuitable or forbidden to be put on the market;
- any autolytic alterations due to delayed evisceration or other reasons;
- any alterations related to consumption of toxic substances;

Meat and viscera of hunted animals are visually inspected, and palpation or incision is performed if needed. Considering that several hot spots of endemic bovine tuberculosis are registered in Vojvodina region, palpation and incision of hepatic lymph nodes should be performed. If any suspicion remains, laboratory testing is recommended (Urosevic et al., 2014).

The diaphragms of all killed wild boars must be examined by pepsin digestion method for estimating the presence of *Trichinella spp* larvae.

The wild boar meat deriving from animal infected with salmonellosis, tuberculosis or trichinellosis is declared unsuitable for human consumption. Standard game meat inspection is effective for identifying and elimination of altered and visually contaminated carcasses, but ineffective in detection of subclinical salmonella carriers (Petrovic et al., 2013b).

4. Control measures during carcass processing

After killing and evisceration, the carcass and matching viscera have to be transported as soon as possible to a game collection facility for inspection, marking and assessment. Quick cooling down is essential for food safety and meat quality, thus, if the environmental temperature is high it is necessary to chill the game meat within a reasonable period of time and no later than 10 hours after killing. This should result in a temperature throughout the meat of not more than +7 °C.

To the purpose of veterinary inspection of meat, Paulsen et al. (2006) categorized the carcasses according to contamination of body cavities and other alterations (such as emaciation, fractures, discoloration) into four categories, with two best categories (i.e., not requiring veterinary intervention) characterized by TVC <6 log cfu/cm² (colony forming units) and *E. coli* counts <1 log cfu/cm². It is important to emphasize, that the time passed from killing to cooling should be as short as possible, because high ambiental temperature is strongly associated with microbial growth and meat contamination, and a period of maximum 12 hours of "pre cooling" is suggested (Paulsen and Winkelmayer, 2004). According to Regulation (EC) 853/2004, the recommended temperature for chilled game meat should be +7 °C and for the offals maximum of +3 °C. For skin removal and subsequent carcass processing, in terms of cross-contamination, similar procedures apply as for cattle (Paulsen et al., 2011).

CONCLUSION

The research on presence of food borne pathogens in wild boar meat is still scarce, and lack sufficient data. The presence of alimentary pathogens in wild pigs' meat is relatively unexplored research area. While the *Trichinella spp*. life cycle is well described and there are relevant data about epidemiology and natural reservoirs of the parasite in this part of Europe, little is known about tuberculosis and salmonellosis prevalence in wild boar population. Thus, implementation of control program, with strictly defined control measures cannot

be overemphasized, and should include veterinary officials as well as hunters and others involved in game meat chain.

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SEARCHING FOR SOLUTIONS IN AQUACULTURE: AQUAPONICS

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Abstract

Aquaponic production combines intensive production with waste recycling and water conservation. Aquaponic join recirculating aquaculture with hydroponics to use nutrient waste from aquaculture as an input to plant growth. Traditional aquaculture systems treat or dispose nutrient-rich wastewater. In aquaponics, the waste products from the fish are converted by a bio-filter into soluble nutrients which are absorbed by the plants, and allow "clean" water to be returned back to the fish. Thus, it produces valuable fish protein with a minimal pollution of fresh water resources, while at the same time producing horticultural crops. Fish in aquaponic production systems can be raised in ponds, tanks, or other containers. Plants are grown separately in hydroponic tanks, submerged in water but suspended in gravel, sand, perlite, or porous plastic films, as well as on floating rafts. Systems vary greatly in design and construction, but most perform the following key functions: finfish and plant production, removal of suspended solids, and bacterial nitrification. This review discusses applications, effects and perspective of aquaponics.

Key Words: fish, aquaponics, aquaculture

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U POTRAZI ZA NAJBOLJIM REŠENJEM: AKVAPONIKA

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Kratak sadržaj

Akvaponika kombinuje intenzivnu proizvodnju sa reciklažom otpadnih materija i očuvanjem vode, i povezuje akvakulturu sa hidroponikom u cilju korišćenja hranljivih materija iz akvakulture za rast biljaka. U tradicionalnoj akvakulturi, otpadna voda bogata hranljivim materijama se ne koristi. U akvaponici, produkti nastali prilikom gajenja riba se pomoću biofiltera konvertuju u rastvorljive materije koje biljke apsorbuju, a "čista" voda se vraća ponovo u proizvodnju. Na ovaj način se omogućava proizvodnja visoko vrednih animalnih proteina, uz minimalno zagađenje vode, dok se istovremeno dobijaju proizvodi hortikulture. Postoji veliki broj sistema za gajenje, različitog dizajna i konstrukcije, ali se svi baziraju na zadovoljavanju ključnih funkcija: proizvodnji riba i biljaka, uklanjanju rastvorenih materija i bakterijskoj nitrifikaciji. U radu su prikazani primena, efekti i perspektive akvaponike.

Ključne reči: Akvaponika, akvakultura, ribe

INTRODUCTION

Aquaponics has been considered as a sustainable agriculture system that amalgamates aquaculture and hydroponics in an enclosed symbiotic environment (Nelson, 2008). The word 'aquaponics' is derived from a combination of 'aquaculture' and 'hydroponics', and refers to the integration of hydroponic plant/vegetable production with aquaculture. It is a bio-integrated system linking recirculating aquaculture with hydroponic production of plants such as vegetables, culinary or medicinal herbs. Aquaponics may provide an effective and efficient means to provide both animal protein (fish) and mineral and vitamin sources (fresh vegetables) to populations where water/and or fertilizer resources are limited with a minimum of environmental pollution.

The basic principals of aquaponics is that fish are fed "waste plant and animal material", which they convert into protein. The waste material from the

fish is then used by plants as the nutrient source, and the water is then recirculated back to the fish tank. An essential component of this is a biofilter (between the fish and the plants) which essentially comprises bacteria which converts the waste products from the fish into soluble nutrients for the plants. An absolutely critical component of this is the conversion of urea (excreted by the fish) into nitrite, and then nitrate because high levels of urea in the water are toxic to fish. The solid waste (fish faeces and unconsumed food) is usually filtered off and converted into soluble nutrients in a separate bypass.

Design

Aquaponic systems are usually designed as an enclosed recirculating system, but a few systems can be open, depending upon environmental factors. Fish or other aquatic organisms are reared in tanks and excrete nutrient-rich waste or effluents into the water. Metabolic byproducts excreted by fish, unionized ammonia NH3-N, ionized ammonia NH4+-N, or combined equal Total Ammonia Nitrogen (TAN) are oxidized and broken down into nitrite (NO2 --N) by nitrifying bacteria of the genera Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus, and Nitrosovibrio. Genera that oxidize nitrite to nitrate (NO3--N) include Nitrobacter, Nitrococcus, Nitrospira, and Nitrospina (Timmons and Ebeling, 2007). These nitrifying bacteria are also known to be light sensitive (Yoshioka and Saijo, 1984). Mineralization also occurs, releasing essential inorganic nutrients into the water for plant uptake (Timmons and Ebeling 2007). These dissolved nutrients accumulate and reach concentrations equal to hydroponic nutrient solutions (Timmons and Ebeling 2007). The water is continuously circulated to hydroponically grown crops that absorb non-toxic nutrients from the water to fulfill growth requirements. The water is then circulated back to the rearing tanks where the process starts again.

There are multiple aquaponic system designs that have been analyzed and utilized for crop production. Depending upon the system scale there are five main components to an aquaponic system: rearing tank, solids removal, biofilter, hydroponic subsystem, and sump (Rakocy and Hargreaves, 1993). Some systems are able to eliminate one or two of the components, and scale and primary production focus are the key factors determining the system design. Some aquaponic systems are able to efficiently operate with the use of hydroponic subsystems acting as a biofilter. This is possible with the aid of media such as hydroton, pea gravel, and expanded shale (Rakocy 1984; McMurtry et al. 1990). Floating raft hydroponics also known as DWC, which utilize polystyrene sheets and net pots for plant support, may also provide adequate biofiltration provided the hydroponic subsystem is large enough (Rakocy 1995). When utilizing media within hydroponic subsystems, care must be taken to prevent an overload of suspended solids; therefore, media filled subsystems are not

ideal for commercial scale production (Timmons and Ebeling 2007).

One of the most important components of an aquaponic system is the hydroponic subsystem: media filled, NFT, and DWC (Lennard and Leonard 2006). A media filled hydroponic subsystem contains a grow bed filled with a soilless medium for plant support.

Popular soilless media include hydroton (expanded clay pebbles), gravel, sand, and perlite. The NFT system consists of troughs that expose suspended plant roots (net cup) to a thin film of water. DWC is similar to the media filled subsystem but instead of using media in the hydroponic bed, a floating raft (polystyrene sheets and net cup) supports the plants.

Currently there are two main irrigation methods for hydroponic subsystems, flood and drain or continuous flow. Flood and drain system uses a siphon to periodically drain water when it reaches a specified level. A continuous flow system allows water to constantly run throughout the system (Rakocy et al. 2006). Lennard and Leonard (2006) found that hydroponic subsystem design and water flow have a significant effect on Green oak lettuce (Lactuca sativa) yield where media>DWC>NFT; NFT systems were 20% less efficient in nitrate removal. Lastly, producers should realize that differing aquaponic or hydroponic methods (system designs) do not alter the genotypic characteristics of plants. Production will not surpass genetic limitations regardless of growing techniques, and plants will reach peak production when optimum requirements are met (nutrient assimilation, light, temperature, etc.).

Fish

There is no real limitation on the types of fish which can be used. Today it is common to grow Nile tilapia (*Oreochromis niloticus*), channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), and various carp species (*Cyprinus sp*.), in aquaponic systems. Tilapia appear to be one of the most popular species of fish reared in aquaponic systems, because the warm temperatures for optimal growth of tilapia are also needed for the growth of plants (Rakocy and McGinty, 1989).

Other species of fish that are reared in aquaponic systems in include largemouth bass (*Micropterus salmoides*), sturgeon (*Acipenser spp*.), baramundi (*Lates calcarifer*), sunfish (Family *Centrarchidae*), bream (*Abramis brama*), pacu (Family Characidae), red claw lobster or crayfish, and ornamental fish such as angelfish (*Pterophyllum scalare*), guppies (*Poecilia reticulata*), tetras (Family Chiracidae), gouramis (Family Belontiidae), swordfish (Family Xiphiidae), mollies (Family Poeciliidae).

Plants

Common plants that do well in aquaponic systems include various lettuce

(Lactuca spp.), tomato (Solanum spp.), spinach, and herb species including sweet basil (*Ocimum basilicum*), mint, watercress, chives, and most common house plants. Species of plants that have higher nutritional demands and will do well only in heavily stocked, well established aquaponic systems include tomatoes, peppers, cucumbers, beans, peas, and squash, among others (Rakocy, 1999).

Many of the fruit vegetables (tomato, pepper, cucumber, melon, etc) appear to require higher levels of nutrients in hydroponics, than the leafy vegetables. Nutrient wastes from tanks are used to fertilize production beds via the water. The roots of plants and associated rhizosphere bacteria remove nutrients from the water. These nutrients, generated from the feces of fish, algae and decomposing feed, are contaminants that could otherwise increase to toxic levels in the tanks. Instead they act as liquid fertilizer for hydroponically grown plants. In turn, the hydroponic beds function as biofilters, and the water can be recirculated to the tanks. Bacteria in the gravel and associated with the roots of the plants have a critical role to play in the cycling of nutrients; without these organisms, the system would stop functioning (Diver, 2006).

Aquaponic plants are subject to many of the same pests and diseases that affect field crops, although they seem to be less susceptible to attack from soilborne pests and diseases. Because plants may absorb and concentrate therapeutic agents used to treat parasites and infectious diseases of fish, these products cannot be used in aquaponic systems.Even the common practice of adding salt to treat parasitic diseases of fish or to reduce nitrate toxicity would be deadly to plants. Instead, non-chemical methods are used, ie., biological control (resistant cultivars, predators, antagonistic organisms), barriers, traps, manipulation of he environment, etc.). It also seems that plants in aquaponic systems may be more resistant to diseases that affect those in hydroponic systems. This resistance may be due to the presence of some organic matter in the water, creating a stable, ecologically balanced growing environment with a wide diversity of microorganisms, some of which are antagonistic to pathogens that affect the roots of plants (Rakocy, 1999).

CONCLUSIONS

Aquaponic system is advantageous compared to other agriculture production systems, and has become very popular today (Rakocy et al. 2006). Since aquaponic systems are designed as enclosed recirculating systems, their agricultural waste and environmental footprints decrease, compared to conventional agriculture practices. Furthermore, utilization of plants as a secondary

crop reduces the pollution load (waste concentration) through nutrient uptake and assimilation (Timmons and Ebeling 2007). Nitrate accumulation has been shown to be reduced by 97% within aquaponic systems compared to regular recirculating aquaculture systems (RAS) (Lennard 2006). Since water within systems is recirculated, the quantity of water needed to run the system is minute compared to most fish and crop production systems. On average, 98% of the water in aquaponic systems is recycled for the duration of operation (Al-Hafedh et al. 2008). The periodical input of water is only necessary when too much water has evaporated from the system. Aquaponic systems decrease the amount of space needed to produce two crops at once. This allows plants and fish to be raised together within a relatively small environment.

Aquaponics can range from an in-home counter top system to large scale commercial systems. Additionally aquaponics on average utilizes less than 1% of land compared to conventional agriculture systems. Along with space, aquaponic systems use fewer resources than average crop and fish production systems due to symbiotic relationships (Treadwell et al. 2010). For example, aquaponics utilizes 90-99% less water than conventional agriculture systems. Also, carbon dioxide (CO_2) from fish rearing tanks can also be used to increase crop production within an indoor facility (Timmons and Ebeling, 2007). Furthermore, aquaponic systems can be deployed in various environments allowing for year round crop production, and potentially a closer farmer-to-consumer interaction. Lastly, successful aquaponic systems utilize secondary crops that are of economic importance or beneficial to the aquatic organisms being produced (Timmons and Ebeling, 2007).

As with all food production systems, there are a few disadvantages with aquaponic systems. First, the ratio of hydroponic growing area compared to fish rearing surface area is relatively large. Ratios have been used ranging from 1:1 to 10:1, which are dependent upon the scale of the system, primary species of focus, and space. Another disadvantage includes the labor involved with plant management. The majority of aquaculturists do not have horticulture experience or knowledge, so additional personnel is often needed. Furthermore, due to the close relationship between fish and plants within an aquaponic system, poor management practices can easily affect the sensitive system. Pesticides cannot be utilized within systems and thus, biological control or natural methods must be used to eliminate plant pests (Timmons and Ebeling 2007). When entering into a competitive market, aquaponic producers should evaluate competitors and their species of production. It has been stated that hydroponics can produce heads of lettuce cheaper than what aquaponic systems can produce (Ako and Baker 2009). Lastly, materials utilized for aqua-

ponic production (hydroton, fish feed, etc) are not considered sustainable. For example, hydroton (clay) is mined from the earth, and fish feed may come from wild caught fisheries or commodity crops. These materials utilize nonrenewable resources for production and may also contribute to environmental pollutants.

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DEVELOPMENT OF A MODEL FOR SURVEILLANCE AND CONTROL OF FLAVIVIRUSES IN HUMAN POPULATION

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Abstract

The aim of this study is development of a model for monitoring and control of vector-transmitted diseases, which manifest increasing tendency during the last few decades. The main infection reservoirs are domestic and wild animals as well as various birds, and the disease is transmitted to humans indirectly - by vectors. Recently detected epidemics of Dengue virus, Chikungunya and West Nile virus in countries where these diseases are not common strongly indicate the expansion of infections transmitted by mosquitoes and other vectors, and are the consequence of climatic changes, international trade and travelling. Currently, there are neither vaccines nor specific antiviral therapy for these infections, while the efforts put on vector control did not halt the rapid increase and global spread of the disease. Serological studies and molecular investigation on humans, mosquitoes, horses and birds have suggested the activity of flaviviruses in Serbia. The obtained information on flavivirus infections in our region are of use in modelling the control and vector monitoring and the prevention of these infections in humans.

Keywords: flavivirus infections, diagnosis, prevention

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IZRADA PREDLOGA MODELA ZA PRAĆENJE I KONTROLU FLAVIVIRUSA U HUMANOJ POPULACIJI

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Kratak sadržaj

Cilj rada je izrada predloga modela za praćenje i kontrolu transmisivnih vektorskih bolesti koje su poslednjih nekoliko decenija u porastu. Rezervoari su divlje i domaće životinje i raznovrsne ptice, a na čoveka se prenose posredno-vektorima. Nedavno uočene epidemije Denga virusom, Čikungunja i West Nile virusom u zemljama u kojima ove bolesti nisu uobičajene, potvrda su sve većeg širenja infekcija komarcima i drugim vektorima, a posledica su klimatskih promena u okruženju, rasprostranjenom trgovinom i putovanjima. Trenutno ne postoje vakcine niti specifična antivirusna terapija za ove infekcije, a napori da se konrolišu vektori nisu sprečili brzi porast i globalno širenje zaraze. Serološke studije i molekularna ispitivanja sprovedena kod ljudi, komaraca, konja i ptica ukazale su na aktivnost flavivirusa u Srbiji. Dobijene informacije o kretanju flavivirusnih infekcija u našoj sredini od koristi su u modeliranju kontrole i praćenju vektora i preveniranju infekcija kod čoveka.

Ključne reči: flavivirusne infekcije, dijagnoza, prevencija

INTRODUCTION

The aim of this paper is development of a model proposed for monitoring and control of vector-transmitted diseases, which demonstrated increased incidence in the last decades. The main infection reservoirs are domestic and wild animals as well as various birds, and the disease is transmitted to humans indirectly - by vectors. Recently detected epidemics of Dengue virus, Chikungunya and West Nile virus in countries where these diseases were not present before, are the signal of a potential threat of the spread of infections transmitted by mosquitoes and other vectors, and are in close connection with climatic changes in the area, international trade and travelling.

In the last decades, mosquito-transmitted flavivirus infections have gai-

ned substantial public health importance because of wide geographical distribution, epidemic emergence outside of the endemic areas and difficult forms of diseases that may cause. *Flaviviridae* family encompasses 53 virus species, among which Dengue and West Nile virus, transmitted by mosquitoes, and Tick-borne encephalitis virus (TBEV), transmitted by ticks, are of particular importance.

Dengue (DEN) is a viral infection caused by an ARBO virus from *Flaviviridae* family. Transmission to humans occurs by a mosquito from the genus *Aedes,* mostly *Aedes aegypti*, and less frequently by *Aedes albopictus.* Dengue has 4 serotypes: 1 - 4, which produce cross-immunity. It causes an illness that ranges from mild to very severe that is manifested by a shock syndrome and hemorrhagic fever with lethal outcome. Currently, there are neither vaccines nor specific antiviral therapy. Dengue is endemic in at least 100 countries all over the world (Asia, Africa, American continent, the Caribbean, Pacific). According to the World Health Organization (WHO), 50-100 millions people in the world are infected by Dengue virus annually. (http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/) Efforts aimed at vector control did not prevent rapid increase of this dangerous disease and its spread throughout the world. Risk factors for the increase of Dengue infection incidence are migration of population from undeveloped endemic countries into developed countries, increase in travelling and tourists' number, urbanization that favours the spread of urban mosquitoes such as *Aedes albopictus*. A danger of Dengue fever is now evident even in Europe, since first autochthonous cases were registered in France and Croatia in 2010 (Gjenero et al., 2011; La Ruche et al., 2010). Serological and entomological investigation on Pelješac peninsula in Croatia, where first autochthonous cases of Dengue were registered, proved the presence of specific Dengue antibodies in healthy residents and the domination of *Aeds albopictus* species in analyzed mosquitoes (Gjenero et al., 2011).

West Nile virus fever is a zoonosis caused by an ARBO virus from *Flaviviridae* family and Japanese encephalitis serogroup. Virus reservoirs in natural environment include hundreds of bird species belonging to 20 families in the order of *Passeriformes,* while vectors involve about 60 mosquito species, mostly from the genus *Culex*. Other vertebrates may be infected with this virus; however, their role in the transmission has not yet been elucidated (Hrnjakovic-Cvetkovic et al., 2014). Under favourable environmental conditions, viral turnaround cycle is intensified and may lead to infection in horses and humans, which are accidental hosts unsuitable for infecting mosquitoes. In most cases, the infection is asymptomatic or proceeds as a mild condition, but

some 1% of infected individuals develop a neurological disease - meningitis, encephalitis and myelitis that rapidly progress to severe conditions with fatal outcomes, especially in the elderly (Mostashari et al., 2001). Sporadic cases and epidemics were recorded in Africa, Middle East, Europe and Asia. Since 1999, when first human cases were recorded in New York, infection has rapidly spread across USA and has become endemic in North America. This spread has instigated intensive research in the area of diagnostics and production of human vaccine. Vaccines for horses have already been developed, whereas those for human use have not yet been registered. Serological studies and molecular investigations in humans, mosquitoes, horses and birds suggested the activity of the virus in Serbia (Petric et al., 2012; Lupulovic et al., 2011; Petrovic et al., 2012). Since 2012, when first epidemics was registered in Serbia (Popović et al., 2012), human cases have been reported every year in the period from August to October. Until October 20, 2014, the Institute of Public Health of Serbia "Slobodan Jovanović Batut" recorded 56 laboratory-confirmed cases of human neuroinvasive diseases caused by West Nile Virus, among which 9 cases of elderly patients with chronic conditions resulted in a lethal outcome (Institute of Public Health of Serbia, 2014).

In the group of mammalian tick viruses, tick-borne meningo-encephalitis virus (TBEV) has been ranked into the family Flaviviridae, genus Flavivirus, with three subtypes: Far East, Siberian and European subtype. Endemic focal points of this viral infection can be found from Western Europe to China and Japan. Those in Europe are primarily located in Austria, Slovenia, Switzerland, *Czech Republic* and some countries of ex-Soviet Union. In the last 30 years in Europe, new focal points and a 400% increase in new cases in endemic areas were registered (ECDC, 2014). Increase in incidence in *Czech Republic* was explained exclusively by a change in climatic conditions (Daniel et al., 2010). Principal vector species in Europe is tick *Ixodes ricinus.* In ticks, virus is transmitted by transovarial route, and all stages from larva, via pupa to adult, may be infected and transmit the infection to vertebrates and humans by bite while feeding. The reservoirs include small rodents, mostly members of the species *Apodemus,* but also some wild and domestic animals, on which adult ticks feed. The disease progress may manifest biphasic course. The European subtype causes milder forms of disease and biphasic course can be noticed in 20-30% of infected population (ECDC, 2014). After the first phase of illness, characterized by unspecific symptoms - fever, fatigue, malaise, muscle pain, headache – the first symptoms of meningitis, meningoencephalitis, poliomyelitis, and polyradiculoneuropathy with Guillain- Barré like paralysis may arise. More difficult forms of the disease frequently affect elderly patients. First

serological studies in Serbia pointed out that the activity of the virus in the territory of Vojvodina, where the seroprevalence of IgG antibody is 7.9% in the residents of South Backa region (Vojvodina, Serbia), while no TBEV-positive serum samples were detected in the region of Nis (Hrnjakovic-Cvjetkovic et al., 2014).

Usutu virus is an ARBO virus from the family Flaviviridae, genus *Flavivirus,* Japanese encephalitis serogroup. It is maintained (like the West Nile virus) throughout the transmission cycle between wild birds and ornithophilic mosquitoes, mostly from the genus *Culex.* While in the African birds infection is asymptomatic, the virus is highly virulent for European birds, and causes lethal encephalitis, necrotic hepatitis and degenerative changes in heart and neural tissue (Bakonyi et al., 2007). USUTU virus infection has been established in many bird species in Italy, Austria, Germany, Switzerland, Spain, Hungary, *Czech Republic* and Poland (Arbeitskreis, 2014). In humans, the virus can cause fever and rash. In immunocompromised patients, pronounced neurological manifestations and a fulminant hepatitis may arise (Cavrini et al., 2009). Medical importance of this ARBO virus in immunocompetent individuals remains to be evaluated. Serological investigation done by ELISA IgG test in humans showed the presence of anti-USUTU virus antibodies in 4.5% samples out of 88 healthy subjects of South Backa region, Vojvodina, Serbia (Hrnjakovic-Cvjetkovic et al., 2014).

DIAGNOSTIC METHODS

A clinician's doubt whether there is a vector-transmitted infection, requires laboratory confirmation. The laboratory methods encompass the direct ones determining the virus isolation in cell culture or amplification of the virus genome, and indirect ones, that is, serological methods that detect the presence of specific antibodies. Flavivirus isolation may be performed from blood or cerebrospinal fluid on different cell lines, but only at the early stage of infection, when a patient is in viraemic phase characterized by high virus titre in the blood. In West Nile virus infection, viraemia is typically present during the first four days of illness (Dauphin and Zientara, 2007). Real-time reverse transcription-polymerase chain reaction (RT-qPCR) is a method of choice for the determination of flaviviruses in human plasma, serum and cerebrospinal fluid.

Serological diagnosis for all flavivirus infections that produce encephalitis in humans has a similar approach. In the last few years, specific ELISA tests have been developed for the detection of IgM and IgG antibodies against certain flaviviruses. IgM antibodies against West Nile Virus emerge 2-8 days

after the onset of illness, similarly to USUTU virus infection. However, a weak point of the ELISA tests is that they cannot completely distinguish between the WNV antibodies and antibodies against other flaviviruses, especially those of the same antigenic complex. Antibodies can persist in the serum for several months after infection. IgM antibodies in WNV infection may be present in serum for as long as a year after the infection (Roehrig et al., 2003). Establishment of an accurate serological diagnosis is difficult, especially in the areas where more than one flaviviruses are circulating, so cross-reactions are possible, as is the case with WNV and TBEV in several European countries. The problem of cross-reaction exists between anti-WNV and anti-USUTU virus antibodies, where expected cross-reaction is higher in IgG than in IgM antibodies. For that reason, it is essential to implement diagnosis of USUTU viral infection and tests that are not yet commercially available for the determination of specific IgM antibodies. By the tests available so far (for IgG antibodies only, for now), both acute and convalescent sera should be analyzed for the presence of USUTU virus, thus monitoring seroconversion of IgG antibodies. Cross-reactions could be resolved by a parallel testing on various flaviviruses using plaque reduction neutralization tests according to the specially defined protocols (Beaty et al., 1995). These tests are more specific as compared to ELISA tests, but they can be performed only in Biosafety level 3 facilities (BSL III). The fact that USUTU virus infection in humans can cause severe neurological syndromes imposes the strong and urgent need for new, accessible and rapid molecular detection methods.

Serological tests are essential for the determination of infection in cases when the viraemic phase is completed. Highly specific tests are required, particularly in countries where the circulation of both USUTU virus and WNV is present (Austria, Belarus, Bulgaria, Czech Republic, Croatia, France, Hungary, Italy, Moldova, Portugal, Romania, Russia, Serbia, Slovakia, Spain and Ukraine). New methods are designed for the identification and differentiation of USUTU virus from other ARBO viruses, particularly members of the Japanese encephalitis serogroup circulating in Europe. Virus monitoring by molecular methods is better with regard to the specificity since there are no cross reactions, but is limited by short viraemic phase. Serology enables the detection of antibodies, which last longer, nonetheless with lower specificity for now, using the inhibition of hem-agglutination or ELISA tests. Each positive serum should be confirmed by plaque reduction neutralization test, a methods that is complex, expansive, time-consuming and accessible only to Biosafety level 3 facilities.

CONCLUSION

Ever more frequent reports on the circulation of mosquitoes and ticks transmitting the diseases characteristic for tropical regions (WNV, USUTU, Dengue, Chikungunya etc.) in the European Union advocate the necessity of a more intense and careful preparedness and control of these diseases by new diagnostic procedures and more specific serological tests even in our country. The surveillance program of flaviviruses in our country exists for WNV and encompasses human, veterinarian and entomological monitoring with the aim of early detection of infections in human population, applying serological tests and detection of a viral genome in blood, cerebrospinal fluid in any suspected case of acute meningoencephalitis. The surveillance in animals has been done by passive and active surveillance of horses, chickens and non-migrating wild birds, and entomological surveillance by weekly and monthly trapping of mosquitoes depending on the determined activity in birds, humans and horses, and testing on specific antibody or virus presence. The obtained information about the fluctuations of flavivirus infections in our environment are valuable for modelling the control and surveillance of vectors. It is expected that informative campaigns will lead to the increase in personal protection, and adequate screening tests would prevent infections in the blood, tissue and organ donors.

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ONE HEALTH – CONCEPT FOR TODAY AND TOMORROW

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Abstract

"One Health" has been defined as "the collaborative effort of multiple disciplines — working locally, nationally, and globally — to attain optimal health for people, animals and the environment". *One Health* is a new initiative, but with the concept that extends back to ancient times. *One Health* is an interdisciplinary concept for complex health challenges from a holistic integrated perspective, more then a divided perspective based on different disciplines. There is no isolation, wild animals, domestic animals, pathogens and diseases do not know of the political borders. The aim of the *One Health* initiative is to form unified solutions applicable for the improvement of health of humans, animals and the environment. A workshop was organized for the representatives of all structures and levels of medical and veterinary services of Serbia. During four tasks, among joint working groups, the most important structure of *One Health* was proposed, introducing possible concept in Serbia.

Key Words: One health, concept, infectious diseases

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JEDINSTVENO ZDRAVLJE – KONCEPT SADAŠNJOSTI I BUDUĆNOSTI

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Kratak sadržaj

Definiciju i osnovnu suštinu "jedinstvenog zdravlja" predstavljaju "zajednički napori više različitih disciplina, koje rade na lokalnom, nacionalnom i globalnom nivou, da bi se postiglo optimalno zdravlje ljudi, životinja i ekosistema, odnosno životne sredine". Jedinstveno zdravlje je nova inicijativa, ali koncept ovakvog razmišljanja datira odavno. Jedinstveno zdravlje je interdisciplinaran koncept za složene promene u javnom zdravlju, sa holističkim integralnim pristupom, koji se bazira na različitim naučno-stručnim disciplinama. Kada je u pitanju javno zdravlje, ne postoji mogućnost razdvajanja populacije, divljih i domaćih životinja, uzročnika (patogena) i bolesti, jer oni ne poznaju administrativne političke granice i ograničenja. Cilj inicijative Jedinstvenog zdravlja je da se formiraju jednoobrazna rešenja koja bi se koristila za unapređenje zdravlja ljudi, životinja i životne sredine. Ministarstvo poljoprivrede SAD (USDA) organizovalo je radionicu o Jedinstvenom zdravlju za predstavnike svih struktura i nivoa zdravstvenih i veterinarskih službi u Srbiji. Tokom rada, zajedničke radne grupe, sačinjene od predstavnika pomenutih službi, su kroz četiri zadatka predložile najvažniju strukturu Jedinstvenog zdravljasa ciljem uvođenja mogućeg koncepta "jedinstvenog zdravlja"u Srbiji. Rešavanjem pitanja organizacije i rukovodstva, službene komunikacije, potrebnih izvora finansiranja i merenja uticaja predstvljena je najvažnija struktura "jedinstvenog zdravlja".

Klučne reči: "jedinstveno zdravlje", koncept, infektivna oboljenja

INTRODUCTION

"*One Health*" has been defined as "The collaborative effort of multiple dis-

ciplines — working locally, nationally, and globally — to attain optimal health for people, animals and the environment". *One Health* is a new initiative, yet with the concept that extends back to ancient times. *One health* initiatives from the past are many. Various emerging health issues are linked to increasing contact between humans and animals, intensification and integration of food production, and the expansion of international travel (Anonymous, 1999). As the number of new infectious diseases emerged in the 20th century, the scientists began to recognize the challenges that societies face regarding these threats that largely come from animals. Of the 1,415 microbes that are known to infect humans, 61 percent come from animals (Taylor et al, 2001).

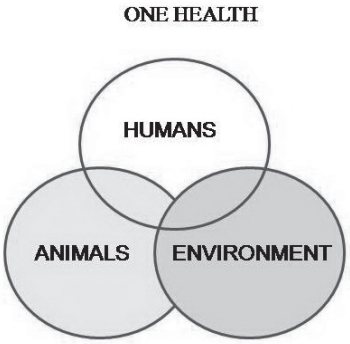
The 1999 West Nile virus outbreak in New York City highlighted the links between human and animal health. In this outbreak, wild crows began dying about a month before the first human cases were identified. The simultaneous outbreaks were not recognized as caused by the same entity until Dr. Tracey McNamara, an astute veterinarian at the Bronx Zoo, tied them together when her exotic birds began getting sick (Drexler, 2002). After recognizing that the outbreaks were caused by West Nile virus, a new entity in the Western Hemisphere, the Centre of Disease Control (CDC), established the National Centre for Zoonotic, Vector-Borne, and Enteric Diseases, now called the National Centre for Emerging and Zoonotic Infectious Diseases (Anonymous, 2011).

More recently, in 2003, there was a hemorrhagic fever outbreak, when a doctor of veterinary medicine said "Health of people, domestic or wild animals cannot be looked into separately. There is only one health and the solutions demand joint work of all of us on different levels". This veterinarian was William B. Karesh, who later on gave series of lectures, with his colleagues on a topic One World-*One Health* (Karesh and Cook, 2005).

The avian influenza (HPAI H5N1) epidemic that began in Hong Kong in 1997, forced the global community to recognize that animal health and human health are linked. The 1997 outbreak affected 18 people, killed 6, and provoked the culling of 1.5 million birds. The HPAI H5N1 virus resurfaced in isolated outbreaks between 1998 and 2003. The idea of „*One Health*" as it became known would assume urgent practical significance in late 2003 with the emergence of highly pathogenic avian influenza (Anonymous, 2010). The World Bank has published a list of steps that should be taken in order to implement the principles of *One Health*, based on pandemic zoonozes. Of course, non-infectious diseases, which affect local and national communities, should also be taken into consideration. First step includes a leader with an authority, champions in the country, mandatory legislation (reporting on diseases, joint decision making) and naming the priorities. Next step includes the frame for

collaboration and communication (memorandum of agreement, joint work groups, permanent teams and partial integration of the services). Step 3 includes incentives (joint budget, special grants) and joint systems (diagnostics and monitoring). The last step includes joint communication, integrative subjects (at Universities on human health, animal health and healthy ecosystem). In order to increase the possibility of success of „*One Health*"initiative, a long term approach, based on risk analysis, is essential. Moreover, a capacity building in all sectors involved in health issues is needed. In addition, understanding between the sectors, which will consequently improve the collaboration and coordination between them is definitely necessary. With the increase of knowledge, all of this could be possible.

One Health is an interdisciplinary concept for complex health challenges from a holistic integrated perspective, more then a divided perspective based on different disciplines. There is no isolation - wild animals, domestic animals, pathogens and diseases do not know of the political borders. The aim of the *One Health* initiative is to find unified solutions for the use of the health of people, animals and environment. New technologies such as internet (social networks) and mobile phones are valuable tools to successfully support this initiative in promoting and spreading information worldwide. Picture 1 shows a very simple schematic structure of *One Health* concept.



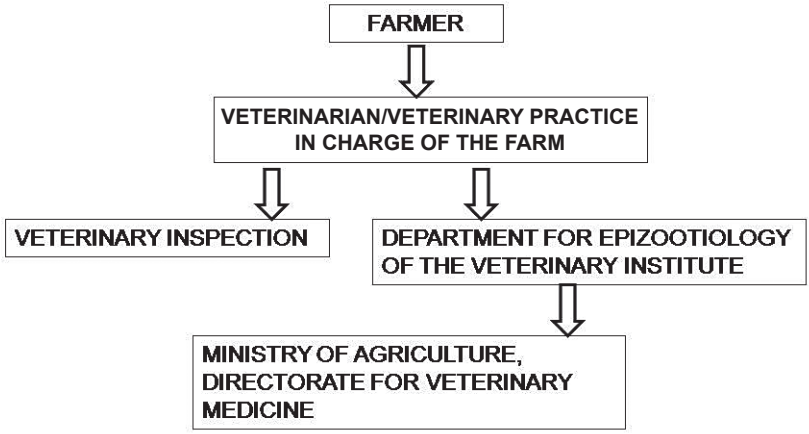
Picture 1 – Schematic view of *One Health* concept

The principals of *One Health* initiative demands purposeful and systematic channels of communication between the services for health protection of people, wild and domestic animals. Maybe the best definition of *One Health* concept, that was already mentioned in he beginning of this article, was given by the working group of American veterinary association in 2008, as a „joint

collaboration of more disciplines on local, national and global level, with the aim of reaching optimal health of people, animals and the environment". In 2010, European Union has published a report named „Estimation of the influence and result of global response to the crisis of avian influence". In this report, it is stated: "EU has already undertaken new initiatives under *One Health* initiative and will continue with actions like this in the future". This report highlights the need for the concept of *One Health* to be transferred into a practical politics and strategy, which will promote the collaboration between agencies and sectors. OIE also supports the *One Health* approach as common and comprehensive way of handling with the protection of public health and animal health on a global level. This collaboration should not be limited to an international level, but should be transferred as new and fundamental paradigm into all national levels.

The society has came to the moment when priorities and values have changed: Increased risks for public health; Increased expectations from the public; Increased expenses of the interventions; Increased expenses of technology; Decreased influence; Decreased institutional funds; Decreased human resources. The change of view towards *One Health* concept demands the existence of a vision, identification of a leadership with a relevant body in charge, thus the vision can evolve. To ensure the real upstart of *One Health* concept, the following parties should be involved: Government, Society, Educators and NGO's, which will have a mutual planning, leadership, financing, partaking and communication (Uhlenhopp, 2014).

Zoonotic diseases are caused by pathogens that can infect both animals and humans, resulting in disease outbreaks, including epidemics in humans and epizootics in animals. These diseases account for 70 percent of emerging infectious diseases. In the absence of timely disease control, zoonotic pathogens can cause pandemics, with potentially catastrophic impacts that are global in scale. Control of a zoonosis requires early and rapid actions. A typical episode may involve a pathogen that originates in wildlife, then passes to livestock, and is then transmitted from livestock to humans. The exposure to a pathogen in animals could be followed by symptoms in animals. Then, an increase of exposure becomes evident in humans, who subsequently could develop symptoms and may seek treatment. The risks of the appearance of food borne diseases differ in the opinion of the experts and in the opinion of the citizens. The experts see the risk in microorganisms, nutrients, contaminants of the environment, natural toxins, and chemicals in agriculture. The citizens on the other hand see the risk of food borne diseases in pesticides, new chemicals in food, additives, fat and cholesterol, and microorganisms (Trajković-Pavlović, 2014).



Picture 2 - Diagram of data collection in cases of zoonotic food borne diseases in animals for food production (Law of veterinary medicine, Official Gazette RS 91/2005)

MATERIAL AND METHODS

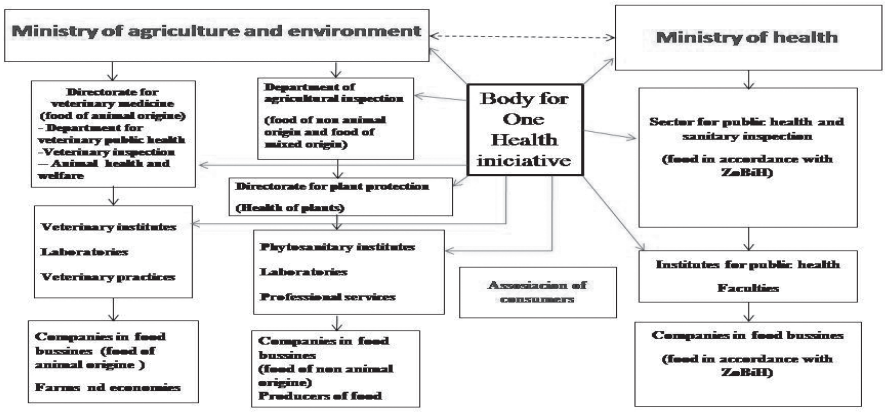
The workshop was organized by the US Department of Agriculture (USDA) with the support of American Embassy in March 2014, for the representatives of all structures and levels of medical and veterinary services of Serbia. During joint work of the groups, four tasks were given to the workgroups in order to form a concept of *One Health* initiative, which could be implemented into the system of supervision and surveillance of public health in Serbia. The aims of the workshop were to increase the awareness of global *One Health*, to develop understanding of the terminology of *One Health*, to participate in a dialog about the strategies of implementation, to identify the resources for the implementation of *One Health* (national and international) and to identify the possibilities and obstacles for implementation of *One health* in Serbia.

RESULTS AND DISCUSSION

One of the main results of the workshop was an increased dialog among the resources, sectors and individuals representing different disciplines and services. An improved support for the inter-resource community was gained. Suggested model for the implementation of *One Health* concept in Serbia was developed. Future possibilities for aiding Ministry of Agriculture were identified.

Currently, the supervision and control of zoonozes within our country is divided between the Ministry of Health (in charge of public health) and Mini-

stry of Agriculture, Veterinary Directorate (in charge of zoonoses in animals). These two Ministries have all the needed services, resources, people and information, but there is not enough dialogue and information exchange. Different actions and procedures have already been done by a *One Health* approach (in detection and control of zoonotic epidemics among humans or animals - Q fever, West Nile, Avian influenza), through the initiatives of separate services, or by the departments of the Ministries. Their obligations and duties in correlation to *One Health* approach should be within the system. There is still room for improvement of mutual collaboration, communication and synchronization of activities within the system. Thus, the proposal of workshop groups was to form a new national body (sector, service), formed of experts, which would be responsible for collecting information, sharing information, communicating and initiating collaboration between Ministries, inspections and other services among different Ministries. Picture 2 shows a draft of possible organization of *One Health* body with in the existing system.



Picture 3 - A proposal of possible organization of the Body for *One health* initiative within the existing system in Serbia

The organizational challenges, actions of the highest priority, the most important issues and responsible entities were identified. Way of communication through the *One Health* body was recognized, but also directly, between the institutions and services, depending on weather a question is a matter of internal or external. The ways of financing the resources of the Body for *One health* approach were proposed, with emphasis on who is in charge for this

component, who are the users, available funds, additional ways of financing and identifying the final point. In addition, the benefit for the society and public health was recognized, as it would lead to improved public health with less people being on sick leaves, less number of annual incidences, less time spent in hospitals, lower risk from zoonoses, that is, less overall expenses. This would lead to an improvement of capacities for disease control, diagnostics and reduction of their influence to the society.

CONCLUSION

The development of society in the Republic of Serbia is prepared for the implementation of *One Health* concept. There are resources (in view of experts and services) for launching the initial organization of *One Health* approach for Serbia, but there is a need for expanded.

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MOISTURE AND ACIDITY AS INDICATORS OF THE QUALITY OF HONEY ORIGINATING FROM VOJVODINA REGION

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Abstract

The color, aroma and flavor are major sensory characteristics of honey, which are mainly determined by the botanical origin of honey as well as by processing and storage conditions. Increased moisture content above the maximum permitted level could result in honey spoilage, which affects its sensory properties. The higher water-in-honey content, the greater possibility of yeast fermentation and thus the change of the flavor and color of honey. Fermentation process results in alcohol formation and, in the presence of oxygen, the alcohol will break down to acetic acid and water, which causes honey to have sour taste. Thus, moisture content of honey is a critical parameter for its quality as it affects the stability of honey and its resistance to microbial spoilage during storage. Physicochemical analysis of moisture content and acidity of honey play an important role in determining the overall characteristic of honey and final assessment of its quality. In this study, the investigation of aforementioned parameters resulted in positive quality assessment for 48 of 50 examined honey samples produced in 2013 in the territory of Vojvodina.

Keywords: honey, quality, moisture, acidity

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SADRŽAJ VODE I KISELOST KAO POKAZATELJI KVALITETA MEDA SA PODRUČJA VOJVODINE

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Kratak sadržaj

Boja, ukus i miris su važna senzorna svojstva meda i najviše zavise od biljnog porekla meda, a zatim i od uslova prerade i čuvanja. Ukoliko je procenat vode u medu veći od dozvoljenog, postoji mogućnost da dođe do vrenja i da se med pokvari, što utiče na njegova senzorna svojstva. Što je veći sadržaj vode u medu veća je verovatnoća da će kvasci fermentisati med i tako prouzrokovati gubitak ukusa. Fermentacijom nastaje alkohol koji se u prisustvu kiseonika razgradi na sirćetnu kiselinu i vodu što takvom medu daje kiseo ukus. Stoga se može reći da je sadržaj vode važan parametar kvaliteta meda obzirom da određuje stabilnost meda i otpornost na mikrobiološko kvarenje tokom čuvanja. Fizičko-hemijske analize parametara kao što su sadržaj vode i kiselost meda imaju značajnu ulogu u definisanju ukupnih svojstava meda i proceni kvaliteta meda. U ovom radu ispitivanjem navedenih parametara procenjen je odgovarajući kvalitet 48 od 50 ispitanih uzoraka meda iz 2013. godine sa područja Vojvodine.

Ključne reči: med, kvalitet, sadržaj vode, kiselost

INTRODUCTION

What is it that makes honey so special foodstuff? An answer to this question is highly complex, same as its extraordinary taste is. Honey is durable food, it never spoils and has virtually *unlimited shelf life. The production of honey involves a wide range of factors acting together in perfect harmony.*

Probably the most important property describing the chemical composition of honey is its diversity, that is, one could not find even two fully identical honey samples (Rogulja et al., 2009). However, huge body or information available to modern consumers results in their increased expectations and demands in view of the safety and quality of food (Prica et al., 2009). Therefore, there is a need to find the answer to the question: What is the quality of honey, how is it assessed and graded? One of the possible approaches to evaluate the quality of honey includes passing of relevant regulations establishing minimum

and maximum levels of particular substances and ingredients in foodstuffs. In Serbia, the quality requirements for honey are stipulated in the Regulation on quality and other requirements for honey, other bee products, products based on honey and other bee products (Sl. list, 2003).

According to the Regulation (Sl. list SCG, 2003), honey is defined as "sweet, dense, crystallized, viscous product produced by honeybees from the nectar of honeyplant flowers or from secretions of living parts (conifer or hardwood species), which the bees collect, transform by combining with specific substances of their own, and deposit in honeycombs to mature". In Codex standard (2001), honey is defined as "natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in the honey comb to ripen and mature". Chemical composition of honey implicates highly complex mixture of more than 200 different substances (Ferreira et al., 2009). Some of these substances are produced by honeybees, some originate from honeyplants, whereas some are produced during the maturation process in the honeycomb (Krell, 1996).

Honey types, as well as the individual samples within particular type, differ by their composition according to their floral and geographic origin, climatic conditions, honeybee species as well as processing and storage conditions (Škenderov and Ivanov, 1986).

The average composition of honeys includes some 17% water, 38.19% fructose, 31.28% glucose, 1.31% saccharose, 7.31% maltose, 7.11% lactose, 0.04% nitrogen and some 0.169% ash. After the carbohydrates, water is the second most important component of honey. Its content ranges between 15 and 23% (Krell, 1996). The moistur content substantially affects some physical properties of honey (crystallization, viscosity, specific weight) and is influenced by climatic factors, bee species, bee-colony's strength, humidity and air temperature in the hive, processing and storage conditions as well as by the honeyplant species. However, there are no substantial differences in water content between individual honey types (Škenderov and Ivanov, 1986).

Honey in its natural form is characterized by extremely low moisture content, thus very few bacteria and microorganisms can survive in such environment, which is essential for its resilience (Geiling, 2013). Yet, honey is highly hygroscopic substance and its moisture content may vary depending on air humidity during storage. The higher moisture-in-honey content, the greater is the possibility that the yeasts will ferment and change the flavor. Namely, fermentation process results in alcohol formation and, in the presence

of oxygen, the alcohol will break down to acetic acid and water, which causes honey to have sour taste and to spoil (Rogulja et al., 2009).

It is well established that molasses, a byproduct of cane sugar, is similar to honey by its properties, yet–although it has a long shelf life molasses can eventually spoil. The durability of honey is partly to be attributed to the bees themselves. Nectar, the first component collected by bees to make honey, is by its nature highly humid with a moisture content ranging from 60-80%. Throughout the process of making honey, the bees dry out much of this moisture by flapping their wings. The chemical composition of bees' stomach significantly contributes to honey's resilience to spoilage. Bees' stomach produces an enzyme called glucose oxidase, which mixes with the nectar, breaking it down into two by-products: gluconic acid and hydrogen peroxide, the latter one being of crucial importance for the maintenance of quality of honey (Geiling, 2013).

Honey is naturally highly acidic. Its pH is extremely low, ranging between 3 and 4.5, which inhibits the growth of bacteria and other spoil-ready organisms (Geiling, 2013). During a long time, formic acid has been considered major (if not the only one) acid in the honey. Nowadays, it is well established that honey contains a wide range of organic acids. Besides the formic acid, honey contains oxalic acid, butyric acid, citric acid, 2,3-dihydroxybutanedioic acid, malic acid, pyroglutamic acid, lactic acid, benzoic acid, maleic acid, gluconic acid, isobutyric acid, succinic acid, pyruvic acid, α-ketoglutaric acid and glycolic acid. Out of these, gluconic acid, a byproduct of enzymatic activity of glucose oxidase, predominates. According to the data from the literature, the content of organic acids in honey ranges between 0.17 and 1.17% (average range 0.57%). Most of organic acids are present in honey in the form of esters, which contributs to its characteristic flavor and aroma. Some of the acids are introduced into honey via the nectar, i.e., their contents depends on the type of the honey, whereas some are produced during storage process and are influenced by storage temperature and processing conditions. The acidity *of honey* can range from 8.7 to 59.5 meq/kg, with an average of 29.1 meq/kg. Increased acidity of honey is an indicator for a fermentation process and transformation of alcohol into organic acid (Rogulja et al., 2009). It is believed that moisture content less than 18% will prevent the fermentation. However, this possibility cannot be absolutely excluded even in honeys with moisture content below 17.1% since the potential effects of yeast content and temperature of honey as well as distribution and availability of water after crystallization have to be taken into consideration (Krell, 1996).

Moisture content can be considered the most important parameter of

honey quality as it determines its stability and resistance towards microbial spoilage (fermentation) during storage (Bogdanov et al., 1999). The influence of acid content on fermentation processes, flavor and aroma as well as bactericidal properties of honey make the total acidity an important indicator of quality of honey. To that end, the objective of this study was to investigate these quality parameters in honey samples collected during 2013 in the territory of Vojvodina.

MATERIAL AND METHODS

To the purpose of determining the moisture content and total acidity, 50 samples of different honeys originating from Vojvodina region were collected. All samples were in their original packages and were transferred to the laboratory and stored in a cold and dark place. The investigated samples included 12 samples of meadow honey, 14 samples of acacia honey, 14 samples of linden honey, 4 samples of multiflower honey, 5 samples of sunflower honey and 1 sample of forest honey.

Moisture content was determined by the refractometric method (Sl. list SFRJ, 1985), using an Abbe refractometer (Model RMT, Optech, Italy). All measurements were performed at 20°C after equilibrium. The corresponding % moisture from the refractive index of the honey sample was calculated by consulting a standard table for this purpose.

The acidity of honey was determined by volumetric method (Sl. list SFRJ, 1985). Ten grams of honey were dissolved in 75 ml of distilled water and alcoholic solution of phenolphthalein was added. The solution was titrated with 0.1 mol/dm^3 NaOH. Acidity (milimol of formic acid per kg of honey) was determined as 10 times the volume of NaOH used in titration.

RESULTS AND DISCUSSION

The obtained results on moisture content and total acidity in the examined honey samples are displayed in Table 1.

Moisture content in the examined samples ranged between 14.2 and 20.2%, with an average of 16.5±1.01%. Pursuant to relevant Regulation in Serbia (Sl. list SCG, 2003), which is harmonized with the EU Directive (EU Council 2002), maximum moisture content in honey put in the market is fixed to 20%. According to the obtained results, moisture content exceeded maximum permitted value in only one sample of sunflower honey.

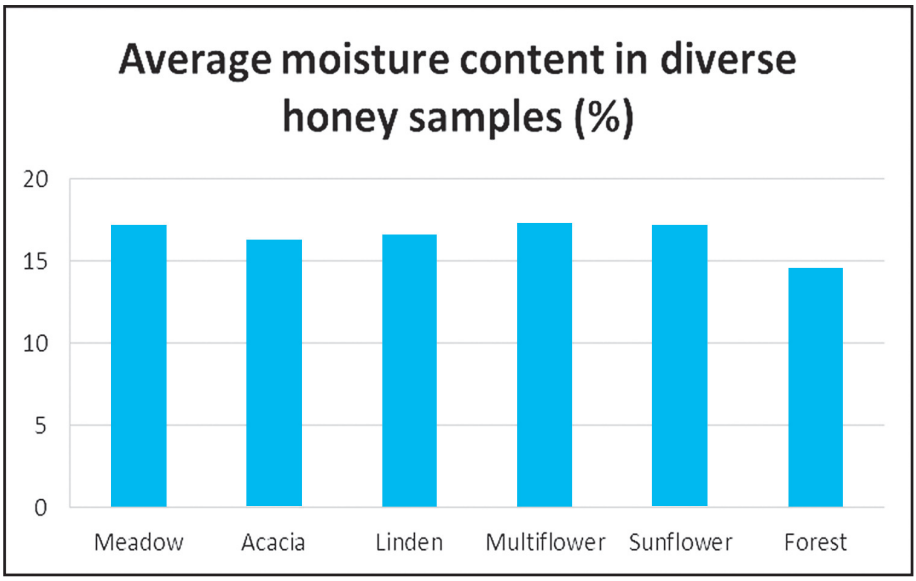
The acidity value in the same samples ranged from 7.75 mmol/kg to 44

mmol/kg, with an average of 17.38±6.79 mmol/kg. Pursuant to EU Council (2002), the maximum permitted acidity of honey is 50 meq/kg (the unit meq/kg is identical with mmol/kg since the acidity is expressed as the content of formic acid). Maximum value permitted by Serbian Regulation (Sl. list SCG, 2003) is somewhat lower, being 40 mmol of formic acid per 1000 g of the sample. Our results revealed that acidity was higher than the maximally permitted level (according to Serbian Regulation) in only one sample of linden honey.

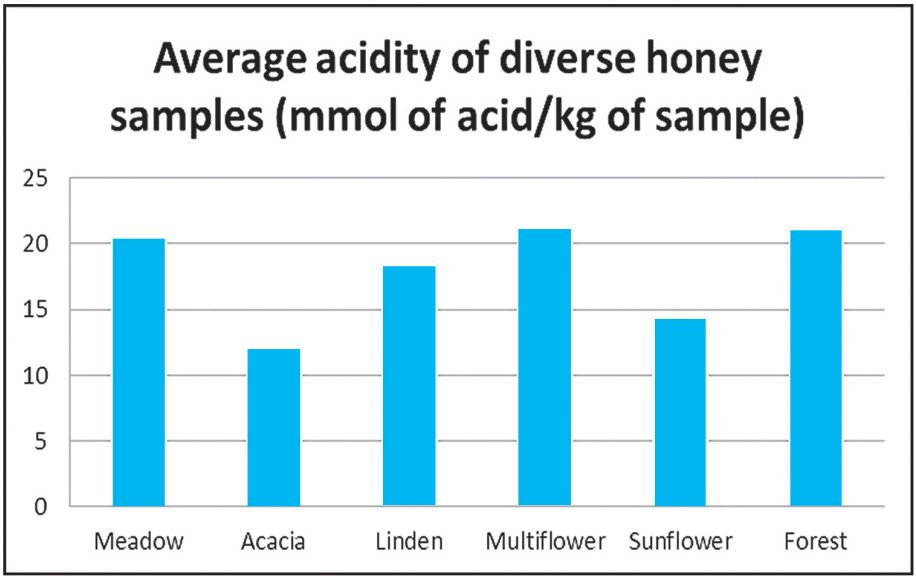
Table 1 Results of determining moisture content and acidity in diverse honey samples

TYPE OF HONEY	No. of samples	Moisture content (%)		Acidity (mmol of acid/1000 g)	
		Range	Average value ±SD	Range	Average value ±SD
Meadow	12	14.6–18.2	17.1±1.3	13.75–26.00	20.39±3.81
Acacia	14	14.2–18.4	16.3±1.3	7.75–18.75	12.08±3,29
Linden	14	14.8–18.6	16.5±7.0	10.50–44.00	18.26±9.90
Multi-flower	4	16.0–19.2	17.2±1.6	12.50–27.70	21.12±6.60
Sunflower	5	16.0–20.2	17.2±1.7	10.00–19.90	14.25±3.54
Forest	1	14.6	14.6	21,00	21.00

The obtained results on moisture content and total acidity of honey samples are presented in Graphs 1 and 2.



Graph 1. Moisture content in diverse honey samples



Graph 2. Total acidity of diverse honey samples

Analysis of the results obtained for the investigated parameters in honey samples revealed the lowest average values for water content and acidity in samples of forest honey and acacia honey, respectively. The highest average values for both parameters were established in multiflower honey samples.

The composition of organic acids in honey has not yet been adequately investigated; however, some evidence (Rogulja et al., 2009) suggest that acacia, chestnut and meadow honeys are characterized by particularly low contents of organic acids, whilst *darker honeys* in general appear to be *higher* in *acidity. Our results also demonstrated low acidity of acacia honey as compared with other examined honey types. The results obtained for meadow honey do not correspond with the aforementioned evidence, yet the acidity was within the proper range.*

Determination of physicochemical parameters in different honeys has been the topic of numerous researches both in Serbia and worldwide. The investigation of different quality parameters in 226 honey samples originating from Braničevo and Podunavlje regions during 2010-2012 revealed that honey in this region of Serbia is of good quality. Namely, all samples were characterized by adequate moisture content, and only one sample of acacia honey demonstrated increased acidity (Milošević et al., 2013). Examination of 201 honey samples originating from the entire territory of Serbia (acacia, sunflower and linden) was performed during 2009. The average moisture content ranged from 16.12% in acacia honey samples to 17.98 in sunflower honey samples. Free acidity differed widely among the three studied botanical samples, ranging from 11.20 in acacia honey samples to 25.65 meq/kg in sunflower honey samples (Lazarević et al., 2012). The investigation of the quality of diverse honeys produced in Montenegro (Đuričković et al., 2012) revealed moisture contents ranging from 17.0% in acacia honey to 19.2 in sage honey. The lowest and highest total acidity was determined in acacia honey (10 mmol/kg) and sage honey (40.0 mmol/kg), respectively.

Moisture content reported for five honey samples from Portugal ranged from 15.9 to 17.2%, whereas free acidity was within the range 16.0–32.0 meq/kg (Gomes et al., 2010). Determination of moisture content in 70 honey samples in Turkey revealed as much as 10% of inadequate samples, whereas the acidity values ranged between 6.94 and 29.6 meq/kg (Kahraman et al., 2010). In honey samples originating from India, the highest average values for water content were obtained for mustard honey (21.75 %), whereas eucalyptus and clover honeys had somewhat lower moisture contents (19.4 and 18.7 %, respectively) (Singh and Bath, 1997). The acidity level of the examined samples ranged between 29.5 and 41.5 meq/kg. By analyzing the samples of multifloral

honey collected in Venezuela during rainy and dry seasons, De Rodriguez et al. (2004) concluded that climatic conditions are of no importance for moisture content in honey. Namely, one of two honey samples with moisture content above 20% originated from dry season. The authors are of the opinion that increased moisture content is more likely associated with insufficient maturity of honey rather than with climatic conditions (De Rodriguez et al., 2004). Similar rates of moisture content in honey were reported in Argentina. The moisture content in 143 analyzed samples was within a range 16.4–18.1 % (Malacalza et al., 2005). Moisture content in Brazilian honey was somewhat higher, ranging between 18.59 and 19.58 % (Azeredo et al., 1999). The investigation including 73 samples of different honey types from Poland revealed moisture contents of 15.93–17.96 % (Popek, 2002).

As obvious from a brief review of quality control of honey in Serbia and worldwide, the analysis of physicochemical parameters is of vital importance in quality assessment. Although the aforementioned researches encompassed different types of honey, our research demonstrated that the quality of honey from Vojvodina corresponds to that of honeys available in international market.

CONCLUSION

The moisture content exceeded the maximum level permitted by the Serbian Regulation in only one of 50 analyzed honey samples. Moreover, in only one sample, the acidity was above the upper limit of 40 mmol of acid per 1000 g of sample (Sl. list SCG, 2003). We can conclude that 96% of investigated samples corresponded with the prescribed quality parameters, which may be taken as indicative of freshness of all honey samples. Nevertheless, potential effects of storage conditions on the quality of honey strongly suggest the necessity of continuous monitoring of the aforementioned parameters throughout the year.

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POTENCY TEST OF COMMERCIALLY AVAILABLE INACTIVATED NEWCASTLE DISEASE VACCINES IN SERBIA

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Abstract

In order to evaluate the efficacy of inactivated oil-emulsion Newcastle disease vaccine (commercially available on market in Serbia) we carried out the potency test in chickens, using hemagglutination inhibition (HI) test for detection of antibodies. The values of HI titers in the serum before and three weeks after vaccination revealed immunoconversion (IC) in chickens, and indicated potential differences in the immunoconversion values between two groups vaccinated with monovalent and polyvalent vaccine. In the period 2010-2012, 27 vaccines were tested, there of 21 polyvalent and six monovalent ones. Three weeks after the vaccination, HI titers of all vaccines were high. The average IC values were calculated as $\log_2$ (HI titer). The values ranged from 2.2 to 8.2 for polyvalent vaccine, whereas average values for monovalent vaccines were in the range from 3.2 to 6.3. Three weeks post vaccination; no statistically significant differences were recorded in the immune response between the tested groups of birds (vaccinated with monovalent and polyvalent vaccines). This supports the fact that both tested vaccines demonstrated good potency to creating immunity against ND in vaccinated birds.

Key words: poultry, New Castle disease, vaccine, potency testing

ISPITIVANJE AKTIVNOSTI INAKTIVISANIH VAKCINA PROTIV NEWCASTLE BOLESTI DOSTUPNIH NA TRŽIŠTU REPUBLIKE SRBIJE

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Kratak sadržaj

U cilju procene efikasnosti inaktivisanih uljnih vakcina protiv Newcastle bolesti (NB) koje su komercijalno dostupne na tržištu Republike Srbije, sproveli smo ispitivanje aktivnosti vakcine, na kokoškama. Za detekciju antitela koristili smo test inhibicije hemaglutinacije (HI). Vrednosti titra hemaglutinacije u serumima, pre vakcinacije i 3 nedelje posle vakcinacije ukazuju na imunokonverziju, i na moguću razliku u vrednostima imunokonverzije izmedju grupe vakcinisanih polivalentnim vakcinama i grupe vakcinisanih monovalentnim vakcinama. U period 2010-2012., kontrolisano je dvadeset sedam vakcina: dvadest jedna polivalentna i šest monovalentnih vakcina. Tri nedelje nakon vakcinacije, HI titar je bio visok kod svih vakcinisanih jedinki. Prosečna vrednost za imunokonverziju je računata preko $\log_2$(HI titer). Vrednosti imunokonverzije su bile u rasponu od 2,2 do 8,2 za polivalentne vakcine, dok je prosečna vrednost za imunokonverziju monovalentnih vakcina bila od 3,2 do 6,3. Tri nedelje nakon vakcinacije, nisu uočene statički značajne razlike u imuno- odgovoru ove dve grupe vakcinisanih jedinki (vakcinisane polivalentnim i vakcinisane monovalentnim vakcinama), što potvrdjuje i činjenica da sve kontrolisane vakcine pokazuju dobru aktivnost u stvaranju imuniteta protiv NB, kod vakcinisanih jedinki.

Ključne reči: živina, atipična kuga živine, vakcine, ispitivanje aktivnosti vakcina

INTRODUCTION

Newcastle disease (ND) is one of the diseases listed in the OIE register. ND is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) of the genus Avulavirus, belonging to the family of Paramyxoviridae. There are ten serotypes of avian paramyxoviruses (APMV-1 to APMV-10) (OIE Terrestrial

Manual 2012). Since the first outbreak, when disease was recognized in poultry and termed ND in 1926, in Java, Indonesia, and in Newcastle-upon-Tyne, England, ND is enzootic in some parts of the world and one of the most important diseases of poultry worldwide.

ND occurs in both extensive and intensive way of farming. Strains of NDV have been grouped into five pathotypes based on the clinical signs seen in infected chickens: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic (Alexander et Senne, 2008). One of the most characteristic properties of different strains of NDV has been their great variation in pathogenicity for chickens. This infection can spread via direct contact with secretions, especially feces, from infected birds or indirect contact through contaminated feed, water, equipment, vehicles, humans, fomites etc.

ND inflicts enormous economic consequences in poultry industry in many parts of the world: big percentage of mortality (it is not rare that all birds from flock die without manifesting of clinical symptoms, especially in the first 3 days of epizootic). Because of all this consequences and huge economic losses, the majority of commercial poultry flocks are involved in the program of control of ND. In Serbia, Newcastle disease has been present for many years. Last epidemic that occurred in our country was recorded in the Province of Vojvodina, during 2006 and 2007 (Milic et al., 2012).

Immunoprophylaxis is the only way in successful fight aganst the outbreaks of this disease. Vaccination programs differ from country to country. Prophylactic vaccination is applied on a large scale in the EU and elsewhere in the world. All member states except Sweden, Finland and Estonia apply a prophylactic vaccination policy. So far, the emergency vaccination was used only once in Italy, during an outbreak in 2001.

In accordance with the Program of animal health protection measures (2014) of the Republic of Serbia, in order to prevent the occurrence, spread and suppression of ND, every farm and backyard with poultry and game birds must be registered and recorded in the Central database. Poultry, game birds and pigeons in all keeping and breeding systems are vaccinated against ND for boosting the immunity, applying vaccines produced from the lentogen forms of virus. Control of the immune status after vaccination is to be performed by serological examination of blood serum. In cases of unfavorable epidemiological situation for poultry and game birds in intensive breeding, the composing Program of immunoprophylaxis is needed, which has to be subjected to the competent veterinary inspection. In broiler chickens in extensive farming conditions, vaccination must be done two times: at the age of one day in the incubator or at the place of destination, by spraying, and from 14 to 18 days

of age in drinking water, by spray or by oculonasal application, according to the manufacturer's instructions. In poultry intended for the production of hatching and consumption eggs in extensive farming conditions, vaccination is performed four times: the first vaccination is carried out by spraying in incubator or at the place of destination, at the age of one day, and the remaining cycles at the age of three, six and twelve weeks, according to the manufacturer's instructions.

Several types of vaccines are available both worldwide and in our market: inactivated vaccines and the vaccines that contain live virus. Each of them has its advantages and disadvantages. Live vaccines are generally used to induce protection in young birds. Inactivated vaccines are used to booster the immune responses in layer flocks and to generate uniform protective antibody titers before the laying period. Inactivated ND vaccines are among the most widely used vaccines in commercial poultry worldwide (Ivo Claassen, 2011).

ND vaccine (inactivated) (also known as avian Paramyxovirus 1 vaccine (inactivated) for vaccines intended for some species) is a preparation of a suitable strain of ND virus (avian Paramyxovirus 1), inactivated while maintaining adequate immunogenic properties (European pharmacopoeia, 6th Edition). At our market, two groups of inactivated vaccines are available: monovalent inactivated vaccines (PEST-OL®, Veterinarski zavod Subotica; BRONED-OL®, Veterinarski zavod Subotica) and polyvalent inactivated vaccines (PESTIKAL + EDS + IB, Genera; POLIVIROL-3, Veterinarski zavod AD Zemun; POLIVIROL-4, Veterinarski zavod AD Zemun) Nobilis® Reo+IB+G+ND, Intervet; Nobilis® IB+ ND + EDS, Intervet; Bronipra ND/IBD, Hipra).

Biological products, such as vaccines, require batch-related quality control to ensure their safety and potency. Part of quality control is based on animal model; consequently, the use of laboratory animals is extensive. The aim of this research was to perform the quality control (potency test) of vaccines against ND, for every batch, before placing them on the market in the Republic of Serbia. Monovalent and polyvalent vaccines were examined, offering an opportunity to compare the differences in immune response of chickens three weeks after the vaccination.

MATERIAL AND METHODS

In the period 2010-2012, twenty-seven vaccines were tested in the Scientific Veterinary Institute „Novi Sad". During these three years (2010-2012), the potency tests were carried out on 21 polyvalent vaccines: PESTIKAL+EDS+IB (8 sample - batch), POLIVIROL-3 (3), POLIVIROL-4 (3), Bronipra ND/IBD

(1), Nobilis® Reo+IB+G+ND (4), Nobilis® IB+ND+EDS (2). Moreover, six monovalent vaccines were controlled during this period: PEST-OL® (3) and BRONED-OL® (3).

Potency testing of vaccines was performed in accordance with the method described in European Pharmacopeia, monograph 01/2008:0870 for inactivated vaccine for Newcastle disease (*Vaccinum pseudopestis aviariae inactivatum*) with certain modifications.

For each batch of the vaccine, a potency test has been carried out by using 10 chickens, 21-28 days old, which did not have antibodies against the causative agent of ND. Each chicken has been vaccinated by the intramuscular application with a vaccine volume equivalent to 1/50 of a dose. Blood samples were taken before vaccination and 21 days after vaccination. The specific antibodies titers were detected by haemagglutination-inhibition (HI) test. The HI test is carried out by using series of the investigated blood serums (two-fold dilution in saline solution). The same volume (four hemaglutination units- 25 μL) of reference ND virus (La Sota strain) is added to every dilution of the serum. After incubation of 30 minutes, 50 μL of 0.5% suspension of red blood cells (derived from younger chickens, free of antibodies against the ND) was added to each dilution of the serum with added virus. The results were read after 45 minutes of incubation. Based on the results of the HI test, we defined the IC values as the difference between the values of the titer before and 3 weeks after vaccination, calculated as $\log_2$(HI titer). Vaccinated animals were monitored daily to determine the health status.

Biostatistical analysis of the results was performed using Student's t-test. To assess the significance of differences between the immune response of chickens vaccinated with monovalent and polyvalent vaccines, two-sided Student's t-test (Ms Office Excel 2010) was used, normally distributed. The standard deviation of the population is not known, thus the standard deviation of the sample was applied.

RESULTS AND DISCUSSION

The examined vaccines were distributed into two groups (depending on the prevalence - antigenicity of the vaccine). This method enabled simpler analysis and comparison of the obtained seroconversion results in the HI test. The first group is a group administered polyvalent vaccine- 77.8% of all tested vaccines (2010- 5 samples; 2011- 8 samples; 2012- 8 samples). The second group encompassed birds receiving monovalent vaccines- 22.2% of all tested vaccines (2010- 2 samples, 2011- 2 samples, 2012- 2 samples).

The results are shown in chronological order (Table 1) through years: 2010, 2011 and 2012, and classified as monovalent and polyvalent.

Table 1. A chronological review of vaccine potency tests (immunoconversion) in chickens

Number of vaccines	Year of testing					
	2010.		2011.		2012.	
	Antigenicity of vaccines / values of immunoconversion (log2(HI titer))					
	polyvalent vaccines	monovalent vaccines	polyvalent vaccines	monovalent vaccines	polyvalent vaccines	monovalent vaccines
1	6.5	6.3	4.5	4.4	2.5	5.6
2	6.2	3.2	8.2	3.7	3.4	5.5
3	6.0		4.1		2.2	
4	6.7		4.9		5.2	
5	3.4		3.9		2.9	
6			2.6		3.4	
7			4.7		3.0	
8			5.3		3.4	
$\bar{x}$	**5.8**	**4.7**	**4.8**	**4.1**	**3.3**	**5.6**
SD	**1.35**	**2.21**	**1.61**	**0.49**	**0.90**	**0.07**

Legend: the average value ($\bar{x}$), standard deviation (SD)

The immunoconversion (IC) values after vaccination are shown in Table 1. Four polyvalent and two monovalent vaccines were submitted for quality control in 2010. During potency testing, values of IC in polyvalent vaccines were within the range from 6.0 to 6.7 and just one of four vaccines had slightly lower serological conversion (3.4). Values of IC in monovalent vaccines were 6.3 and 3.2. The average values for IC in polyvalent and monovalent vaccines were 5.8 and 4.7, respectively. In 2011, eight polyvalent and two monovalent vaccines were tested for quality control. Values of IC in polyvalent vaccines, during

potency testing, were in the range from 3.9 to 5.3. Two of eight polyvalent vaccines had IC values out of this range: one had somewhat lower value (2.6), and the other one had slightly higher value (8.2). The results obtained of IC in monovalent vaccines were 4.4 and 3.7. The average value for IC in polyvalent vaccines was 4.8 and the average value for IC in monovalent vaccines was 4.1. In 2012, eight polyvalent and two monovalent vaccines were examined for quality control. During potency testing, results of IC in polyvalent vaccines were in the range from 2.5 to 3.4. Two of eight polyvalent vaccines had values out of this range: 2.2 and 5.2. The differences from initial to the final HI titers of two monovalent vaccines were 5.6 and 5.5. The average value for IC in polyvalent vaccines was 3.3 and the average value for IC in monovalent vaccines was 5.6.

Chickens vaccinated with polyvalent vaccines in 2010 and 2011 had higher values of IC, while in year of 2012, immune- response in chickens vaccinated with monovalent vaccines was more noticeable. The results of IC values were higher than 2 in all tested vaccines (27), indicating a sufficient activity of the vaccines (European pharmacopoeia, 6th Edition). During this research, all chicken were without any clinical signs of ND or any other disease.

The values from Table 1 form two groups with different numbers of data: polyvalent vaccines (N1 = 21) and monovalent vaccines (N2 = 6). T-value (t = 0.58) is calculated based on mean values and standard deviations. The obtained result is compared with the values from the Student table of critical values.

When using HI to evaluate the immune response after vaccination, it should be taken into account that HI titers are greatly influenced by the quality of the vaccine, the route and method of administration, environmental and individual factors, but they also depend on health status of the individual birds. The quality of the vaccine and its efficacy is determined by all aforementioned factors.

Vaccine antigens are often combined to reduce the number of injections. It is well known that some vaccine antigens, such as the whole cell pertussis, enhance the potency of other vaccine antigens. Most of the vaccines include a number of substances, such as an adjuvant and a preservative, which may or may not affect the quality of the product. This unknown quality makes each batch of vaccine a unique product, and strict controls must be in place to ensure the safety and potency of each batch. A major part of the tests used for quality control is based on animal models, and consequently, the use of laboratory animals is extensive, particularly for vaccine quality control (Hendriksen CF, 2009). Due to the substantial number of animals used annually for the release of veterinary vaccines, global regulatory agencies actively encourage the evaluation, development, and implementation of novel approaches that reduce, refine, and replace (3Rs) the use of animals in evaluating vaccine safety and

potency product release testing. The development of an in vitro potency assay for inactivated NDV vaccines would save time, expenses, and use of experimental animals (K.A. Liljebjelke et al., 2008).

The most widely used tests for quality control of vaccines include *in vivo* potency tests. Typically, those tests evaluate the protective or immune response to the complete vaccine, including antigenic material (e.g., adjuvants, excipients). *In vitro* antigen quantification has some scientific and psychological limitation, because that is only a measure of antigen quantity, and not necessarily of biological activity (Hendriksen CF, 2009). Many typically used adjuvants, such as mineral oil and aluminum salts, may interfere with *in vitro* quantification methods. The challenges caused by the adjuvants that are present in many veterinary vaccines present one of the key technical issues. Therefore, these adjuvants should be separated from the antigenic component of the vaccine before *in vitro* potency testing. Since the adjuvant is a critical component for developing the appropriate protective response for inactivated vaccines, additional *in vitro* tests may be required to ensure their quality. Regardless, when antigen quantification methods are developed, the effect of an adjuvant on the immunogenicity of the protective antigen also needs to be investigated (Jodie Kulpa-Eddy et al., 2011).

Another approach, which still relies on the use of animals, is the replacement of the challenge procedure by serology. Unfortunately, *in vitro* serological methods are not feasible alternatives in vaccine – induced protection cases, based on both humoral (antibody) and cellular responses (Hendriksen CF, 2009).

For many veterinary vaccines, regional differences affect the availability and implementation of *in vitro* replacement assays. For example, the USDA published an *in vitro* ELISA potency test for inactivated swine erysipelas vaccine (*Erysipelothrix rhusiopathiae*), while the European Directorate for the Quality of Medicines & HealthCare (EDQM) published a mouse–based serology test in the European Pharmacopoeia (Ph. Eur.). The EDQM has developed, validated, and approved an *in vitro* test for inactivated Newcastle disease vaccine that is not a standard requirement in the United States.

Due to the successful international communication, number of Replacement methods for veterinary vaccines that will be available and accepted for use may increase.

However, although these results are satisfactory, it is evident that despite vaccination, the epidemic continues to emerge. Recurrent outbreaks of ND despite vaccination have raised the question whether currently used ND vaccines are still adequate, not only for the protection against clinical disease, but also for the inhibition of virus transmission (Kapczynski et King, 2005).

However, the increasing development of molecular biology favors the development of an effective vaccine, such as a recombinant vaccine that would initiate protective immunity at a satisfactory level.

Poultry vaccines are of highest priority for further development of alternative replacement methods for quality control testing. The growing role of international organizations such as the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) and the World Organization for Animal Health (Office International des Epizooties - OIE) is apparent. The harmonization of guidelines and reference standards for broad use of the vaccines would probably increase the interaction between those organizations and the national regulatory groups. This harmonization will make the implementation of the 3Rs for vaccine product release more realistic. Although the vaccine companies must develop and validate product-specific assays, the reference standards would provide the basis for their further development and validation (Jodie Kulpa-Eddy et al., 2011).

CONCLUSION

All vaccines (both polyvalent and monovalent) that were included in potency testing (2010-2012) provide good protection of chickens from ND.

In the year of 2010, the average value for IC in polyvalent vaccines was 5.8 and the average value for IC in monovalent vaccines was 4.7. In the year of 2011, the average value for IC in polyvalent vaccines was 4.8 and the average value for IC in monovalent vaccines was 4.1. In the year of 2012, the average value for IC in polyvalent vaccines was 3.3 and the average value for IC in monovalent vaccines was 5.6.

Realized value of the Student T-test ($t = 0.58$) is less than tabular values for confidence level $P = 95\%$, as well as the confidence level $P= 99\%$. Thus, we may conclude that there is no statistically significant difference between the values obtained for immunoconversion results of polyvalent and monovalent vaccines. Consequently, there was no statistically significant difference between the values obtained after vaccination with polyvalent and monovalent vaccines.

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Case report UDK 619:611.69:636.2

RARE CAUSATIVE AGENTS OF MAMMARY GLAND INFECTION: *CANDIDA LAMBICA* -CASE REPORT-

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Abstract

A brief case report on bovine subclinical mastitis caused by yeast species *Candida lambica* is presented in this article. Basic cultural, microscopic and biochemical traits of this rare agent implicated in bovine mammary infection are described. Identification of isolates was performed using an *Integral System Yeasts Plus* test, a commercial kit for identification of yeasts of importance in medicine. The available literature offers only sporadic reports on *C. lambica* infection in both humans and animals.

Key words: bovine mastitis, *Candida lambica, Integral System Yeast Plus* test

RETKI UZROČNICI INFEKCIJE MLEČNE ŽLEZDE KRAVA: *CANDIDA LAMBICA* -PRIKAZ SLUČAJA-

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Kratak sadržaj:

U radu je dat kratak prikaz slučaja subkliničkog mastitisa krave izazvanog kvasnicom *Candida lambica.* Prikazane su osnovne kulturelne, mikroskopske i biohemijske karakteristike ovog retkog uzročnika infekcije

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mlečne žlezde krava. Identifikacija izolata izvedena je primenom *Integral System Yeasts Plus* testa, komercijalnog sistema za identifikaciju medicinski značajnih kvasnica. U dostupnoj stručnoj literaturi, postoje samo sporadični izveštaji o infekcijama koje kod ljudi i životinja izaziva *C. lambica.*

Ključne reči: bovine mastitis, *Candida lambica, Integral System Yeast Plus* test

INTRODUCTION

Wide application of antibiotics and corticosteroids in both human and veterinary medicine throughout the period since the 1950s resulted in an increased incidence of yeast infections. The fungi, particularly yeasts, are considered opportunistic pathogens for mammary gland. Previous administration of antibiotics, treatment with contaminated antibiotic preparations, as well as syringes are prerequisites for the occurrence of the infection (Krukowski and Saba, 2003; Zaragoza *et al*., 2011). Prevalence of mycotic mastitis is usually low, 1-12% off all mastitis cases (Krukowski and Saba, 2003). However, if the infection remains unidentified and the factors implicated in its development are not eliminated on time, yeast mastitis may reach even epizootic proportions (Krukowski and Saba, 2003; Costa *et al*., 2012). During the past several years, increased incidence of subclinical and clinical mastitis caused by yeasts from the genus *Candida* has been reported (Zaragoza *et al*., 2011; Dworecka-Kaszak *et al*., 2012). Microbiological examination of milk samples is inevitable in the diagnostics of mycotic mastitis, and identification at species level is performed according to morphologic features (formation of chlamydoconidium, pseudohyphae and germinal tube development), growth in the presence of 0.1% cyclohexamide, acidic pH tolerance and carbohydrates assimilation and/or fermentation.

Mastitis in dairy cows is associated with a variety of fungal species. Though the yeasts of the genus *Candida* are most commonly isolated species (Zaragoza *et al*., 2011; Milanov *et al*., 2014) there are only sporadic reports on the isolation of *C. lambica* (Spanamberg *et al*., 2008; Zaragoza *et al*., 2011). In human medicine, only several cases of *Candida lambica* infections have been reported so far: bloodstream infections (Pfaller *et al*., 2004; Vervaeke *et al*., 2008), infection in patients with hematologic malignancies (Kruger *et al*., 1998), arthritis, probably acquired from a contaminated wound, in a patient with chronic alcoholism (Trowbridge *et al*., 1999).

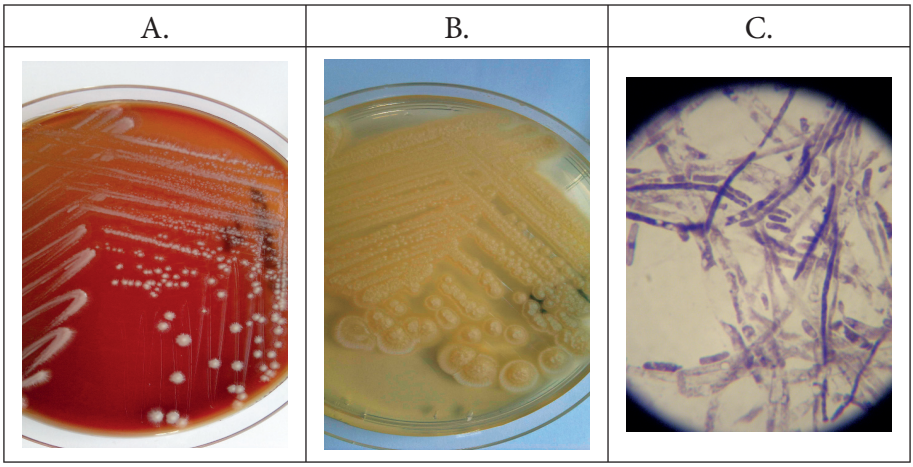
CASE REPORT

Regular monthly examination of milk sample using California Mastitis Test revealed positive finding in the single infected udder quarter in a Holstein-Friesian cow aged 3.9 years. The colour and consistency of the milk were not significantly changed, but the presence of small patches was evident in the sample. Before milk sampling for microbiological examination, the cow underwent two unsuccessful antibiotic treatments that included parenteral administration of enrofloxacin, amoxicillin and penicillin as well as intramammary administration of *cefquinome*, bacitracin, neomycin, tetracycline and prednisolone. The time period between the last antibiotic administration and collection of milk sample was more than one month. In July, when the sampling was performed, the somatic cell count per one mL of milk from infected udder was 1679000. The sampling was performed on 170th day of second lactation, and average seven-day milk yield was a 27 Litres.

Milk samples for microbiological examination were obtained separately from each of the four quarters. Disinfection of teats was performed using 70% ethanol, and the milk was collected into sterile plastic tubes, cooled and transported to the laboratory. To the purpose of bacterial isolation, aliquots of 50μL were inoculated onto Columbia blood agar base (Oxoid, Basingstoke, UK, CM0331) with 5% defibrinated ovine blood, MacConkey agar (Oxoid, CM0007) and onto Sabouraud dextrose agar (Oxoid, CM0041) and incubated during 2-3 days at 25°C and 37°C.

From the milk sample collected from the right anterior quarter, massive amounts of pure culture yeast were isolated. The growth was noticed after 24-hour incubation at all nutritive media and at both incubation temperatures (25°C and 37°C), with largest colonies observed on Sabouraud dextrose agar. Colonial appearance on blood and Sabouraud dextrose agar after 72h incubation at 25°C is presented in Figure 1 (A and B). Preparations made of 7-day old cultures grown on Sabouraud dextrose agar at 37°C. The preparations were stained according to Giemsa method and examined using light microscopy (1000x, immersion) (Figure 1, C).

Figure 1: Yeast colonies at blood agar (A) and Sabouraud dextrose agar (B) after 72h incubation at 25°C and microscopic appearance (C)



Biochemical characteristics of the isolate were examined using *Integral System Yeasts Plus* test (Liofilchem, Italy, Ref. 71822) according to manufacturer's instructions. *Integral System Yeasts Plus* test is a system for identification of most clinically important yeasts. Presumptive identification is based on assimilation reactions of sugars (glucose, maltose, saccharose, lactose, galactose, melobiose, cellobiose, inositol, xylose, raffinose, trehalose and dulcitol). Based on its assimilation features, the isolate was identified as *Candida lambica* (Table 1). Microscopic examination revealed abundant, moderately branched pseudohyphae (Figure 2, C). True hyphae *C. lambica* are not formed (http://www.doctrofungus.org/ the fungi/Candida_ lambica.php).

Table 1. Assimilation ability of isolate *Candida lambica* in *Integral System Yeasts Plus* test

Identification	Glu	Mal	Sac	Lac	Gal	Mel	Cel	Ino	Xil	Raf	Tre	Dul	ID code
C.lambica	+	-	-	-	-	-	-	-	+	-	-	-	1040

After the first microbiological examination of the samples and isolation of the yeast, the sampling was repeated for finding confirmation. Namely, *C.*

lambica was found in dairy products, water, and fruits and thus may be present in the sample as a contaminant. Second sampling was performed under maximum aseptic conditions, at the very end of milking. Such procedure minimizes the probability of contamination with yeasts that are commonly present on the skin of the udder and teats (Krukowski and Saba, 2003). The repeated sampling has confirmed the previously obtained result.

C. lambica is a rare causative agent of mastitis in dairy cattle. Similar to other yeasts of the genus *Candida*, it readily grows on nutritive media commonly applied in bacteriology labs, such as blood agar, MacConkey agar and Sabouraud dextrose agar. According to cultural and biochemical traits, it is similar to the species *C. krusei,* which is more commonly isolated from the milk of cows with mycotic mastitis (Türkyılmaz and Kaynarca, 2008; Wawron *et al*., 2010; Dworecka-Kaszak *et al*., 2012). By using chromogenic agars and a commercial phenotyping gallery, *C. lambica* might be misidentified as *Candida krusei* (Vervaeke *et al*., 2008). Neither *C. lambica*, nor *C. krusei* grow on media containing cycloheximide (which differentiates them from *C.lipolytica*). Besides molecular methods that enable most reliable differentiation between these two related species, some simpler and readily available methods such as maximum growth temperature and biochemical traits determination could be successfully applied. Both species grow at incubation temperatures 25°C and 37°C; however, contrary to *C. krusei*, *Candida lambica* does not grow at 42°C. Commercial test *Integral System Yeasts Plus* used in this research enables differentiation between these two species based on their assimilation abilities (Table 2).

Table 2. Assimilation ability of *Candida lambica* and *C. krusei* in *Integral System Yeasts Plus* test

Identification	Glu	Mal	Sac	Lac	Gal	Mel	Cel	Ino	Xil	Raf	Tre	Dul	ID code
C.lambica	+	-	-	-	-	-	-	-	+	-	-	-	1040
C.krusei	+	-	-	-	-	-	-	-	-	-	-	-	1000

Some of our previous investigation of yeasts isolates from milk of cows with subclinical and clinical mastitis using the *Integral System Yeasts Plus* demonstrated that biochemical profile of the achlorophyllous alga *Prototheca zopfii* is identical to that of C. *krusei* (Milanov *et al*., 2014). Since this test is not applicable for the identification of *P. zopfii* (contrary to *P. wickerhamii*), the simi-

larity of cultural features of these organisms makes microscopic examination of the preparation unavoidable for final identification at species level.

Even though there are some therapeutic options for the management of yeast mastitis, it is rarely practiced in everyday practice. Therapeutic options in *Candida* infections include amphotericin B, fluconazole, tioconazole, miconazole and nystatin (Krukowski and Saba, 2003). Regrettably, in this case as well as in the number of other cases, yeast mastitis remains undiagnosed. It enhances spread of the infection within the herd, and commonly practiced antibiotic therapies only aggravate the situation. Milk samples are submitted for microbiological examination only after several unsuccessful treatments with diverse antibiotic classes.

CONCLUSION

The incidence of yeast mastitis in dairy cattle has been showing increasing tendency. Timely identification, control of infection spread within the herd and adequate management require microbiological examination of milk samples. Everyday practice in our veterinary clinical laboratories does not implicate identification of yeasts to the level of species. Moreover, specific test designed for application in veterinary medicine are not commercially available. *Integral Systems Yeasts Plus* test is designed for identification of yeasts of medical importance and is highly applicable for the identification of *Candida* species involved in the mastitis of dairy cows.

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UPUTSTVO AUTORIMA ZA PRIPREMANJE RUKOPISA

ARHIV VETERINARSKE MEDICINE je časopis Naučnog instituta za veterinarstvo"Novi Sad" u Novom Sadu. Časopis objavljuje originalne, stručne i pregledne radove, priloge iz prakse, izveštaje sa kongresa i stručnih sastanaka, prikaze knjiga, radove iz istorije veterinarske medicine

Sve primljene rukopise Uređivački odbor šalje recenzentima radi stručne procene. Ukoliko recenzenti predlože izmene i dopune, tada se kopija recenziranog rukopisa dostavlja prvom autoru s molbom da tražene izmene unesu u tekst ili pak u protivnom da argumentovano izraze svoje neslaganje sa datim primedbama recenzenta. Konačnu odluku o prihvatanju rada za štampu donosi glavni i odgovorni urednik zajedno sa uređivačkim odborom.

Časopis se štampa na srpskom jeziku, a kratak sadržaj se prevodi na engleski. Radovi stranih autora se štampaju na engleskom jeziku sa kratkim sadržajem na srpskom.

Molimo saradnike da svoje radove pišu u skladu sa sledećim uputstvima.

Opšta uputstva

Tekst rada se kuca u programu za obradu teksta Word, latinicom, fontom Times New Roman, veličina slova 12 tačaka (12 pt), dupli proredom. Levu i desnu marginu podesiti na 20 mm, a gornju i donju na 30 mm, na A4 strani. Ukoliko se u tekstu koriste specijalni znaci (simboli), koristiti font Symbol. Rukopis rada dostaviti odštampan jednostrano papiru, ali i u elektronskoj formi. Paginacija na desnoj strani lista, počevši od naslovne strane. Reference u tekstu treba da navedu ime autora, iza kojeg se stavlja zarez i godina. Ukoliko ima više od dva autora, tada se u zagradi piše samo prezime prvog autora uz dodatak «i sar.,» pa godina (Vidić i sar., 2004).

Ukoliko je rad iz programa nekog projekta na kraju rada navesti finansijera projekta i evidencioni broj.

Naslovna strana

Na prvoj stranici treba napisati sledeće:

- naziv članka, odnosno rada treba pisati velikim slovima bez podvlačenja i bez skraćenica
- imena autora pisati ispod naslova punim imenom i prezimenom, razdvojena samo zarezom.

Iznad prezimena se brojem označava ustanova u kojoj radi autor (autori):

- navesti punu adresu ustanova u kojima autori rade; navoditi onim redosledom koji odgovara redosledu autora u radu;
- na dnu stranice treba navesti ime e-mail jednog od autora, radi korespondencije.

Kratak sadržaj

Na posebnoj stranici uz rad treba priložiti i kratak sadržaj rada, obima 300 reči. Pored naslova i imena autora i ustanova, kratak sadržaj treba da sadrži najvažnije činjenice iz rada. Takođe, ispod kratkog sadržaja treba navesti 3-8 ključnih reči.

Pisanje teksta

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

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

Grafikoni se takođe označavaju arapskim brojevima (ispod grafikona) po redosledu navođenja u tekstu, sa nazivom na srpskom jeziku. Koristiti font Times New Roman i veličinu slova 12 pt, sa jednostrukim proredom i bez uvlačenja. Ukoliko se koriste skraćenice, treba ih objasniti u legendi ispod grafikona.

Sheme (crteži) se označavaju arapskim brojevima (ispod shema) po redosledu navođenja u tekstu, sa nazivom na srpskom jeziku. Koristiti font Times New Roman i veličinu slova 10 pt, sa jednostrukim proredom i bez uvlačenja.

Ukoliko se koriste skraćenice, treba ih objasniti u legendi ispod sheme.

Fotografije se označavaju arapskim brojevima (ispod fotografije) po redosledu navođenja u tekstu, sa nazivom na srpskom jeziku. Primaju se isključivo originalne fotografije (crno-bele ili u boji) na sjajnom (glatkom, a ne mat) papiru. Na poleđini svake fotografije treba napisati redni broj i strelicom označiti gornji deo slike. Za svaki primerak rukopisa dostaviti po jednu sliku.

Poglavlja rada

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

U uvodu treba ukazati na najvažnije, odnosno najnovije činjenice i poglede vezane za temu rada, sa kratkim obrazloženjem cilja sopstvenih ispitivanja.

Materijal i metode rada. U ovom poglavlju treba opisati uslove pod kojima su ogledi izvedeni, navesti pun naziv metoda koje su korišćene u ispitivanjima, materijal i životinje na kojima su izvedena ispitivanja.

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

Diskusija. U ovom poglavlju se prikazuju uporedna analiza dobijenih rezultata sa rezultatima i mišljenjima drugih autora sa isticanjem značaja ispitivanja ali bez donošenja zaključaka.

Zaključak. U ovom poglavlju autor iznosi svoja zaključna razmatranja.

Literatura. U ovom poglavlju autor treba da iznese literaturne podatke, odnosno radove, koje je koristio u toku izrade svog rada. Poželjno je da korišćena literatura bude što novija. Reference treba pisati jednu ispod druge (numerisati ih arapskim brojevima) i abecednim redom prema prvom slovu prezimena prvog autora. Broj referenci nije u principu ograničen ali se preporučuje da ne bude veći od 15. Reference članaka koji su prihvaćeni za štampu treba označiti kao «u štampi» i priložiti dokaz o prihvatanju rada.

Primeri navođenja referenci:

1. **Članak u časopisu:**

Stojanović D., Maličević Ž., Ašanin R.: The use a new model for the investigation of sepsis. Acta Veterinaria, 52, 2/3, 125-131, 2002

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

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

4. **Članak u zborniku radova sa naučno-stručnog skupa:**
Valčić M., Lazić S., Rašić Z.: Mesto i uloga terenskog veterinara u epizootiološkom radu.
U: Dragiša R.Trailović, urednik, Zbornik radova, X regionalno savetovanje iz kliničke patologije i terapije životinja, 1-5. septembar, Kragujevac, Beograd: Fakultet veterinarske medicine, 2008, 75-82.

Napomena

Rad koji ne ispunjava sve gore navedene uslove neće biti poslat na recenziju i biće vraćen autorima da ga dopune i isprave.

Adresa časopisa

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NOTE FOR CONTRIBUTORS

ARHIVE OF VETERINARY MEDICINE is a journal of the Scientific Veterinary Institute "Novi Sad" in Novi Sad. The journal publishes original, expert and review papers, case reports, reports from symposia and other meetings, book reviews, cases from history of veterinary medicine.

All manuscripts are sent for a review and evaluation. In the case the reviewer suggests additional changes, the manuscript will be sent to the first author with a kind request to change the manuscript. In the case the author does not want to change, appropriate argumentation should be given. Final decision on accepting the manuscript is given by the editor in chief, together with editorial committee.

The journal is published in the Serbian language, followed by an abstract in English. The papers of foreign authors are published in English followed by an abstract in Serbian.

The manuscript should be written according to the following instructions:

General notes

The paper should be in Word program, Latin characters, size 12 pt, Times New Roman, double spaced. Left and right margins 20 mm, top and foot margins 30 mm, paper size A4. If special symbols are used, use font Symbol. The manuscript should be submitted on paper size A4, but also in electronic form. Pagination on the right side, starting from the title page. References and notes are cited in the text by authors' names, followed by the year of publication. If there are more than two authors, only the name of the first author is written followed by the abbreviation "i sar." (Vidić i sar., 2004).

If the paper is part of a project, name the financier and the project number at the end.

Title page

On the title page the following should be written:
- the title of the paper in capital letters, without underlining and abbreviations
- the names of the authors (first and second name, followed by a comma).

Above the second name place a number that denotes the institution where the author works:
- full name of the institutions should be given.

- at the bottom of the page write E-mail address of one author, for correspondence.

Summary

Every paper should be followed by a summary (300 words). Beside the title, name of the authors and institutions, it should contain the most important facts from the paper and three to eight key words.

Text

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

Tables number with the Arabic numerals (above the table). Use Times New Roman, 12 pt, single space, without indentation. If abbreviations are used, give an explanation bellow the table.

Graphs number with the Arabic numerals (below the graph). Use Times New Roman, 12 pt, single space, without indentation. If abbreviations are used, give an explanation bellow the graph..

Scheme number with the Arabic numerals (bellow the scheme). Use Times New Roman, 10 pt, single space, without indentation. If abbreviations are used, give an explanation bellow the graph.

Photographs number with the Arabic numerals (bellow the photo). Only original photographs can be used (black and white). On the back side write ordinal number of the photo and mark the top of the photo.

Headings

Headings in the paper are**: Introduction, Material and Methods, Results, Discussion (or Results and Discussion), Conclusion and Literature.**

Introduction points on the most important, i.e. most recent data regarding the topic with a short presentation of the aims of this research.

Material and Methods. Here describe the conditions in the experiment, name the used methods, material and animals.

Results. The results are displayed through tables or graphs, numbered with ordinal numbers and with an explanation what the photo, table or graph shows.

Discussion. Here give analyses of the obtained results comparing to the results and opinions of other authors, pointing the importance of this research, without giving a conclusion.

Conclusion. Here the authors gives his final conclusions.

Literature. The author should list the references, preferably the most recent one. References should be numbered with Arabic numerals, one under the other, written in alphabetical order according to the surname of the first author. In general, the number of references is not limited, but it is advisable to write 15 references.

Examples of references:

1. **Articles in journals:**

Stojanović D., Maličević Ž., Ašanin R.: The use a new model for the investigation of sepsis. Acta Veterinaria, 52, 2/3, 125-131, 2002

2. **Books:**

Qinn P.: Clinical Veterinary Microbiology. London, Mosby, 1998

3. **Chapters in books:**

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

4. **Articles in proceedings:**

Valčić M., Lazić S., Rašić Z.: Mesto i uloga terenskog veterinara u epizootiološkom radu.

U: Dragiša R.Trailović, urednik, Zbornik radova, X regionalno savetovanje iz kliničke patologije i terapije životinja, 1-5. septembar, Kragujevac, Beograd: Fakultet veterinarske medicine, 2008, 75-82

Note

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

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