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DERMANYSSUS GALLINAE ERADICATION APPROACH – APPLICATION OF INERT COMPOUNDS AND INTEGRAL ANIMAL HEALTH PROTECTION

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Abstract

The generally accepted approach to *D. gallinae* control is based on the control of infestations. The reduction of *D. gallinae* infection to an acceptable level can temporarily prevent its harmful effects on the health status of poultry. However, the reduced number of *D. gallinae* continues to persist in poultry environment resulting in an intensive increase of infestation rate and consequent transmission of infectious and contagious diseases. Contrary to suppression, the eradication approach completely eliminates *D. gallinae* thus improving general health status of the flock and providing the control of infectious and contagious diseases associated with this organism and eliminating other adverse effects. Biological efficiency and selection of inert compounds was performed in laboratory conditions. The previous experience with application of SiO2 formulations in practice confirmed the possibility of successful eradication in 8 cases (combined application of liquid and powdered form). New generation of inert compounds (P 547/17) showed a range of superior properties as compared with SiO2 formulations, especially in view of high and long-lasting residual effects on non-absorbent surfaces. In laboratory conditions, after 7 months, the layer formed by a 20% working emulsion on a metal substrate exhibited efficiency of 92% after 1-hour exposure. In clinical conditions, the presence of mites was not detected even after 6.5 months of the settlement of the flock. Clinical tests of inert oils are still ongoing, but their high potential for the eradication of *D. gallinae* The eradication of *D. gallinae* is highly complex procedure, which

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cannot be performed at all times and in all conditions. The conditions for the eradication of *D. gallinae* using inert compounds include adequate hygienic preparation of the facilities while still empty, adequate application of selected compounds, and then adequate ambient temperatures which leads to increase of mites activity. The program of *D. gallinae* control includes procedures for the preparation, implementation, eradication checks, and prevention of re-infestation.

Keywords: *Dermanyssus gallinae*, inert formulations, eradication

PRISTUP ERADIKACIJE DERMANYSSUS GALLINAE, PRIMENA INERTNIH JEDINjENjA I INTEGRALNA ZDRAVSTVENA ZAŠTITA ŽIVINE

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Kratak sadržaj

Opšte uvažen pristup kontrole *D. gallinae* zasniva se na suzbijanju infestacija. Suzbijanje *D. gallinae* smanjuje infestaciju na prihvatljiv nivo i privremeno sprečava značajan uticaj na zdravstveni status živine. Međutim, smanjeni broj *D. gallinae* nastavlja da perzistira u ambijentu i infestacija intenzivno raste, ostavljajući i dalje mogućnost prenosa infektivnih i zaraznih bolesti. Za razliku od suzbijanja, pristup eradikacije u potpunosti eliminiše *D. gallinae,* a time i uticaj na opšti zdravstveni status jata, ostvaruje važnu pretpostavku u kontroli infektivnih, posebno zaraznih bolesti (koje ona prenosi), ali i otklanja sve druge štetne posledice. U laboratorijskim uslovima utvrđena je biološka efikasnost i izvršen izbor inertnih jedinjenja. Dosadašnje iskustvo praktične primene SiO2 formulacija, potvrdila su mogućnost uspešne eradikacije u 8 slučajeva (kombinovana aplikacija tečnog i praškastog oblika). Nova generacija inertnih jedinjenja (P 547/17) pokazuje niz naprednijih osobina od SiO2 formulacija, posebno visoko i dugotrajno rezidualno dejstvo na ne upijajućim površinama. Sloj

koji je formirala 20% radna emulzija na limenoj podlozi, u laboratorijskim uslovima, nakon 7 meseci ispoljava efikasnost od 92%, u ekspoziciji od 1 sat. U kliničkim uslovima, i nakon 6,5 meseci od naseljavanja jata nije detektovano prisustvo grinja. I ako su klinička ispitivanja nove generacije inertnih jedinjenja još u toku, već sada je evidentno da imaju potencijal za eradikaciju. Eradikacija *D. gallinae* je zahtevan postupak kojeg nije moguće izvesti u svakom momentu i svim uslovima. Uslovi za eradikaciju *D. gallinae* inertnim jedinjenjima je higijenska priprema praznih objekata, adekvatna primena izabranih jedinjenja, i zatim adekvatna ambijentalna temperature koja dovodi do povećane aktivnosti grinja. Programska kontrola *D. gallinae* obuhvata postupke pripreme, sprovođenja, provere eradikacije i sprečavanja reinfestacije.

Ključne reči: *Dermanyssus gallinae*, inertne formulacije, eradikacija

INTRODUCTION

More than ever before, poultry farming is faced with the problem of poultry red mite (*D. gallinae*) control. When it comes to the effect on poultry, *D. gallinae* (Figure 1.) directly endangers the health status and at the same time transfers a number of infectious and communicable diseases.

Directly, feeding on blood, *D. gallinae* causes stress and anaemia, disrupts the immune response, transfers diseases, contributes to cannibalism and diminishes the general health status. Stress is clinically visible through the agitation of poultry, and it can also resemble nervous disorder, if *D. gallinae* enters the outer ear canal (Simic and Zivkovic, 1958). Somatic and psychogenic stress has been diagnosed through a blood analysis (Kowalski and Sokol, 2009). The manifestation of anaemia depends on the intensity of the *D. gallinae* infestation, age of the flock, nutrition and general health status of the flock. It has been established that with medium infestation, the number of mites per hen is 25000 - 50000 with the possibility of reaching 250000. A hen infested with *D. gallinae* can lose 3% of the total amount of blood every night, and as far as 5% with an extremely high number of *D. gallinae* (Emous, 2005). A blood analysis of infested poultry has established a severe decrease of erythrocytes, from 3.1 million to 1.2 million (Babic et al., 1956) and damages to humoral immunity (Kowalski and Sokol, 2009).

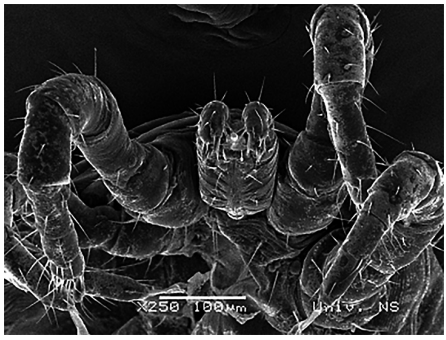

Figure 1. *D. gallinae* (SME, Photo Bokorov and Pavlićević)

Figure 2. Hen mortality caused by co-effect of *D. gallinae* and *Salmonella spp.* (Photo Pavlićević)

The role of *D. gallinae* as a vector is complex: it is mechanic, trans-stadial and trans-ovarian (Moro et al., 2005). So far, it is known that *D. gallinae* transfers Fowl pox virus (Huong et al., 2014), St Louis encephalitis *(SLE)* and Eastern equine encephalitis (*EEE)* (Durden et al., 1993); Western equine encephalitis virus (*WEE)* (Durden et al., 1993; Mullen and Durden, 2002; Moro et al., 2005); Newcastle disease virus, tick borne encephalitis virus or hantaviruses, and type A influenza virus (Sommer, 2011); *Salmonella galinnarum* and *S. enteritidis* (Moro et al., 2007, 2009) (Figure 2.); *Escherichia coli* and *Erysipelothrix rhusiopathiae* (Chirico et al., 2003); *Pasteurella multocida* (Petrov, 1975); *Mycoplasma synoviae* and *Mycoplasma gallisepticum* (Huong et al., 2014); *Rickettsia spp.*, *Spirocheta spp*., *Chlamydia spp.* and *Mycobacterium spp.* (De Luna et al., 2008); *Coxiella Burnetii*, protozoa and filaria (Moro et al., 2005).

The widely recognized approach to the control of *D. gallinae* is based on suppressing the infestation. Suppression of *D. gallinae* temporarily prevents substantial impact on the health status and reduces infestation to an acceptable level. However, the decreased number of *D. gallinae* continues to persist in the environment and the infestation grows intensely, leaving the possibility of transfer of infectious and communicable diseases. Therefore, the harmful impact of *D. gallinae* on the flock re-emerges.

The aim of this research is application of selected inert substances to establish the feasibility and importance of poultry red mite eradication in laboratory and practical conditions of intense poultry keeping, and to explain its potential contribution to integrated health protection.

MATERIALS AND METHODS

Laboratory examination was performed using Petri dish and tin boxes method on laboratory mite specimens. Clinical examination encompassed controlled application and expert monitoring, by conducting detection measures (Pavlićević, 2007a, 2017a) on a monthly basis until the end of the production year. Examinations have been conducted in the period between 2016 and 2018.

RESULTS

The results of laboratory examination of selected inert formulation are presented in Table 1. In Table 2, the results of clinical examination for selected inert formulations on farms of layers of consumer eggs in cage systems are shown.

Table 1. The results of laboratory examination of selected inert formulation: natural diatomaceous earth (DE), SiO2 formulations for liquid application in plastic Petri cups and formulations with inert oils (P 547/17) in tin boxes.

No	Formula, other	Exposure 1 (min./hr)	Day of results collection, efficacy (%)				
			1.	2.	3.	4.	5.
1	Natural DE, powdered form	min.	12	25	79	98	-
		hr.	33	46	89	98	-
2	Natural DE, powdered form	min.	92	100	-	-	-
		hr.	99	100	-	-	-
3	SiO2 product, liquid form	min.	85	91	-	-	-
		hr.	37	78	88	97	100
4	P 547/17	min.	100	-	-	-	-
		hr.	100	-	-	-	-
5	P 547/17 residual effect 4 months	hr.	99	-	-	-	-
6	P 547/17 residual effect 4 months	hr.	92	-	-	-	-

Table 2. The results of some clinical examination for selected inert formulations on farms of layers of consumer eggs in cage systems: Numbers: 1-11 combined application of powdered and liquid forms of SiO2 formulations, No.: 12-14 inert oils P 547/17

No.	Capacity of facility	Product	Duration of suppression (months)	Finding (-/+) (months)
1	2500	Comb. SiO2, no. (1+3)*	12	12 (-)
2	25000	Comb. SiO2, no.(2+3)	12	12 (-)
3	25000	Comb. SiO2, no. (2+3)	12	12 (-)
4	25000	Comb. SiO2, no. (2+3)	12	12 (-)
5	25000	Comb. SiO2, no. (2+3)	12	12 (-)
6	25000	Comb. SiO2, no.(2+3)	12	12 (-)
7	25000	Comb. SiO2, no.(2+3)	12	12 (-)
8	25000	Comb. SiO2, no. (2+3)	12	12 (-)
9	12000	Comb. SiO2, no. (1+3)	12	12 (+)
10	40000	Comb. SiO2, no. (2+3)	12	12 (+)
11	18000	Comb. SiO2, no. (2+3)	12	12 (+)
12	2000	P 547/17 (15 %)	Pending	6,5 (-)
13	4500	P 547/17 (15 %)	Pending	6,5 (-)
14	20000	P 547/17 (20 %)	Pending	6 (-)

* Represent No. of products from Table 1, which is used for combined application.

DISCUSSION

Although this examination has been primarily focused on the poultry health status aspect of *D. gallinae* control, it also includes the basic elements of the general review of the topic.

The first task in setting down *D. gallinae* control measures is determining the aim of this control. When reviewing the sources of information in *D. gallinae* control, we have noticed that this issue has not been given adequate attention, or that is has been simply claimed that eradication is not possible. With the lack of adequate guidance, the generally accepted practice of *D. gallinae* control is based on suppression.

If the suppression is set as a goal of *D. gallinae* control, it is associated with the following: (1) regular annual expenses for farmers for *D. gallinae* control, which also grow each year (Emous, 2005, 2017), while postponing other harms (best case) or simply both of these together (probably most common); (2) constant presence of parasites, i.e., vectors in the facility; (3) an evident toxicological risk (uncritical control); (4) spreading (prevalence points to a long-term adverse tendency of the disease).

Eradication of *D. gallinae* from production facilities (1) eliminates the toxicological risk that arises from inadequate *D. gallinae* control; (2) eliminates the role of *D. gallinae* vectors; (3) eliminates the harmful effect of *D. gallinae* on the health status of the flock; (4) greatly protects the farmers' economic interest; (5) greatly protects the health and economic interests of the consumers; (6) greatly protects the interests of farm staff; (7) prevents the development of resistance; (8) prevents further expansion of dermanyssosis. These reasons justify eradication.

The feasibility of *D. gallinae* eradication in practical conditions was proven in 2000 in Serbia. Eradication was possible with selected synthetic chemical neurotoxic compounds, acaricides (insecticides). Apart from the basic biological efficacy, it was important to define the level of chemoresistance (Pavlićević et al., 2016). Synthetic chemical neurotoxic compounds are associated with toxicological risks. In order to eliminate them it was necessary to find another option, which would meet the requirements of biological efficacy and rational application. Theoretical analysis as well as comparative laboratory and clinical testing have established the prospective of using selected inert substances in the control of *D. gallinae*.

In laboratory environment, inert substances are selected according to the requirements of biological efficacy and a particularly pronounced residual effect. Apart from this, we have also established their deficiencies and limitations. The optimized use has been achieved by combining the powdered and liquid forms of SiO2, and has successfully confirmed the possibility of eradication by using inert formulations (Figure 3.). Potential limitations of inert formulations for achieving biological efficacy in clinical practice are poor environmental conditions. With the improvement of the product (P 547/17) can be expected to reduce the environmental impact on product efficiency.

Eradication of *D. gallinae* using the aforementioned inert substances is not possible at any time and in all conditions. The conditions for eradication using inert substance include the following: (1) an empty facility; (2) selection of appropriate and efficient formulation, which should be properly applied; (3) proper hygienic conditions; (4) rest period for facility and (5) adequate

temperature conditions. The approach of introducing all necessary measures for *D. gallinae* control to an empty facility and preventing any harmful effect on the flock is immensely important issue of preventive veterinary medicine. Inert formulations differ according to their biological efficacy, so their selection ought to be established in advance. Hygienic conditions (Figure 4.) and an empty facility allow for ideal direct contact with *D. gallinae* population, but also an ideal contact with the surfaces on which a layer with a prolonged effect is to be formed.

Figure 3. Example of a successfully prepared facility by means of a combined SiO2 treatment (Photo Pavlićević).

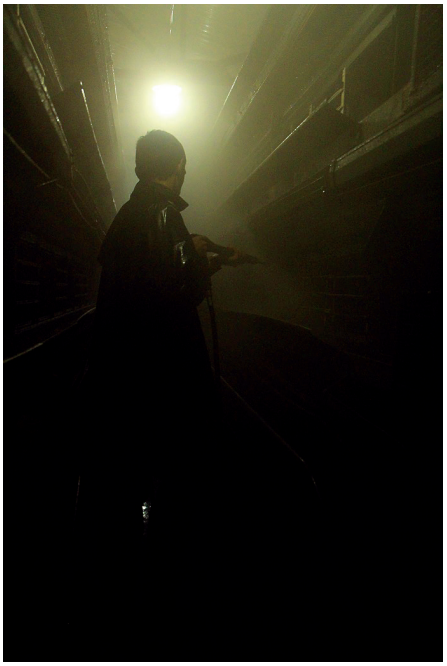

Figure 4. Hygienic preparation, washing the facility with puromate (Photo Pavlićević).

The use of inert substances is highly demanding in terms of hygiene conditions. The currently used technology of poultry keeping is of utmost importance for the success of disinfection. Facility rest period in temperature conditions for mite activity incites *D. gallinae* to leave its hiding places in search of food, thus becoming exposed to the prolonged effect of the inert layer. In time, the infestation is exhausted and finally exterminated. Higher temperatures enhance the procedure and also reduce the necessary break period.

The new generation of inert substances is applied in the form of water emulsion (P 547/17) (Figure 5.). Their use highly improves the most important aspect of *D. gallinae* control, that is, residual effect on non-absorbent surfaces. To the best of our knowledge, such strong residual effect on *D. gallinae* (92% efficiency with 1 hour exposure, after 7 months in laboratory conditions) has not been reported so far. The first test suggests eradication potential, but for absorbent surfaces (e.g. concrete) repeated treatment is required, because on these surfaces, it is not possible to achieve an extended effect with one treatment.

Figure 5. An example of a facility prepared with the new generation of inert substances (P 547/17) (Photo Pavlićević).

Figure 6. An example of the successful eradication of *D. gallinae* by combined SiO2 method, the farm "Boksiti, Milići", Republic of Srpska, Bosnia and Herzegovina (Photo Pavlićević).

The safety is thus ensured in a twofold manner, with the application itself and the type of formulation. Application in an empty facility eliminates the possibility of direct exposure to application and stress of the poultry, and minimizes contact level. In this way, neither the diatomaceous earth has any

significant influence. Inert substances of the new generation are practically non-toxic (Perić, 2018). Eradication of *D. gallinae* completely eliminates (present and future) toxicological risks, which may arise from uncritical control, and especially the use of synthetic neurotoxic chemical compounds.

The test results of inert substance application (in 8 facilities) have confirmed the possibility of successful eradication. The time period of an entire annual cycle and the envisaged exploitation detection, has undeniably confirmed the reliability of eradication findings. In certain cases (in 3 facilities) there was no eradication, but these still presented examples of efficient suppression with mere preparation of the facility that could be eradicated with very little attention.

Clinical examinations are influenced by a multiplicity of factors, which we were not able to uniformly ensure, control or strictly define on this occasion. According to the results of laboratory examination, the very factor of facility rest period of one year is not sufficient for *D. gallinae* eradication (Pavlićević, 2007), but a one-month rest period in the summer period along with the appropriate application of selected inert formulations can enable eradication. Since the influencing factors will need to be more closely defined, the efficient suppression ought to be the first step in the systematic approach to the control program. This way, the toxicological risk is removed; health and economic damages are reduced and gradually, as possibilities are created it would be possible to perform eradication in the facilities, i.e., farms.

To successfully conduct eradication and maintain its full potential, it is necessary to take a comprehensive and thorough program-approach. The program for the control of *D. gallinae* includes the following components: data base, analysis and definition of a logic control approach; rational selection of methods and products; active role of farmers; preparations for the procedure; optimisation of application; eradication control; eradication audit as well as prevention of the re-infestation by introducing biosecurity measures; affirming a wide and preventive information base. Biosecurity measures are important within the farm and especially in relation to external environment. Eradication conducted using SiO2 formulations will be invalidated if a new flock or transportation cages are infested on a high level.

An important part of the problem is the statement that farmers' orientation towards selecting a product can be exclusively economically motivated (Anon, 2017). This situation demands the veterinary professionals to educate farmers about the technological process for the preparation of a new flock, which they will be required to adhere to, thus ensuring an adequate basis for the control of *D. gallinae* and protection of general health status of poultry. An opportunity for the latter is introducing the control of *D. gallinae,* being an

important vector, to the program control measures for related communicable diseases. In this way, the facility rest period applied as a measure in the control of communicable diseases could simultaneously be used for the control of *D. gallinae* (Pavlićević et al., 2017).

Apart from this, eradication should be one of the criteria for classification of communicable diseases which should be reported. Required reporting of *Dermanyssosis* would enable: (1) the protection of not infested farms by implementing relevant biosecurity measures; (2) interest-driven direction of farmers; (3) establishing and monitoring the exact prevalence (if applicable with intensity and extensity of *D. gallinae* infestation); (4) improving toxicological risks prevention; (5) contributing to the systematic control approach; (6) affirming integrated health protection.

Mandatory reporting and implementation of measures and recommendations for technological procedure into the program for communicable diseases (for which it is a vector), would create the conditions for a systematic application of the control program. Eradication of *D. gallinae* from production facilities would lead to the cessation of adverse tendencies of *Dermanyssosis*, health risks and great economic damages. From an economic perspective, the suggested approach can easily be reviewed if we consider the example of a producer with the capacity of 100.000 hens and an estimation of an annual expense of 0.60 Euro per hen (Emos, 2017). In a ten year period, the money savings would be 600.000 Eur.

So far, clinical examinations have been conducted on farms with conventional cages (Figure 6.). Pending examinations are in facilities with enriched cages, which require modification of the procedure. Examinations in aviaries have not yet been conducted, and specific modifications of the program can give successful results. However, organic poultry production and contact with wild birds pose specific challenge. Only future examinations could provide an insight in such situations. For micro producers, who keep poultry in the open, we recommend an alternative approach to *D. gallinae* control rather than eradication (P- 2017/0762).

CONCLUSION

Making eradication the final aim of *D. gallinae* control is technically conceivable. Inert formulations enable a safe and manifold useful procedure. The emphasis is laid on preventive veterinary medicine and its capacity of preventing *Dermanyssosis* tendencies in poultry farming by eliminating harmful consequences and sustaining the integrated health protection.

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PREVALENCE OF THE GIANT LIVER FLUKE (*Fascioloides magna*, Bassi, 1875) IN RED DEER (*Cervus elaphus*) IN THE REGION OF FLOODPLAIN FORESTS OF NORTHERN SERBIA.

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Abstract

This is the first study offering insights into the prevalence of giant liver fluke in the population of red deer in the territory of Serbia. Giang liver fluke (*Fascioloides magna*, Bassi, 1875) is the most important liver parasite among wild ruminants in Europe, especially in the region of floodplain forests along the upper watercourse of Danube river. The main objective of this research was establishing the prevalence of giant liver fluke in the region of floodplain forests of northern Serbia. In the observed regions (hunting grounds), the population prevalence rates ranged from 0 to 80% with an average prevalence in positive herds being 70.6%. The total population of red deer, from the observed hunting grounds, exposed to the giant liver fluke includes 47.9% of red deer population in Serbia, which is 0.7% of the total hunting area of Serbia. Giant liver fluke is present in north-western regions of Serbia in a narrow area of floodplain forests along the watercourse of Danube and Sava rivers next to the border with Croatia. The red deers populating the wetland basin of "Gornje Podunavlje" migrate freely through the tri-border area of Hungary, Croatia and Serbia making a consistent epizootical unit. Moreover, the game migrates freely between Croatia and Serbia in the area of *Posavina* forests along the river Sava. All data obtained in this research are essential for further activities aimed at preventing the spread of this parasite within red deer population and thus decreasing consequent damages and losses.

Keywords: giant liver fluke, red deer, prevalence, Danube, Sava

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PREVALENCIJA AMERIČKOG METILJA (Fascioloides magna, Bassi, 1875) KOD EVROPSKOG JELENA (Cervus elaphus) NA PODRUČJU PLAVNIH ŠUMA SEVERNE SRBIJE.

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Kratak sadržaj

Ovo je prvi rad koji pruža uvid u prevalenciju prisustva američkog metilja u populaciji evropskog jelena na prostoru Srbije. Američki metilj (*Fascioloides magna*, Bassi, 1875) je najznačajni parazit jetre divljih preživara Evrope, a pogotovo na području podunavskih plavnih šuma u gornjem toku. Ovaj rad je imao za cilj da utvrdi prevalenciju američkog metilja kod evropskog jelena (Cervus elaphus) na području plavnih šuma severne Srbije. Prevalencija u posmatranim regionima (lovištima), u populacijama evropskog jelena se kretala od 0-80%, sa prosečnom prevalencijom kod pozitivnih populacija od 70,6%. Ukupna populacija evropskog jelena, poreklom iz posmatranih lovišta, izložena američkom metilju je 47,9% ukupne populacije evropskog jelena na teritoriji Srbije, koja se nalazi na 0,7% ukupne lovne površine Srbije. Američki metilj je prisutan u severozapadnim delu Srbije, u uskom pojasu podunavskih i posavskih plavnih šuma, uz granicu sa Hrvatskom. Jeleni koji obitavaju u basenu plavnih šuma "Gornje Podunavlje" slobodno migriraju u trouglu Mađarska, Hrvatska i Srbija i čine jedinstvenu epizootiološku celinu, takođe kao i na području posavskih šuma jeleni se slobodno kreću u pograničnom pojasu Hrvatske i Srbije. Svi dobijeni podaci su neophodni za buduće aktivnosti u cilju prevencije širenja i smanjivanja šteta u populaciji evropskog jelena.

Ključne reči: američki metilj, evropski jelen, prevalencija, Dunav, Sava

INTRODUCTION

Giant liver fluke (*Fascioloides magna*, Bassi, 1875) is nowadays considered the most important liver parasite in wild ruminants in Europe, especially in the regions along the watercourse of Danube river (Králová-Hromadová et al., 2016). Giant liver fluke is a parasitic organism of the class *Trematoda* family *Fasciolidaese*. Mature fluke usually measures 4 – 10 cm in length, 2 – 3.5 cm in width and are 0.2 - 0.45 cm thick (Figure 1.) (Erhardová, 1961). The organism is highly invasive species, which is mainly due to the wide spectrum of potential definitive and intermediate hosts, its pronounced ability to adapt to new hosts, extensive spatial distribution and potential to colonize new territories (Králová-Hromadová et al., 2016; Pybus, 2001).

The development and life cycle of giant liver fluke *requires* an aquatic *snail* (*Lymnaea* spp., *Radix* spp.) as an intermediate host (Erhardová-Kotrlá, 1971; Pybus, 2001). The development in the interemediate host lasts some 2.5 months, while the metacercariae remain infectious for the host during 2 – 2.5 months (Králová-Hromadová et al., 2016; Pybus 2001). In case of *Fascioloides magna*, further development is determined by host species, which are divided into definitive, *dead-end* and *aberrant* ones (Pybus, 2001). Upon ingestion of infectious metacercariae, the prepatent period in definitive hosts can extend from 3 (Erhardová-Kotrlá, 1971) to even 7 months (Foreyt and Todd, 1976). In Europe, red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) are the most common definitive hosts that facilitate completing of the life cycle of *Fascioloides magna* in liver pseudocyst and release of eggs via the billiary liver system into the intestines (Králová-Hromadová et al., 2016; Pybus, 2001). Adult flukes can survive in definitive hosts even up to five years (Erhardová-Kotrlá, 1971).

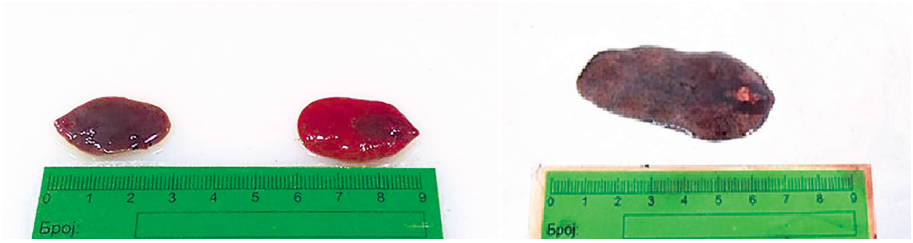

Figure 1. Giant liver fluke (*Fascioloides magna*, Bassi, 1875).

The parasite is originally native to North America, but has been introduced to Europe during import of North American deers. The first case recorded in Europe was in the wapiti stag (*Cervus elaphus nelsoni)* imported in 1865 from

Wyoming (USA) into the Royal National Park La Mandria in northwestern Italy (Pybus, 2001). Besides the territory of Italy, two further infection foci were identified in the region of Czech-Polish border and floodplain forests along the Danube in Central Europe (Králová-Hromadová et al., 2011, 2016). The first reports on substantial spread of giant liver fluke in *Danube* River Basin have been published during last 25 years, confirming its presence in Slovakia (Rajský et al., 1994), Hungary (Majoros and Sztojkov, 1994), Austria (Winkelmayer and Prosl, 2001), East Croatia (Marinculić et al., 2002) and northwestern parts of Serbia (Trailović et al., 2008).

The most common pathoanatomical finding in the liver associated with giant liver fluke infection includes presence of fibrous pseudocysts containing usually two parasites (pairs), free migrating of immature flukes, marbled black pigmentation (hematin) of the parenchyma or below the capsule, rupture of the capsule and perihepatitis (Pybus, 2001). Giant liver fluke infection in definitive host usually results in poor growth, decreased productivity and (rarely) mortality (Pybus et al., 2015; Trailović et al., 2008).

Only two members of the family *Fasciolidae*, *Fasciola hepatica* and *Fasciola gigantica*, manifest zoonotic potential, while human infections associated with *Fascioloides magna* have not been reported so far (Mas-Coma, 2005; Pybus, 2001). Hygienic safety of meat and internal organs is evaluated using the same method as in the case of domestic ungulate *fascioliasis, depending on the invasion rate and apparent changes* (Herenda et al., 2000).

The objective of this research was to establish the prevalence of giant liver fluke (*Fascioloides magna*, Bassi, 1875) in the region of floodplain forests of northern Serbia. The data obtained in this research are indispensible for further activities aimed at preventing the spread of this parasite within red deer population and thus decreasing consequent damages and losses.

MATERIAL AND METHODS

The research was conducted in the period September 2017 - January 2018 in the territory of northern Serbia (Vojvodina region) encompassing total 3.275 ha in the provinces of Bačka, Srem and Banat. In the province of Bačka, we observed the hunting areas: "Kozara", "Apatinski rit" and "Plavna", in the province of Srem "Bosutske šume", "Kućine" and "Karakuša", and in the province of Banat hunting ground "Deliblatska peščara" (Table 1.). The investigation sites are located along the watercourse of Danube and Sava rivers, mostly in floodplain forest habitats containing most dense red deer population. Observed red deer are grouped into three separate populations (Bačka, Srem, and

Banat) based on territorial (regional) separation or degree of mutual contact (Figure 2.).

Table 1. Investigated hunting grounds, surface area and density of red deer (*Cervus elaphus*) population.

Region	District	Municipality	Hunting ground (ID number)	Number of individ. animals	Surface area of the hunting ground (ha)	Population density (number of animals / 100 ha)
Bačka	West Bačka	Sombor	„Kozara“ *(2332)*	1.426	11.507,63	12,39
		Apatin	„Apatinski rit“ *(2331)*	411	6.335,76	6,49
	South Bačka	Bač	„Plavna“ *(2334)*	118	3.629,29	3,25
	Σ			*1.955*	*21.472*	*9,10*
Srem	Srem	Šid	„Bosutske šume“ *(3015)*	402	14.912,18	2,70
			„Kućine“ *(3018)*	89	1.986,48	4,48
		Ruma	„Karakuša“ *(3016)*	165	8.125,22	2,03
	Σ			*656*	*25.023*	*2,62*
Banat	South Banat	Kovin	„Deliblatska peščara“ *(2710)*	664	31.036	2,14
	Σ			*664*	*31.036*	*2,14*
Bačka+Srem				*2.611*	*46.495*	*5,62*
Bačka+Srem+Banat				*3.275*	*77.531*	*4,22*

The total area encompassed by the sampling was 60.396 ha with an estimated population of 3.275 deer game animals. A total of 79 samples of red deer liver was examined for the presence of the giant liver fluke (*Fascioloides magna*, Bassi, 1875). All animals encompassed by this research were shot during regular hunting. After shooting, the livers were collected, labeled and individually packet into the plastic bags and stored at 4°C. Subsequently, parasitological and pathomorphological examination were performed. Parasitological and pathomorphological examination revealed the presence of parasites and the

invasion rate and pathoanatomical liver changes were recorded. The obtained data were statistically analyzed using IBM SPSS Statistics 20 (IBM, Armonk, NY, USA), and for figure graphic processing we used software desktop QGIS 3.2.1 (OSGeo, USA) and CRS MGI 1901/Balkans zone 7, EPSG:3909.

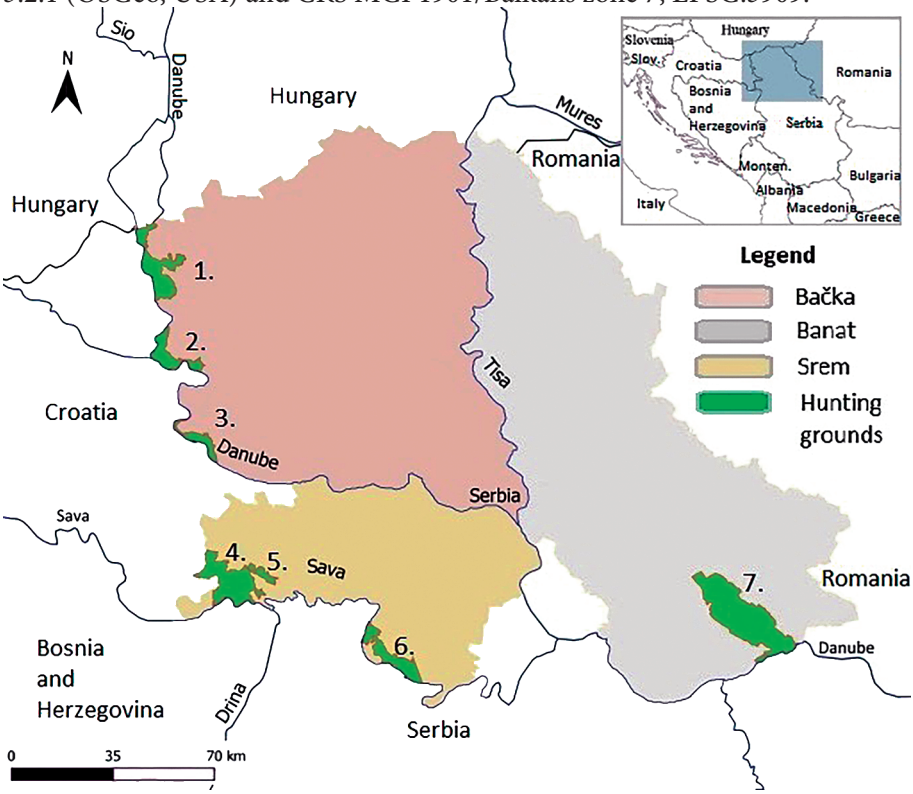

Figure 2. Location of the hunting ground (1. „Kozara“, 2. „Apatinski rit“, 3. „ Plavna“, 4. „Bosutske šume“, 5. „Kućine“, 6. „Karakuša“, i 7. „Deliblatska peščara“).

RESULTS

Total number of 79 livers from three distinct red deer populations was investigated as following: 25 livers from the hunting grounds in Bačka region, 26 livers from hunting grounds in Srem region and 28 livers from the hunting grounds in the Banat region revealing the overall prevalence of 45.6% (36/79) of positive animals. Among the red deer population on observed hunting grounds in the region of Bačka, the presence of parasite was confirmed in 80% (20/25) animals out of which 35% had 10≥ parasites in the liver, whereas the findings from hunting grounds in the region of Srem revealed parasite pres-

ence in 61.5% (16/26) animals and 75% with 10≥ parasites in the liver. An average prevalence in populations with confirmed presence of giant liver fluke was 70.6% (36/51) animals, out of which 52.8% contained 10≥ parasites in the liver. On the observed hunting ground in the territory of Banat, the presence of giant liver fluke has not been confirmed in the population of red deer. All animals positive for the presence of giant liver fluke originated from floodplain forests along the upper watercourse of Danube and Sava rivers in Serbia.

Table 2. The prevalence of giant liver fluke within red deer population and liver invasion rate.

Region	Number of samples	Prevalence	Invasion rate		
			High (10≥)	Medium (3-9)	Low (≤2)
Bačka	25	20 (80)	7 (35)	4 (20)	9 (45)
Srem	26	16 (61.5)	12 (75)	3 (18.8)	1 (6.2)
Banat	28	0	0	0	0
Total	*79*	*36 (4.,6)*	*19 (52.8)*	*7 (19.4)*	*10 (27.8)*

Giant liver fluke was identified in 36 livers. The observed pathoanatomical changes revealed presence of cysts filled with dark-coloured substance containing parasites, which in case of large number of individuals protrude above the liver surface and alter its outer appearance (Figure 3.). Liver parenchyma was marbled with dark pigmented stripes and spots in 63.9% (23/36) of cases, whereas perihepatitis was observed in 69.4% (25/36) livers.

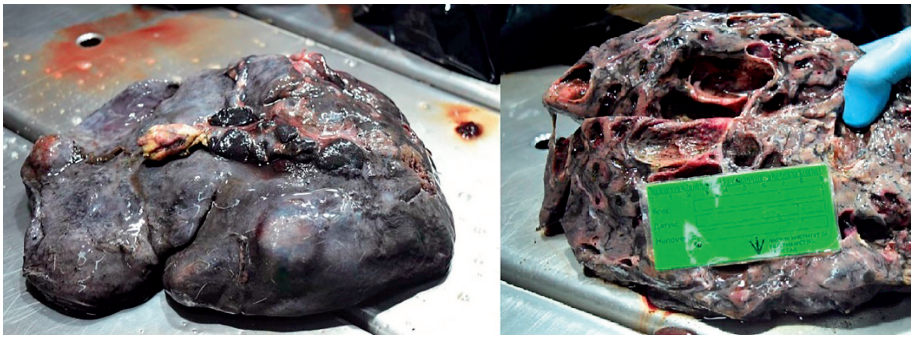

Figure 3. The presence of pseudocysts in the liver of red deer associated with giant liver fluke (*Fascioloides magna*, Bassi, 1875) infection.

DISCUSSION

First reports on the presence of giant liver fluke in Serbia date back to 2008. The parasite was indentified in fallow deer (*Dama dama*) originated from fenced hunting ground in South- Bačka District (Trailović et al., 2008, 2016). The data on the presence and/or distribution of giant liver fluke infection in red deer (*Cervus elaphus*) have not been available so far. Some available literature data on the presence of giant liver fluke among the red deer populations in neighboring countries such as Hungary and Croatia (Majoros and Sztojkov, 1994; Pybus, 2001; Marinculić et al., 2002; Janicki et al., 2005) suggested potential presence of the parasite in Serbia, especially in northwestern regions bordering Hungary and Croatia. In countries of the *Danube* River Basin, the programs for monitoring giant liver fluke in deer game have been implemented and are mainly based on the identification of parasites and/or their eggs in the faeces.

The prevalence of giant liver fluke recored in the observed population of red deer in the area of floodplain forests of northern Serbia ranged from 0 to 80.0% with an average prevalence in positive herds being 70.6% (61.5 – 80.0%), which corresponds with the results in the countries of the *Danube* River Basin such as Slovakia, Austria, Hungary and Croatia, where prevalence rates sometimes exceeded 60% (Rajský et al., 2002; Špakulová et al., 1997; Giczi, 2008; Králová-Hromadová et al., 2016; Slavica et al., 2006). The invasion rate in positive livers ranged 6.3 - 45% for low invasion rate (≤2 parasites), 18.8 - 20% for medium invasion rate (3-9 parasites), and 35 - 75% for high invasion rate (10≥ parasites) (Table 2.).

The dominant population of large game in the northern part of Serbia includes roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*). In the territory of Serbia, hunting grounds occupy 7.132.368 ha, out of which 1.964.957 ha are in Vojvodina Region (Statistical bulletin, 2016). The population of red deer in Serbia is estimated to 5.522 animals, out of which 4.337 animals are in Vojvodina Region (Statistical bulletin, 2016). In this research, we identified and defined the area in which the presence of giant liver fluke has been confirmed, that is, 46,495 ha of the total 60,396 ha that makes 0.7% and 2.4% of the total hunting ground surface in Serbia and Vojvodina, respectively, and with an average density of the investigated population being 5.6 animals / 100 ha. Total population exposed to the giant liver fluke infection includes 2,611 animals from observed hunting area of 46,495 ha, which makes 47.9% and 60.2% of red deer population in Serbia and Vojvodina Region, respectively.

CONCLUSION

The data on the prevalence of giant liver fluke within the observed hunting grounds in the area of floodplain forests of northern Serbia, provides important insights into the high rate of exposure and hazards to the population of red deer. Moreover, the broad transboundary epizootical area strongly suggest the necessity of continuous monitoring of distribution of giant liver fluke with an aim of preventing its spreading and preventing the damages and losses in the population of red deer in this area.

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ELECTRONIC RAT-CONTROL DEVICES – SOLUTION OR SCAM? RESULTS OF FIELD TRIALS AND A SUMMARY OF THE LITERARY DATA

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Abstract

The electronic rat-control devices are humane means of controlling harmful rodents without toxic substances. They are relatively inexpensive and very easy to use and have gained increasing popularity in recent years. Although they have been introduced long ago in the practice of deratization, scientific information about their real effectiveness is scarce and at the same time very controversial. The purpose of this study was to evaluate the repellent efficiency of an electronic device using the combined action of ultrasonic waves, light signals, and electromagnetic field change in practice. Two field trials were carried out on a cattle-breeding farm and a feed warehouse inhabited by brown rats (*Rattus norvegicus*) and roof rats (*Rattus rattus*). Repellent efficacy was determined by comparing the indicators evaluating the presence and activity of rodents during the pre-testing period before the inclusion of the device and after its activation during the test period. A lack of repellent effect was found in both field studies. The results obtained are supported by an analysis of the scientific literature confirming the inadequate effectiveness of electronic rat control devices in practical conditions. Emphasis is placed on the deficiencies and the need for regulatory adjustments governing the control and admission of electronic devices to control rats on the market.

Keywords: deratization, ultrasound, electromagnetic, electronic device, rat control.

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ELEKTRONSKI UREĐAJI ZA KONTROLU PACOVA – REŠENJE ILI PREVARA? REZULTATI TERENSKIH ISPITIVANJA I PREGLED LITERATURNIH PODATAKA

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Abstract

Elektronski uređaji za kontrolu pacova predstavljaju humano sredstvo za kontrolu štetnih glodara bez toksičnih supstanci. Ovi uređaji su relativno jeftini, veoma lako se koriste i postaju sve popularniji tokom poslednjih nekoliko godina. Iako su već odavno prisutni u praksi, naučni dokazi o njihovoj efikasnosti su šturi, a istovremeno i vrlo diskutabilni. Cilj ove studije bila je evaluacija efikasnosti repelentne zaštite elektronskog uređaja koji primenjuje kombinovano dejstvo ultrazvučnih talasa, svetlosnih signala i promene elektromagnetnog polja u praksi. Sprovedena su dva terenska ispitivanja – jedno na farmi goveda, a jedno u skladištu stočne hrane koje su naseljavali sivi pacovi (*Rattus norvegicus*) i crni pacovi (*Rattus rattus*). Efikasnost repelentne zaštite odredjivana je na osnovu poređenja indikatora za prisustvo i aktivnost glodara tokom perioda pre istraživanja i uvodjenja uredjaja i nakon aktivacije uređaja tokom eksperimentalnog perioda. Odsustvo repelentnog efekta ustanovljeno je u obe terenske studije. Dobijene rezultate potkrepljuje i analiza naučne literature koja potvrđuje neadekvatnu efikasnost elektronskih uređaja za kontrolu pacova u praktičnim uslovima. Naglasak je stavljen na manjkavosti i potrebu za zakonskim usklađivanjem u oblasti kontrole i puštanja u promet elektronskih uređaja za kontrolu pacova.

Ključne reči: deratizacija, ultrazvuk, elektromagnetni, elektronski uređaj, kontrola pacova.

INTRODUCTION

The electronic rat-control devices are an alternative to the chemical method of controlling harmful rodents and have gained increasing popularity in recent years. It is believed that these are humane means of reducing the number of rodents on a given territory as they create an environment with unfavourable conditions for their development but without causing a strong pain response, agony or death. Due to the absence of toxic substances they are considered to be environmentally safe. The devices are relatively inexpensive and very easy to use (Bomford and O`Brien, 1990). Electronic rat-control devices use different principles to repel rodents - some emit ultrasonic waves that negatively affect rodents, while others modify the electromagnetic field around electric conductors or emit sudden and strong light signals. Despite a wide variety of different commercially available models, as well as extensive media advertising, many users report unsatisfactory performance in practice. The information on their effectiveness in the scientific literature is scarce and at the same time there is a serious contradiction between the results of tests carried out in laboratory and field conditions (Maclean, 1970; Shumake et al., 1982; Shumake et al., 1984; Shumake, 1997). This motivated us to conduct our own research to assess the suitability of these agents for controlling synanthropic rodents in real-world practice.

MATERIALS AND METHODS

The repellent efficacy in field conditions of an electronic device (Pest X Repel, model PR-500.3, Microsys Co Ltd., Bulgaria) with a combined action was tested. The device emits ultrasonic signals at a frequency of 15 - 36 kHz and changes the electromagnetic field in the objects. At the same time, with the built-in flash lamp, it also emits sudden, powerful light signals at a frequency of 75 units per minute. The device is programmed to permanently change the shape and frequency of the transmitted pulses in order to avoid rodents accustoming to them. According to the manufacturer, one appliance is sufficient to repel rodents on an area of 200 m^2, with the initial repellent effect occurring within 1-2 weeks, and the maximum effect is achieved after the third week. During this period, rodents should leave their hiding places and the protected site (Annonimous).

The studies were conducted in two areas inhabited by rodents:

Site №1 - a cattle-breed farm infested by brown rats (*Rattus norvegicus*) with high density (50 - 100 specimens/100 m^2). The device was placed near an

electrical distribution board in a barn of about 180 m^2.

Site №2 - a feed warehouse infested by roof rats (*Rattus rattus*) with medium density (10 - 50 specimens/100 m^2). The device was placed near an electrical distribution board in an area of 50 m^2.

The repellent efficiency of the electronic device in the objects was determined by comparing indicators that evaluated the activity and the number of rodents during the pre-test period before the inclusion of the device and after its activation during the test period. The following was determined: the number of food sources visited, the average daily consumption of non-toxic food bait, the number of dusty track plates visited and the intensity of the traces left, as well as the number of active holes (only in the site №1, infested by brown rats with digging activities). These indicators were determined every 5 days during the ten days pre-test period and during the test period, which was 35 days in the cattle-breeding farm and 60 days in the feed warehouse.

The statistical data analysis was processed using GraphPad software. Comparison of results between the groups was done using unpaired t-test. Differences were defined as statistically significant in values of $P < 0.05$.

RESULTS

The results of the field trials are presented in table 1. In both sites, no statistically significant decreases in rodent density and activity were detected after activation of the electronic device.

Table 1. Dynamics in rodent density and activity during the pre-test and test period.

Table 1. Dynamics in rodent density and activity during the pre-test and test period.

		Pre-test period (days)			Test period (days)											
		5th	10th	Average ± SD	5th	10th	15th	20th	25th	30th	35th	40th	45th	50th	55th	60th	Average ± SD
Cow-breeding farm	A1	10	10	10	10	10	10	10	10	10	8	-	-	-	-	-	9.7
	B1	650	720	685 ± 49.5	680	621	705	625	670	695	740	-	-	-	-	-	676.57 ± 42.77
	C1	10	10	10	10	10	10	10	10	10	10	-	-	-	-	-	10
	D1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++
	E	15	19	17	17	20	16	23	15	15	18	-	-	-	-	-	17,7
Feed warehouse	A2	6	8	7	5	5	8	5	10	5	8	7	7	8	10	5	9,62
	B2	115	138	126.5 ± 16.26	155	140	165	180	130	148	136	177	125	146	175	136	151.1 ± 19.13
	C2	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	D2	++	++	++	++	+++	++	++	++	++	++	++	++	+	++	++	++

Legend: A - number of visited food sources (n=10), B - daily consumption of non-toxic food baits (g/24h), C - number of visited dusty track plates (n=10), D – intensity of traces, E - number of active holes, SD – standard deviation.

DISCUSSION

The scientific literature data on the effectiveness of electronic repellent devices revealed a significant contradiction between the results of tests carried out in controlled laboratory conditions with small groups of synanthropic rodents and those performed in real practice in field studies. For example, in the laboratory tests performed by Maclean (1970) to evaluate the repellent properties of an ultrasound device, satisfactory efficacy was found in the repulsion of rats and mice. However, he found higher repellent efficiency in cases where he gave rodents free access to alternative sources of food and water in ultrasonic free spots (Maclean, 1970). Similar results in laboratory conditions were obtained from Shumake et al. (1982), who studied the impact of three ultrasound devices (20, 20-30 and 40kHz) on the behaviour and intake of food in Philippine black rats (*Rattus rattus mindanensis*). They found that when the food was plentiful, the rats significantly reduced the intake of food in ultrasound chambers, preferring to feed in the chambers without ultrasonic waves. However, when the food was limited, insufficient to meet the physiological needs of rats, only 20-30 and 40 kHz chambers showed a decrease in dietary intake compared to the control chamber. The authors note that the achieved repellent effect was partial - despite lower food intake, the cameras were still visited by rodents, which in practice does not exclude epidemiological risks and in some cases proved to be insufficiently effective, such as in the food industry (Shumake et al., 1982).

Unlike comparatively positive results in laboratory tests, the performance data of electronic repellent devices in real-world practice conditions are definitely negative. Already in 1962, Marsh et al. reported a lack of efficiency of a 15 kHz ultrasonic generator in a grain storage facility (Marsh et al., 1962). Similar are the results of Sprock et al. (1967), who studied the efficiency of an electronic generator emitting sound and ultrasonic waves in the range of 1.8 - 48 kHz in domestic mice and rats.

Lavoie and Glahn (1977) conducted field trials in brown-rat sites with two types of ultrasound devices, one with a frequency variation, and the other with a constant frequency of 20 kHz. For both sets, manufacturers claimed to have high efficiency in rodent repulsion. After 3 weeks, no statistically significant reduction in rodent activity and food consumption was achieved in the first type of device. In the second type of device, the initial reduction in rodent activity was followed by recovery to baseline levels after the first week, indicating rapid adaptation of rodents. Howard and March (1985) also found that ultrasound devices are not enough effective in practical conditions.

The large-scale tests on the effectiveness of 6 ultrasound devices available from the United States, conducted by Shumake (1997), found that 5 of them did not exhibit even a minimal repellent effect, and one of the devices had a partial temporary effect - just 3 days after the start of the tests, restoration of the initial parameters of the number of rodents started. Based on the obtained results, these devices have been identified as practically ineffective. The same author note that 25 years after the ultrasound device was started, there is still no conclusive scientific evidence demonstrating their effectiveness in the repulsion of synantropic rodents (Shumake et al., 1984).

Scientific data on the other type of electronic devices (electromagnetic) are similar. Thus, in a US study on the effectiveness of an electromagnetic repellent device in a feed stock house infested by house mice, it was found that 2 weeks after activation, traces of rodents were observed in 50% of the tracking sites. After 3 weeks, the number of sites has increased to 75%, including those in close proximity to the device, which is an indication of an increase in rodent activity and insufficient efficiency of the electromagnetic device (Fitzwater, 1978). Rooney and McKeen (1977) also did not detect a statistically significant decrease in the number of domestic mice inhabiting a poultry farm in California (USA) after an electromagnetic device was inserted. The effectiveness of the same device was previously investigated by Kruger in 1975, who even found an increase in the number of rodents in a poultry farm during the testing of the device (Fitzwater, 1978). Similar results in tests with another electromagnetic device were obtained by Steve Palmateer (1977, EPA Animal Biology Laboratory, Beltsville, Maryland, USA), who found very small, statistically insignificant differences in food consumption, breeding and activity of brown rats from the control and experimental group (Fitzwater, 1978).

In our field studies with a combined electronic device emitting ultrasonic waves, strong light signals and electromagnetic field changes, similar results were obtained to the above results. No statistically significant differences in rodent density and activity were found in both the cattle-breed farm, infested by brown rats and the feed warehouse, infested by roof rats. On the basis of the results obtained, it can be concluded that the electronic device has an unsatisfactory repellent efficiency in the practice conditions of objects inhabited by these two species of rodents.

In support of our results and conclusions, Bomford and O'Brien (1990) studies aim to analyze and summarize the published research on the effectiveness of ultrasonic devices in the control of harmful animal species. Summing up dozens of different scientific reports, they found that ultrasound devices were either ineffective in practice, or their effect was partial and temporary,

and concluded that they were unsuitable for practice. Shumake (1997) makes a similar summary of electromagnetic efficiency, based on a large-scale study by the US Environmental Protection Agency (Annonimous, 1980), which conclusively demonstrated the absence of any negative impact on the devices in the feeding, water intake, reproduction and displacement of brown rats. Consequently, based on the results obtained, the EPA fined the manufacturers of these devices and filed lawsuits (Shumake, 1997).

The low efficiency of electronic devices in practice causes the European and Mediterranean plant protection organization (EPPO, France) to declare that the use of these devices is not a good GPP in rodent control (OEPP/EPPO, 1998).

Why do these devices continue to advertise and market? The answer to this question may lie in the fact that in 1982 only in the United States the sales of ultrasonic repellent devices amounted to 17 million dollars and in the next decades they have increased many times. It is clear that it is a highly profitable business, suggesting that the problem can only be solved if much stricter and radical legislative measures are introduced (Bomford and O`Brien, 1990; Mix, 1984). In this regard, the Federal Trade Commission (FTC, USA) establishes that many manufacturers produce repellent devices that claim to be highly effective in repelling rodents, but these claims are often not supported by research results. That's why, in 2001, the FTC warned more than 60 companies in the USA that produced, advertised and sold electronic pest-control devices whose efficiency was not supported by any research (Federal Trade Commission, 2001).

At the same time, it has to be noted that many users report the success of rodent evasion and high efficiency of electronic devices. These assessments are in most cases subjective and could not serve as a basis for general conclusions, but should not be overlooked either. Unfortunately, at present, there is no list of producers and trademarks for which there is scientific evidence or reliable reviews of the practice for their high efficiency as well as those that are a typical counterfeit. Therefore, ordinary users and deratization professionals are very often involved in misleading misconduct by mass media advertising (Federal Trade Commission, 2003).

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DYNAMICS OF MICROBIAL CONTAMINATION IN A POULTRY HATCHERY

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Abstract

The hatcheries may become contaminated with pathogenic bacteria which could spread in the hatchery through the air. That is why the implementation of an effective cleaning and disinfection program and the maintenance of good hygiene are extremely important for the normal course of production and for reducing the spread of infectious agents. In this study, data on the degree and dynamics of bacterial contamination in a poultry hatchery are presented. In the incubation sector, bacterial contamination on the surfaces was found to be low-level ranging from 0.25 to $4.43x10^1$ CFU/cm^2 but in the air it was strongly influenced by the hatching. In the hatchery sector, bacterial contamination on the surface and in the air was high, with the highest values found on the egg shells ($1.77x10^6$ CFU/cm^2), on the floor ($3.2x10^4$ CFU/cm^2) and in the air ($1.77x10^5$ CFU/cm^3) of hatcher cabinets during hatching. The results obtained show that the most important source of microbial contamination in the hatchery is the hatchery sector, especially during hatching, when highly contaminated materials as fluff, shells and dried secretions are released. In case of poor organization of working process, the bacteria could spread by air and contaminate the other sectors of the hatchery. The study confirms the importance of a different approach in the development of preventive measures, depending on the degree of risk in different zones in the hatchery, which is the basis for the effective management practice aimed at decreasing microbiological hazards in hatcheries.

Keywords: poultry, hatchery, microbial contamination, hygiene, risk

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DINAMIKA MIKROBIOLOŠKE KONTAMINACIJE U INKUBATORSKIM STANICAMA

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Kratak sadržaj

U inkubatorskim stanicama može doći do kontaminacije patogenim bakterijama koje se dalje šire putem vazduha. Iz tog razloga implementacija efikasnog programa čišćenja i dezinfekcije kao i pravilno održavanje higijene su od ključnog značaja za održavanje normalnog toka proizvodnje i ograničavanja širenja infektivnih agenasa. U ovoj studiji prezentivani su podaci o stepenu i dinamici bakterijske kontaminacije u inkubatorskoj stanici. U sektoru za inkubaciju ustanovljen je nizak nivo bakterijske kontaminacije na površinama, koji se kretao u rasponu od 0,25 do 4,43x10[1] CFU/cm[2] ali je samo leženje imalo veliki uticaj na nivo kontaminacije u vazduhu. U sektoru za leženje pilića ustanovljen je visok nivo bakterijske kontaminacije na površinama i u vazduhu sa najvišim vrednostima izmerenim na ljusci jajeta (1,77x10[6] CFU/cm[2]), na podu (3,2x10[4] CFU/cm[2]) i u vazduhu (1,77x10[5] CFU/cm[3]) u inkubatorima tokom procesa leženja. Dobijeni rezultati ukazuju na to da je najvažniji izvor mikrobiološke kontaminacije u inkubatorskim stanicama sektor za leženje pilića, naročito tokom samog procesa leženja, kada dolazi do oslobađanja visoko kontaminiranih materijala kao što je paperje, ljuske i osušeni ekskreti. Ova studija je potvrdila značaj različitog pristupa u razvijanju preventivnih mera u zavisnosti od stepena rizika u različitim zonama inkubatorskih stanica, što predstavlja osnovu za efikasno upravljanje sa ciljem da se smanje mikrobiološki hazardi u inkubatorskim stanicama.

Ključne reči: živina, inkubatorska stanica, mikrobiološka kontaminacija, higijena, rizik

INTRODUCTION

The hatcheries are animal husbandry facilities which may become contaminated with pathogenic microorganisms. Because of the constant air flow in hatchery there is a risk of disease transmission, especially during hatching process (Ayubi and Karadzhov, 1994a).

It is established that the extent of bacterial contamination in poultry hatcheries is one of the main factors influencing the egg hatching, the embryonic death and the vitality of newly hatched chicks (Mauldin, 2008; Qureshi, 2002). Thus, the implementation of an effective cleaning and disinfection program, and the maintenance of good hygiene is extremely important for the normal course of production and to reduce dissemination of pathogenic microorganisms (Ayubi and Karadzhov, 1994a; 1994b).

The aim of the present survey was to determine the level and the dynamics of bacterial contamination during the production process in a poultry hatchery. This would allow to identify the sources of bacterial contamination and to implement relevant preventive measures in different areas of the hatchery, which is an important prerequisite for the high efficiency of the disinfection program and application of the effective management systems in hatcheries.

MATERIAL AND METHODS

The study was carried out in a poultry hatchery located in South Bulgaria. A disinfection program was performed and it included fumigation of incoming eggs with formalin (53 mL formaldehyde, 35 g potassium permanganate per 1 m^3), aerosol disinfection of incubator cabinets filled with eggs during the first day of incubation (30 mL formaldehyde per 1 m^3), and daily prophylactic spray disinfection of the floor and walls in the hatcheries with aqueous disinfectant solution containing glutaraldehyde and quaternary ammonium compounds. The same disinfectant, in shape of foam is used for disinfection of the equipment as well as the empty hatchers and incubators after chickens have been removed.

The level and the dynamic of microbial contamination in each facility during the various technological stages were estimated by conventional techniques of microbiological testing. Thus, samples of different surfaces and the air were taken.

The samples of the surfaces were taken by microbiological swab method. A sterile cotton swab, soaked in sterile saline solution, was rubbed in a surface of 25 cm^2 by using sterile metal template. The samples were put in tubes, con-

taining 10 mL sterile saline solution and refrigerated during transportation to the laboratory. Sample homogenization and content extraction was performed by vigorous shaking in a vortex for 3 minutes, after which the tampons were removed from the tubes. Ten-fold dilutions (from 10^{-1} to 10^{-7}) were prepared in sterile saline solution. For the quantification of the total number of mesophilic aerobic and facultative anaerobic microorganisms, aliquots of 0.1 mL from the primary dilution and the corresponding decimal dilutions were sowed in Tryptone Soya Agar (HiMedia, India). The samples were incubated at 37° C for 48h. The grown colonies were counted twice - on the 24th and 48th hour of the incubation, and final result was presented as the highest colony amount. The counting was performed by using a digital colony counter (Colony counter LA660, HiMedia, India).

The amount of microorganisms per 1 cm^2 of the control surfaces was calculated depending on the number of grown colonies on the agar and the corresponding rate of dilution. For every control surface, 6 cotton swab samples were examined and the average results are presented.

The air samples were obtained by the passive sedimentation method. Petri dishes, containing Tryptone Soya Agar (HiMedia, India), with a diameter of 8.5 cm were used. The plates were arranged at different heights in the hatchery. They were opened for a certain period of time (from 2 to 10 min.) pursuant to the expected microbial contamination. The samples were incubated at 37° C for 48h. The grown colonies were counted twice-on the 24th and 48th hour of the incubation, and the highest number of bacteria cells was taken as a final result. Depending on the number of grown colonies on the agar and the exposure of the Petri dishes, a total bacterial count of 1 m^3 was determined (Stryjakowska-Sekulska et al., 2007). For each studied zone, 6 Petri dishes were put on different levels, and the average results were taken.

The statistical data processing was performed using GraphPad software. The results were presented as the average amount of 6 tests ± Standard deviation. Comparison of the results between two groups was performed by unpaired t-test. The differences were considered as statistically significant when P value was less than 0.05 ($P< 0.05$).

RESULTS

The results of the microbiological examinations of the samples are presented in Table 1.

Table 1. Dynamics of bacterial contamination in the incubatory and hatchery sector.

	Subunit / Surface	Technological stage							
		Incubation period					Hatching period		
		1 Day	6 Day	12 Day	18 Day	Average	20 Day	21 Day /Hatching/	Average
Incubatory/ Hatchery hall	Wall	$0.4x10^{1}\pm0.36$	$0.93x10^{1}\pm0.75$	$1.07x10^{1}\pm0.55$	$0.87x10^{1}\pm0.59$	$0.82x10^{1}\pm0.6$	$1.7x10^{1}\pm0.49$	$3.4x10^{2}\pm0.42^{*H}$	$1.78x10^{2}\pm1.71^{*IH}$
	Floor	$1.0x10^{1}\pm0.9$	$0.47x10^{1}\pm0.47$	$1.06x10^{2}\pm0.39^{*I}$	$5.8x10^{1}\pm3.19$	$4.43x10^{1}\pm4.81$	$2.42x10^{2}\pm1.02$	$2.36x10^{4}\pm1.8^{*H}$	$1.19x10^{4}\pm1.72^{*IH}$
	Air	$8.82x10^{2}\pm3.7$	$7.36x10^{2}\pm5.28$	$8.7x10^{4}\pm1.87^{*I}$	$9.4x10^{2}\pm6.86$	$2.24x10^{4}\pm3.91$	$1.77x10^{3}\pm0.77$	$1.58x10^{5}\pm0.2^{*H}$	$8.0x10^{4}\pm8.29$
Incubator / Hatcher cabinets	Wall	0	$0.27x10^{1}\pm0.21^{*I}$	$0.4x10^{1}\pm0.36$	$0.33x10^{1}\pm0.3$	$0.25x10^{1}\pm0.28$	$0.75x10^{2}\pm0.59$	$1.74x10^{3}\pm1.16^{*H}$	$9.07x10^{2}\pm11.7^{*IH}$
	Door	$0.4x10^{1}\pm0.36$	$0.07x10^{1}\pm0.16$	$0.13x10^{1}\pm0.21$	$0.4x10^{1}\pm0.36$	$0.25x10^{1}\pm0.31$	$0.77x10^{2}\pm0.51$	$3.4x10^{3}\pm1.1^{*H}$	$1.74x10^{3}\pm1.89^{*IH}$
	Floor	$0.2x10^{1}\pm0.22$	$0.27x10^{1}\pm0.33$	$4.53x10^{1}\pm3.64^{*I}$	$0.6x10^{1}\pm0.22$	$1.4x10^{1}\pm2.52$	$8.09x10^{2}\pm1.19$	$3.2x10^{4}\pm2.63^{*H}$	$1.64x10^{4}\pm2.41^{*IH}$
	Shelf surface:								
	- vertical	$0.2x10^{1}\pm0.22$	$0.33x10^{1}\pm0.3$	$0.27x10^{1}\pm0.33$	$0.27x10^{1}\pm0.33$	$0.27x10^{1}\pm0.28$	-	-	-
	- horizontal	0	$0.33x10^{1}\pm0.39$	$0.6x10^{1}\pm0.22$	$1.57x10^{1}\pm0.9^{*I}$	$0.63x10^{1}\pm0.76$	-	-	-
	Fan blade	0	$0.47x10^{1}\pm0.39^{*I}$	$0.53x10^{1}\pm0.33$	$0.53x10^{1}\pm0.33$	$0.38x10^{1}\pm0.36$	$1.83x10^{1}\pm1.66$	$8.2x10^{2}\pm0.68^{*H}$	$4.19x10^{2}\pm4.21^{*IH}$
	Eggs surface	$10.4x10^{1}\pm11.5$	$0.65x10^{1}\pm1.59^{*I}$	$1.3x10^{1}\pm2.01$	$3.9x10^{1}\pm3.49$	$4.07x10^{1}\pm6.96$	$3.7x10^{3}\pm1.95$	$1.77x10^{6}\pm2.27^{*H}$	$8.87x10^{5}\pm1.79^{*IH}$
	Air	$2.52x10^{2}\pm1.82$	$1.47x10^{3}\pm0.8^{*I}$	$4.07x10^{4}\pm0.36^{*I}$	$1.18x10^{3}\pm0.4^{*I}$	$1.08x10^{4}\pm1.77$	$7.51x10^{3}\pm1.28$	$8.77x10^{5}\pm3.24^{*H}$	$4.53x10^{5}\pm2.87^{*IH}$

Legend: The results were presented as the average amount of 6 tests ±standard deviation; the superscript ^{*I} indicate statistically significant difference during the incubation period, the superscript ^{*H} indicate statistically significant difference during the hatching period, the superscript ^{*IH} indicate statistically significant difference in average values between the hatchery and the incubation period.

DISCUSSION

In this experiment, significant differences in the degree of microbial contamination between the incubator and the hatchery sector were established. In the incubator sector, the bacterial contamination of the eggs during the incubation period was relatively low ($4.07x10^1$ CFU/cm^2) and no significant deviations were observed during the entire technological period. The residual microflora on the eggs when placed in incubators was low – $10.4x10^1$ CFU/cm^2. It was further reduced to $0.65x10^1$ CFU/cm^2 after the aerosol disinfection with formaldehyde on the incubators on the first day of incubation. In contrast, Ayubi et al. (1996) noticed significantly high rate of bacterial contamination on the egg shell during the first day of incubation – $1.6x10^2$ CFU/cm^2 in their study. Ours results show that the disinfection measures in the hatchery are highly effective. The high efficiency of the aerosol disinfection of hatching eggs with formalin is confirmed by Ayubi et al. (1994) in the laboratory tests and by Kim and Kim (2010) under field conditions.

The microbial contamination on the surfaces in the incubator sector was also relatively low (from $0.25x10^1$ to $1.4x10^1$ CFU/cm^2) and no significant variations were observed during this technological stage. The bacterial contamination of the air in the incubator sector was considerably higher - from $1.08x10^4$ in the incubators to $2.24x10^4$ CFU/cm^3 in the hall. The microbial contamination in the incubators was found to be significantly lower than that of the incubator hall. This particularity suggests that the source of microbial contamination in the incubator sector were not the eggs, therefore, it is most probably sought from outside. In connection with this, a dramatic increase of the bacterial air contamination in the incubation hall was found on the 12th day of incubation ranging from $7.36x10^2$ to $8.7x10^4$ CFU/cm^3. According to us, this paradox can be explained by the fact that this period coincides with the end of the hatching process of the previous batch of eggs. During the preparation of the samples in the adjacent hatchery sector, the hatcher cabinets were emptied from the newly hatched chicks. Due to the lack of a separate buffer zone and a sanitary filter between the incubation and hatchery sector, there is an opportunity for easy transfer of contaminated air and materials between the two sectors.

In contrast to the incubator sector, significantly higher microbial contamination was found in the hatchery sector, and substantial variations were observed during the technological stage. It was established that microbial contamination increases dramatically during hatching. The highest increase levels were observed in microbial contamination on egg shells (478 times - from $3.7x10^3$ to $1.77x10^6$ CFU/cm^2), in the air of hatchers (116 times - from $7.51x10^3$

to $8.77x10^5$ CFU/cm^3), on the floor of hatching hall (97 times - from $2.42x10^2$ to $2.36x10^4$U/cm^2), in the air of hatching hall (89 times - from $1.77x10^3$ to $1.58x10^5$ CFU/cm^3), as well as the floor of hatchers (39 times - from $8.09x10^2$ to $3.2x10^4$ CFU/cm^2).

The reports of other authors investigating microbial contamination in poultry hatcheries revealed that the high level of microbial contamination was determined in the hatchery sector and that microbial contamination increases drastically at the beginning of mass hatching (Ayubi and Karadzhov, 1994a; 1994b). The dramatic increase of microorganism population during hatching suggests that bacteria could penetrate inside fertilized eggs. It is well known that eggs can be contaminated with bacteria before lying, for example in hens infected with *Salmonella*, or secondarily after laying - during storage, transportation or incubation. Microorganisms that enter the egg surface may enter the egg through the pores of the shell (Lucore, 1994; Spitzer, 2015). During the incubation of the eggs, favourable conditions (appropriate temperature and humidity) are created to allow multiplication of the infiltrated microorganisms and their accumulation inside the eggs. Depending on the type of infiltrating microorganisms and their amount the result can be embryonic death or hatching of infected and / or diseased chicks, but in all cases there is a sharp and dramatic increase in microbial contamination during hatching (Ayubi and Karadzhov, 1994b; Kozhemyaka, 2010; Wilson, 1997).

The spread of the microorganisms from the hatchery sector to other parts of the facility can be done in different ways - via the air, equipment or staff. Qureshi (2002) puts the accent on the role of contaminated fluff released during hatching, which can easily be spread in the whole hatchery via the workers, the inventory or the air movements, leading to increase of the microbial contamination in all sectors in the hatchery.

Based on the obtained results we conclude that the most contaminated and high-risk area in the hatchery is the hatchery sector at the end of the hatchery process, which is the main source of microbial contamination of the entire hatchery. It is therefore necessary to establish a buffer zone and a sanitary filter between the hatchery and the incubator sector, restrict the passage of workers, hatchery equipment and tools to the incubation sector and also introduce disinfection program that takes into account the specificities of each area.

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STUDY RESULTS OF THE PRESENCE OF *Culicoides* spp. IN SERBIA DURING 2017

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Abstract

During 2017, 784 insect samples were examined and the presence of *Culicoides* spp. was established in 25.51% of samples. Earlier research has found that the dominant population of *Culicoides* spp in Serbia belongs to *Obsoletus* complexes, established in 60.05% of analyzed samples. Out of the entire insect population analyzed, males were found in 22.84%, unpigmented (young) females in 67.97%, females who took blood in 7.39%, whereas 1.35% were gravid females. *Culicoides* spp. from the *Pulicaris* complex was established in 38.85% of examined samples. Males were found in 18.91%, unpigmented (young) females in 71.72%, females who took blood in 9.09%, and 1.11% were gravid females. Other types of culicoids have been established in less than 10% of the examined samples. During examination, the most prevalent species were *Culicoides obsoletus, C. picturalis, C. lupicaris, C. scoticus* and *C. fascipennis.*

Keywords: *Culicoides* spp., epizootiology, Serbia

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REZULTATI ISPITIVANJA PRISUSTVA *Culicoudes* spp. U SRBIJI TOKOM 2017. GODINE

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Kratak sadržaj

Tokom 2017. pregledano je 784 uzorka insekata a prisustvo *Culicoides* spp. je ustanovljeno u 25,51%. Dosadašnja istraživanja potvrdila su da je u Srbiji dominantna populacija *Culicoides* spp. iz Obsoletus kompleksa koji su ustanovljeni u 60,05% analiziranih uzoraka. Mužjaci su nađeni u 22.84% ispitanih uzoraka insekata, nepigmentisane (mlade) ženke u 67,97%, ženke koje su uzele krv u 7,39%, a 1,35% su bile gravidne ženke. *Culicoides* spp. iz Pulicaris kompleksa ustanovljeni su 38.85%. Mužjaci su nađeni u 18.91%, nepigmentisane (mlade) ženke u 71,72%, ženke koje su uzele krv u 9,09%, a 1,11% su bile gravidne ženke. Ostale vrste kulikoida su ustanovljene u manje od 10% pregledanih uzoraka. Tokom ovih pregleda dominantne su bile sledeće vrste: *Culicoides obsoletus, C. picturalis, C. lupicaris, C. scoticus* i *C. fascipennis*.

Ključne reči: *Culicoides* spp., epizootiologija, Srbija

INTRODUCTION

Entomofauna of Serbia is rich and diverse. Among this abundance of arthropods and insects, some species were not a topic of interest of the experts since they did not stand out as potential vectors, or simply did not come to the point that experts were dealing with them (Krunić, 1986). This was the case with the family of Ceratopogonidae, which belongs to the family Culicodina. Insects of the genus *Culicoides* spp. have not been studied in Serbia, so there were contradictory opinions about their persistence in our area (Pavlović et al., 2002). Only with the outbreak of bluetongue disease in 2006, the study of this kind of insects became important and the first research was started in order to determine the presence and extent of these insects (Pavlović et al., 2002). The

research carried out during 2006-2007 confirmed their presence throughout the territory of Serbia. Research in the period 2011-2012 was focused on the study of *Cullicoides* species that are present in Serbia (Pavlović et al., 2009). Finally, in 2014, the re-emergence of bluetongue disease emphasized the need for continuous control of the presence of these insects (**Maksimović Zorić et al., 2016**). Since then, continuous annual monitoring of these insects has been conducted that gave an insight into their seasonal dynamics as well as the most dominant species (Pavlović et al., 2014; 2016a; 2016b; 2017).

MATERIAL AND METHODS

Based on the instructions of Veterinary Directorate on performing entomological and virological tests for the monitoring and surveillance of bluetongue disease (BTD) in the Republic of Serbia No. 323323-02-10787/2016-05 dated 12/02/2016 in the period from 01/01/2017 to 31/12/2017 entomological tests were carried out in order to control bluetongue disease.

In that period, a total of 784 entomological check-ups were made. *Culicoides* spp. samples were collected from all epizootic areas in Serbia. Determination of *Culicoides* spp insects was made by morphometric method recommended by the Italian National Reference Centre for Exotic Diseases (National Reference Centre for the study of Exotic Animal Diseases (CESME)) and OIE Reference Laboratory for Bluetongue Istituto Sperimentale Zooprofilattico dell'Abruzzo e del Molise "G. Caporale" (IZSAM) from Teramo, Italy. Species definition of *Culicoides* spp. has traditionally been based on the morphology of adult insects (Goffredo and Meiswinkel, 2004). Adult individuals of *Culicoides* spp. are notable for their characteristic wing pigmentation pattern and distribution of wing microtrichia, which in certain species can be used as the principle diagnostic feature. In practice, however, the requirement is that specimens should be slide mounted, image-captured, measured and analysed, which is time consuming and therefore the use of morphometries for identification purposes in high-throughput systems such as surveillance programs is recommended (Weeks et al., 1999; Mathieu et al., 2012).

RESULTS

Of the total number of insect samples, the presence of *Culicoides* spp. was established in 25.51% (200/784).

In the epizootic area of Belgrade, *Culicoides* spp. was found in 24.39% (10/41) samples, while in Central Serbia, *Culicoides* spp. was detected in

Požarevac in 28.57% (24/84) samples, Kraljevo in 45.75% (70/153), Niš in 47.64% (81/170), Zaječar in 28.42% (27/95) and in Šabac in 78.84% (41/52) of tested samples. No presence of *Culicoides* spp. (0/0) was detected in the epizootic area of Jagodina.

In the Vojvodina Province, the presence of *Culicoides* spp was found in 38.46% (10/26) samples in Novi Sad, in Pančevo in 44.18% (19/43), Subotica in 59.25% (32/54) and in Sombor in 40.90% (9/22) of tested samples. We did not detect the presence of *Culicoides* spp. in the epizootic area of Zrenjanin (Central Banat District) (0/0).

Earlier research has found that the dominant population of *Culicoides* spp in Serbia belongs to *Obsoletus* complexes, which was established in 60.05% of samples. Males were found in 22.84% samples, non-pigmented (young) females in 67.97%, females taking the blood at 7.39%, while 1.35% were pregnant females.

Culicoides spp. from Pulicaris complexes were found in 38.85% samples. Among tested samples, males were found in 18.91% samples, non-pigmented (young) females in 71.72%, females who took blood in 9.09% and 1.11% were pregnant females.

Other types of Culicoides are set up in less than 10% of the examined samples.

DISCUSION

During the previous examinations, 22 species of *Culicoides* were found and the following species were dominant in 2017: *Culicoides obsoletus, C. picturalis, C. lupicaris, C. scoticus* and *C. fascipennis*. As compared with the period 2015-2016, a change in a faunistic sense since the dominant species in the previous period included *C. obsoletus, C. pulicaris, C. parrots* and *C. nubeculosus* (Pavlović et al., 2017).

Increasing spread of Culicides, the emergence of new genera in some areas and the large number of these insects is a consequence of climate change. In the last century, the mean air temperature in the world increased by 0.5 °C (Blackwell, 2001; Wilson and Mellor, 2008). The temperature and relative humidity of the air have the most important influence on the short-term fluctuations of *Culicoides* (sudden increase in number) and then on their long-term spread. World-wide studies have established that majority of these insects do not appear when the average air temperature is below 13 °C and above 35 °C (Mehlhorn et al., 2007; Purse et al., 2015; Pavlović et al., 2016b). This is also reflected on the biodiversity of present species in certain regions. Thus, *C. imicola* appeared and soon it was the dominant species of eastern Spain, southern

France, northern Italy, northern and southern Greece, the coastal part of Albania, Montenegro, Bosnia and Herzegovina and Croatia (Patakakis et al., 2009; Omeragić et al., 2009; Bosnić, 2011). On the other hand, the Culicoides species from Obsoletus Complex and Pulicaris Complex dominate in the western Balkans (Serbia, Bulgaria, and Romania) (Ioniţă et al., 2009; Bobeva et al., 2013; Pavlović et al., 2016a).

Culicoides's seasonal dynamic is directly correlated with temperature and humidity. *Culicoides* from both groups - Obsoletus and Pulicaris complex, were reaching maximum abundance in spring and autumn, which is normal for this species (Conte et al., 2007; Pavlović et al., 2016a). High temperatures favour their development, while very high temperatures can reduce the survival of adult insects. This correlation was observed during the monitoring of the seasonal dynamic of *Culicoides* in Serbia in the period 2006-2007, 2011-2012 and from 2015 until today (Pavlović et al., 2017). The average season for the appearance of these insects is from March to October, depending on the area being tested. Seasonal dynamics of the presence of *Culicoides* spp. were monitored during one year.

During January, February and November 2017, *Culicoides* spp. were not found in any of the tested samples. During March, their prevalence was 1.58% (1/63), during April 31.73% (27/85), in May it was 49.29% (35/71), June 66.66% (60/90), July 77.89% (74/95), August 66.21% (49/74), September 66.66% (35/71), October 49.29% (35/71) and December 3.44% (2/58).

Culicoides are also subjected to molecular assays (RT-PCR) to analyze the presence of blue tongue viral genome. The genome of blue tongue disease virus was identified in one sample from the territory of the Mionica municipality in September 2016, which is an expected finding considering the outbreak of this disease just in the month of September.

CONCLUSION

During 2017, a total of 784 entomological check-ups were made. Of the total number of insect samples, the presence of *Culicoides* spp. was established in 25.51% (200/784). During the examination period, there was a change in view of dominant species of Culicoides species. In our examination dated 2017, the dominant species were *Culicoides obsoletus, C. picturalis, C. lupicaris, C. scoticus* and *C. fascipennis*. As compared with the period 2015-2016 there was a change in the faunistic sense, that is, the dominant species in the previous period were *C. obsoletus, C. pulicaris, C. parrots* and *C. nubeculosus*. This was influenced by microclimatic and other biotic factors.

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POISONING OF DOMESTIC CARNIVORES BY BANNED PESTICIDES IN SOUTH BAČKA DISTRICT

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Abstract

Cases of deliberate, illegal animal poisoning are widely documented in the literature. Recently, there has been an increase in number of cases of poisoning of domestic and wild animals with highly toxic pesticides in the Republic of Serbia. During the two-year period (2016-2017), in total 40 autopsies of dogs and 2 cats from the territory of the South Bačka District were performed at the Department of Pathology at Scientific Veterinary Institute "Novi Sad" to determine the cause of death. Reasonable suspicion of poisoning in 13 dogs and 2 cats was made based on anamnestic data. The expertises were performed on request of the Republic veterinary inspector in 5 cases, and on the request of the owner in 8 cases. After autopsy, liver, kidneys and stomach content were sampled for toxicological analysis. The presence of carbofuran was determined in three dogs and one cat and the presence of 4,6-dinitro-*ortho*-cresol in one dog by method of gas-mass chromatography. In these cases of poisoning, on the basis of anamnestic data, clinical picture and autopsy finding it was concluded that poisonings were deliberate. Although the number of confirmed cases of domestic carnivores poisoning during the two-year period is relatively low, it is assumed that the number of undetected and undiagnosed poisoning cases is much higher. Abuse of highly toxic pesticides can have severe consequences for both public health and the overall biodiversity.

Keywords: poisoning, domestic carnivores, banned pesticides, carbofuran, 4,6-dinitro-*ortho*-cresol

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TROVANJA DOMAĆIH KARNIVORA ZABRANJENIM PESTICIDIMA NA TERITORIJI JUŽNO-BAČKOG OKRUGA

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Kratak sadržaj

Slučajevi namernih, nezakonitih trovanja životinja se mogu naći u većem broju literaturnih izvora. U novije vreme u Republici Srbiji beleži se porast slučajeva trovanja domaćih i divljih životinja visoko toksičnim pesticidima. U periodu od dve godine (početak 2016. do kraja 2017. godine) u cilju utvrđivanja uzroka uginuća, na Odeljenju za patologiju Naučnog instituta za veterinarstvo „Novi Sad" obdukovano je ukupno 40 pasa i 2 mačke, sa teritorije Južnobačkog okruga. Na osnovu anamnestičkih podataka, osnovana sumnja na trovanje postavljena je kod 13 pasa i 2 mačke. Ekspertize su obavljane na osnovu zahteva Republičkog veterinarskog inspektora u 5 slučajeva, dok je po zahtevu vlasnika zabeleženo ukupno 8 slučajeva. Nakon izvršene obdukcije, izvršeno je uzorkovanje organa: jetra, bubrezi i sadržaj želuca u cilju toksikoloških analiza. Metodom gasno-masene hromatografije utvrđeno je prisustvo karbofurana u sadržaju želuca i jetri kod tri psa i jedne mačke kao i prisustvo 4,6-dinitro-*orto*-krezola kod jednog psa. U navedenim slučajevima trovanja je na osnovu anamnestičkih podataka, kliničke slike ili nalaza prilikom patomorfološkog pregleda zaključeno da se radi o slučaju gde je trovanje namerno. Iako je broj potvrđenih slučajeva trovanja domaćih karnivora u toku dvogodišnjeg perioda relativno mali, može se pretpostaviti da je broj neotkrivenih i nedijagnostikovanih slučajeva trovanja životinja znatno veći. Zloupotreba visoko toksičnih pesticida može imati ozbiljne posledice kako na javno zdravlje tako i na celokupan biodiverzitet u regionu.

Ključne reči: trovanje, domaći karnivori, zabranjeni pesticidi, karbofuran, dinitro-orto-krezol.

INTRODUCTION

Cases of acute pesticide poisonings of wildlife and domestic animals have been documented worldwide since the middle of the 20th century (Cramp, 1973; Fleischli et al., 2004). The pesticides most frequently involved in animal poisoning are insecticides and rodenticides (Segev et al., 2006; Wang et al., 2007; Yas-Natan et al., 2007; Berny et al., 2010; Anastasio and Sharp, 2011; Caloni et al., 2012; Waddell et al., 2013). Animal poisoning cases by herbicides, molluscicides and fungicides have also been reported but less frequently (Berny et al., 2010; Caloni et al., 2012). There are many data in the literature on unintentional or accidental poisonings with pesticides used in agriculture in target and non-target species. Wild animals are most frequently exposed to accidental poisoning (Kwon et al., 2004; Berny and Gaillet, 2008; Martínez-Haro et al., 2008; Slaninova et al., 2009; Wagner et al., 2013; Ogada, 2014). It is estimated that pesticides were illegally used in 68% of animal poisonings (Berny, 2007).

Restrictions have been made in the European Union on the use of certain chemical preparations used in agriculture, due to their high toxicity (EC 2003, EC 2006). Non-selective and unsafe use of pesticides, as well as the use of poisonous baits for killing non-target species, are some of the most serious problems for public health and biodiversity. Also, there is a growing problem of the conservation of endangered animal species due to the illegal use of pesticides (Ruiz-Suárez et al., 2015). The ban on the use of highly toxic pesticides can reduce their availability and hence the possibility for poisoning of domestic and wild animals. For example, after 1998 the percentage of poisoning of cattle with organochlorine pesticides declined significantly because of the ban on use of these pesticides (Guitart et al., 2010; Caloni et al., 2012). However, intentional or accidental cases of poisoning of animals with banned pesticides are still present in a large percentage worldwide (Tennakoon et al., 2009; Berny et al., 2010; de Siqueira et al., 2015; Ruiz-Suárez et al., 2015). An increased percentage of cases of deliberate, illegal poisoning of domestic and wild animals has been recorded in Republic of Serbia. Although the literature data on the incidence of pesticide poisoning in domestic animals in our country is very scarce, it is known that this number is significantly higher than statistical data show (Aleksic et al., 2014). Killing, causing of injuries, torture or any other form of animal abuse is a criminal act and is regulated by the Criminal law of the Republic of Serbia, Article 269 (Sl. glasnik RS", No. 85/2005, 88/2005 - ispr., 107/2005 - ispr., 72/2009 i 111/2009). The aim of this article is to describe cases of poisoning of dogs and one cat by forbiden highly

toxic pesticides (carbofuran and 4,6-dinitro-ortho-cresol) on the territory of the South Bačka District, in the period from the beginning of 2016 to the end of 2017.

MATERIAL AND METHODS

From January 2016 till the end of 2017, due to a reasonable suspicion of poisoning and to determine the cause of death, autopsies of 13 dogs and 2 cats were performed at the Department of Pathology at the Scientific Veterinary Institute Novi Sad. Autopsies were performed according to the standard protocol of Scientific Veterinary Institute "Novi Sad". Data about breed, age, gender, estimated time of death, body condition and in some cases anamnestic data from the owner about the possible circumstances that led to the death, were collected. In only 3 cases, a suspected bait was found in the immediate proximity of the body, which was also subjected to toxicological analysis. Macroscopic changes in the organs were photo-documented, and samples of stomach contents as well as parenchymal organs (liver, kidney) were collected for toxicological investigations. Quick, easy, cheap, effective, rugged, safe (QuEChERS) sample preparation was used for toxicological analysis and it was adapted from the Association of Analytical Communities (AOAC) Official method 2007.01 for extraction and clean up.

Reagents and Chemicals

All chemicals and reagents used were of analytical grade with high purity.

Standard Solutions

Standard solutions were prepared using a carbofuran and 4,6-dinitro-*ortho*-cresol standard, manufacturer by Dr. Ehrenstorfer Lot number 10910, Germany and Lot number 41217.

Sample Preparation

According to Kartalović et al. (2016) adapted method of sample preparation for pesticides (OCP), polychlorinated biphenyl's (PCB) and polyciclic aromatic hydrocrbons (PAHs) was used. This modified method was based on the extraction with acetonitrile (ACN, Sigma-Aldrich) in the presence of anhydrous magnesium sulfate (MgSO4; *Merck*, Darmstadt, *Germany*) and anhydrous sodium acetate (CH3COONa; *Merck*, Darmstadt, *Germany*). Sample (3 g) was measured and transferred into centrifuge tube, 3 ml of water and 3 ml of Acetonitrile were added. After intensive stirring on a vortex, 3 g of anhydrous magnesium sulfate and 1 g of anhydrous sodium acetate were added. Exother-

mic reaction occurred within 1 min after the intense stirring on vortex. The sample was then centrifuged until 5 min at 3000 rpm. One milliliter of upper acetonitrile extract is transferred into the 5 ml tube, which contained 150 mg of anhydrous magnesium sulfate, 100 mg of Primary and Secondary Amine (PSA), Merck manufacturer (Darmstadt, Germany), and 50 mg of C18, Merck manufacturer (Darmstadt, Germany), (Anastassiades et al., 2003). The tube content was centrifuged for 5 min at 3000 rpm. After centrifuging, purified and clear extract was obtained. Then, 0.5 ml of the extract was evaporated in nitrogen and reconstituted with hexane. A sample prepared in this way was ready for the analysis on GCMS (Agilent 7890B/5977A, USA).

GCMS Analysis

Carbofuran and 4,6-dinitro-*ortho*-cresol identification were based on comparison of the retention times of the peaks and target ions, with those obtained from standard (standards supplied by instrument manufacturer).

Quantification was based on matrix calibrations curves prepared from the standard solution of carbofuran and 4,6 - dinitro-*ortho*-cresol. The coefficients of determination (r2) for the carbofuran and 4,6-dinitro-*ortho*-cresol standard calibration plots were more than 0.99.

Instrumentation

Agilent 7890B/5977A MSD, gas - mass chromatography was used for analysis. The GC operating conditions were as follow: fused silica column [30m*0.25μm film of HP-5M (thickness)]; injection temperature was set at 280 °C using splitless mode and volume injected was 4 μL. The column temperature was programmed as following: hold at 50°C for 0.4 min; 50-195 °C at 25 °C/min, hold 1.5 min; 195-265 at 8 °C/min and maintained at 315 °C for 1.25 minutes on 20 °C/min, MSD temperature was 280 °C. Verification of peaks was carried out based on retention times and target ions, compared to those of external carbofuran and 4,6-dinitro-*ortho*-cresol. Procedural blank and solvent blanks were analyzed and quantified, but no carbofuran and 4,6-dinitro-*ortho*-cresol were found in these blanks.

RESULTS

From January 2016 till the end of December 2017, in total 40 autopsies of dogs and 2 cats were performed at Department of Pathology at Scientific Veterinary Institute „Novi Sad“ to determine the cause of death. Reasonable suspicion of poisoning in 13 dogs and 2 cats was made based on anamnestic

data, the appearance of nervous system symptoms as well as the finding of a suspected substance in the proximity of the bodies. The chemical-toxicological analysis of the sampled material was performed in only 7 cases. The expertises were performed on the request of the Republic veterinary inspector in 5 cases, and on the request of the owner in 8 cases.

The finding of toxic pesticides was confirmed in four dogs and one cat by method of gas-mass chromatography. The presence of carbofuran was confirmed in three dogs (two pulin and šarplaninac breed) and one domestic cat, while the presence of 4,6-dinitro-*ortho*-cresol in gastric content and parenchymal organs was confirmed in one dog (Hungarian viszla breed). In all the cases with carbofuran poisoning, death occurred within 15-30 minutes after the onset of the first clinical symptoms. Clinical signs included convulsions, rotation in circles, appearance of foam in the mouth. There were no anamnestic data about dog poisoned with 4,6-dinitro-*ortho*-cresol. Two dogs were poisoned in public areas, and other two dogs and the cat in house yards. All poisoned animals had owners. In all these cases of poisoning based on anamnestic data, clinical picture and autopsy findings, it has been concluded that poisonings were done deliberately.

Autopsy findings

All bodies of dogs and cat that died due to the ingestion of toxic bait were in good body condition. The gross pathology finding of the dog poisoned with 4,6-dinitro-*ortho*-cresol was greatly unspecific. Hair around the muzzle was colored in yellow. The dominant macroscopic finding in the stomach was the presence of particles of round shape and intense yellow color, mixed with stomach content. Mucus of the stomach was extremely wrinkled and hyperemic (Figure 1).

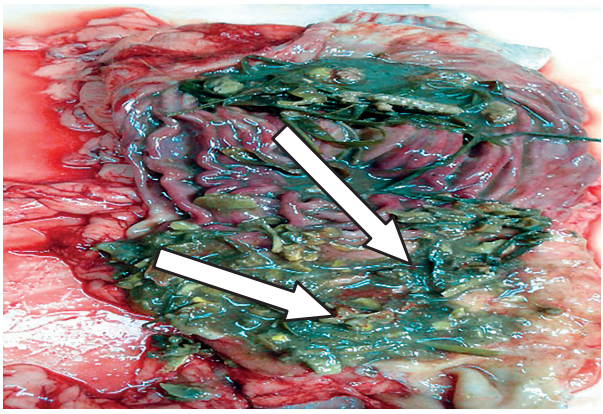

Figure 1: Stomach of the dog. Intense yellow particles are seen in the lumen (arrow).

In 3 dogs and a cat poisoned with carbofuran, the main macroscopic lesions were in the form of bowel congestion (4/4), presence of haemorrhagic exudate in the abdominal cavity (3/4), and foamy-haemorrhagic content in the lumen of the trachea (4/4). In the lumen of the stomach, the presence of partially digested poisonous bait of intense pink color was determined (Figure 2).

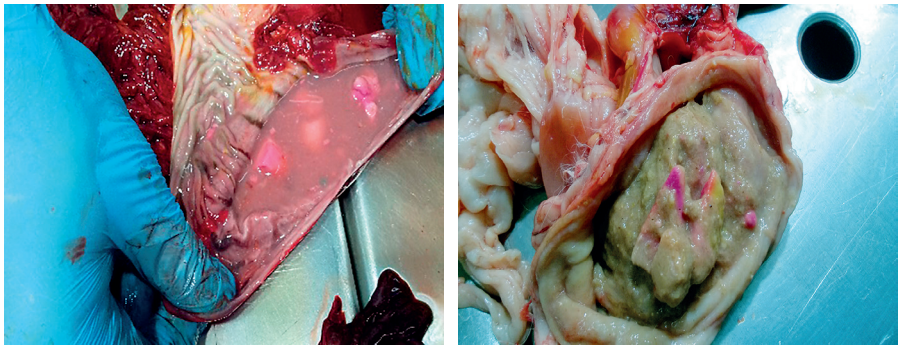

Figure 2. a) Stomach content of the cat. Partially digested poisonous carbofuran bait of intense pink color; b) Stomach content of the dog with poisonous bait inside.

Method for validation of carbofuran and 4,6-dinitro-ortho-cresol

Validation plan included determination of precision, reproducibility, accuracy, linearity, LOQ, LOD and uncertainty (Kartalović et al., 2016). The method precision was evaluated by repeatability using the meat fortified with carbofuran concentrations injected in triplicate (50.0 mg kg^{-1}, n=20). Accuracy was calculated by recovery. Linearity of detector was tested in range of 5 to500 mg kg^{-1}, and was satisfactory in all range. Limit of detection (LOD—standard deviation equal to 3) and limit of quantification (LOQ— standard deviation equal to 10) were calculated using the excel program. LOD values ranged 1.6 mg kg^{-1}, and the LOQ was 5mg kg^{-1} for carbofuran and 4,6-dinitro-*ortho*-cresol. Method preparation and method determination for carbofuran and 4,6 dinitro-*ortho*-cresol is the same like for pesticides and PCB and because of that in calculating measurement uncertainty the contributions PT (FAPAS-Pesticide Residues in Olive Oil, July - September 2014 Round 0598), the contribution of reproducibility and contribution of bias are taken into account. Calculation of faithfulness for expanded measurement uncertainty for pesticides and polychlorinated biphenyl's in the observed matrix was 38.7%, what satisfies the conditions that are recommended by SANCO 2014 (Kartalović et al., 2016).

Table 1. The average values of precision, reproducibility, accuracy, linearity, LOQ and LOD for carbofuran and 4,6-dinitro-*ortho*-cresol

Contaminent	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (r^2)[a]	LOQ (μg kg^{-1})	LOD (μg kg^{-1})
Carbofuran	11.3	6.33	95,02	0,99853	1,21	0,36
Dinitro-4,6-*ortho*-cresol	13.2	7.24	94.21	0.99893	1.91	0.56

([a]r^2 – correlation coefficient)

DISCUSSION

Three cases of acute poisoning of dogs and a cat with carbofuran, as well as one case of poisoning of the dog with 4,6-dinitro-*ortho*-cresol in the territory of South Bačka district during two year period (2016-2017) are described in this paper. In recent years there has been an increase in the number of cases of deliberate poisoning of domestic and wild animals with prohibited pesticides in the Republic of Serbia (Mihaljev et al., 2013; Aleksic et al., 2014). When it comes to poisonings of domestic animals in Europe, reports from many countries show that the highest percentage of poisonings is recorded in dogs, then in cats, horses and small ruminants (Berny et al., 2010). The most commonly used poisons included carbamate insecticides such as aldicarb and carbofuran (Tennakoon et al., 2009; Berny et al., 2010; Novotný et al., 2011; Ruiz-Suárez et al., 2015)181 birds, mammals and baits were analysed over the period 2004\ u201309 for possible intoxication by carbamates. Intoxication by carbamate carbofuran was diagnosed in 89 cases, and in another 19 cases (nine Wild Boars and 10 Bisons.

Carbofuran is one of the most toxic carbamate pesticides. The active substance is 2,3-dihydro-2,2-dimethyl-7-benzofuran-1-methylcarbamate. Carbofuran acts as a reverse inhibitor of acetylcholine esterase enzyme. It is easily consumed by target species because its formulation is suitable for mixing with pet food, meat, fish (De Siqueira et al., 2015). Animal owners mostly find their pets dead or with a severe clinical picture typical for a cholinergic crisis involving dyspnoea, diarrhea and convulsions (Khan, 2012). Such conditions require urgent veterinary intervention. Brain and skeletal muscles (target tis-

sues) are mostly affected by the toxic effect of carbamate insecticides, while cardiovascular, respiratory, reproductive and immune systems can also be affected. Atropine sulphate is used for the control of muscarinic receptors as the only specific physiological antidote for carbamate toxins (Tse et al., 2013). In most animals that died due to carbamate pesticide poisoning, the macroscopic and histopathological findings were mostly unspecific, and the changes were usually in the form of systemic congestion and hemorrhage in most organs (De Siqueira et al., 2015), which match our findings. Histopathologic changes in the form of neuron death in several parts of the brain (hippocampus, cortex, thalamus, amygdala) are described in acute carbamate poisoning in rats. In severe forms of poisoning necroses of skeletal muscles are also described (Gupta, 2007). Since 2007, the European Union has prohibited trade of products containing carbofuran (EC, 2007). Although the ban on the use of this pesticide is expected to reduce the number of animal poisonings, researches worldwide suggest that poisonings are still common (De Siqueira et al., 2015; Bille et al., 2016; Caloni et al., 2016;). Carbofuran is forbidden for use in Serbia since 2014, due to its high toxicity and negative ecotoxicological effects.

4,6-Dinitro-*ortho*-cresol (DNOC) is a cresol derivative, yellowish crystalline solid. It is used in agriculture as larvicide, insecticide and ovicide. DNOC is classified in class Ib, 'highly hazardous', in the WHO Recommended Classification of Pesticides by Hazards (WHO, 1999). Although the use of DNOC as a pesticide is prohibited in many countries, significant quantities of unused pesticides exist, especially in developing countries (World Health Organization, 2000). Dinitro-*ortho*-cresol can be absorbed through the skin as well as by ingestion or inhalation of aerosols. Workers in the agricultural and chemical industries are most often exposed. In acute exposure to dinitro-*ortho*-cresol by ingestion, inhalation or through skin, there are signs of poisoning in the form of difficult breathing, increased thirst, accelerated breathing, nausea, anorexia, and intense yellow pigmentation occurs. Liver, kidney and central nervous system damage has been reported in acute poisoning of people. Chronic exposure to DNOC results in the occurrence of the same symptoms. According to Dere et al., DNOC causes cellular damage at vital organs such as liver, kidneys, and lungs, thus inducing changes in the physiological and metabolic activities (Dere et al., 2007). Acute oral exposures to DNOC resulting in toxicity and death have been reported in rats, mice, cats and pigs at relatively similar doses.

CONCLUSIONS

Intentional and accidental poisonings of animals with high toxic pesticides pose a threat to human health as well as to general public health and safety. Pesticides such as carbofuran and 4,6-dinitro-*ortho*-cresol are forbidden for usage in the European Union as well as in Serbia, but poisonings continue to occur both in domestic animals and wildlife. Toxico-epidemiological data from most European countries show that pet poisoning is a very important and frequent veterinary problem. The results obtained in this paper show that poisonings of companion animals by banned pesticides such as carbofuran and 4,6-dinitro-*ortho*-cresol are still present in our country. Considering that this study covers a short period of time (2 years) and population of carnivores from a small geographical area (South Backa District), more detailed country-wide studies need to be carried out to obtain an insight into the incidence of poisoning of domestic animals in our country.

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SOME ADVERSE EVENTS FOLLOWING IMMUNIZATION IN VETERINARY MEDICINE

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Abstract

Vaccines are a very effective tool for the prevention and eradication of infective diseases in both veterinary and human medicine. Although for safety reasons, vaccines undergo very strict controls before being placed on the market, the risk of adverse reactions is not eliminated. According to the World Health Organization (WHO), adverse event following immunization (AEFI) is any untoward medical occurrence that follows immunization and which does not necessarily have a causal relationship with the usage of the vaccine. Adverse reactions may arise as a direct consequence of immunization due to the specificity of the vaccine itself, the quality of the vaccine or the immunization errors. In addition, adverse reactions may also be the result of a coincidental relationship between the effect and immunization. However, biological mechanisms of AEFI are very complex. During the mass vaccination campaigns, when a large number of animals are vaccinated in a short period of time, adverse reactions are expected to be the most frequently reported. In Serbia, livestock is currently being vaccinated against Lumpy Skin Disease, Bluetongue, and Classical swine fever.

Keywords: vaccine, adverse event, mass vaccination

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PROCENA NEŽELjENIH REAKCIJA NAKON VAKCINACIJE U VETERINI

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Kratak sadržaj

Vakcine su veoma efikasan alat za prevenciju i iskorenjivanje infektivnih bolesti u veterinarskoj i humanoj medicini. Iako iz sigurnosnih razloga vakcine prolaze kroz vrlo stroge kontrole pre stavljanja u promet, rizik od neželjenih reakcija nije eliminisan. Prema Svetskoj zdravstvenoj organizaciji (SZO), neželjeni događaj nakon imunizacije je svaka neugodna medicinska pojava koja prati imunizaciju i koja ne mora nužno imati uzročnu vezu sa upotrebom vakcine. Neželjene reakcije mogu nastati kao direktna posledica imunizacije usled specifičnosti same vakcine, kvaliteta vakcine ili grešaka prilikom imunizacije. Pored toga, neželjene reakcije mogu biti i posledica slučajnog odnosa između nastalog efekta i imunizacije. Međutim, biološki mehanizmi nastanka ovih reakcija su veoma složeni. Tokom kampanja masovnih vakcinacija, kada se veliki broj životinja vakciniše u kratkom vremenskom periodu, očekuje se da se neželjene reakcije najčešće prijavljuju. U Srbiji se stoka trenutno vakciniše protiv bolesti kvrgave kože, bolesti plavog jezika i klasične svinjske kuge. Pošto nijedna od ove tri vakcine nije DIVA, praćenje neželjenih događaja koji se mogu pojaviti je od izuzetnog značaja.

Ključne reči: vakcina, neželjena reakcija, masovna vakcinacija

INTRODUCTION

Vaccines are a very effective tool for the prevention and eradication of infective diseases in both veterinary and human medicine. Vaccines are, also, used to improve the welfare of companion animals as well as to treat the non-infectious diseases such as allergies or cancer, even to increase production and fertility of livestock (Meeusen et al., 2007). Immuno-contraception of pests is also based on vaccine use (Hardy et al, 2006). Effects of vaccinations are

remarkable and measurable, particularly with regard to the cost. Thanks to the massive vaccination, among the other applied measures, the Rinderpest was declared as eradicated worldwide by the OIE and FAO in May and June 2011 (Roeder et al, 2013). Classical swine fever (CSF) has been successfully eradicated from some countries including EU member states, Canada, United States, Australia and New Zealand, where the use of the vaccine was of the highest contribution (Greiser-Wilke and Moening, 2004). World Health Organization (WHO, 2018) announces the elimination of human rabies transmitted by dogs by 2030 through the affordable human vaccines and antibodies and mass dog vaccination supported by increased communication, awareness and education.

Today, in veterinary medicine, the most used vaccines are modified live vaccines (MLV), killed (KV), and toxoid type vaccines, each characterized by advantages and disadvantages. While live vaccines typically stimulate more rapid, stronger, and longer-lasting immunity than killed vaccines, using the killed vaccines there is no risk of the vaccine organism spreading between animals as well as the risk of causing abortion is minimal (Jorge and Dellagostin, 2017). MLVs are mainly available for diseases caused by viruses, such as bovine herpesvirus 1, bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), and parainfluenza-3 virus (Pl3). KVs usually contain adjuvants, or added substances, that further stimulate the immune system to respond to the vaccine challenge. Even more, the majority of KVs are safe to use in any animal, including pregnant ones (Jorge and Dellagostin, 2017).

Apart from the vaccine formulation, the administration route and the method of vaccination influence on the efficacy and safety of a vaccine.

Usually, in the veterinary medicine, the vaccines are administered parenterally via intramuscular, subcutaneous and intradermal injection. The mucosal immunization includes intraocular, intranasal and/or oral administration. Mucosal application of attenuated vaccines via drinking water or spray, being very successful, is now routinely applied in poultry (Makoschey, 2015). The oral administration of rabies vaccine in vaccination of wild carnivores has had a great contribution to the rabies control and eradication (Lupulović et al, 2015). Oral vaccine for classical swine fever, after having been used since years for the CSF control and eradication of CSF in wild boar, is now recommended for domestic pigs vaccination, particularly for those kept extensively, in backyards (Milićević et al., 2013; Dietze et al., 2013).

ADVERSE EVENT FOLLOWING IMMUNIZATION (AEFI)

Although for safety reasons, vaccines undergo very strict controls before being placed on the market, the risk of adverse reactions is not eliminated. According to the World Health Organization (WHO, 2018), adverse event following immunization is any untoward medical occurrence that follows immunization and which does not necessarily have a causal relationship with the usage of the vaccine.

AEFIs are grouped into five categories: vaccine product-related reaction, vaccine quality defect-related reaction, immunization error-related reaction, immunization anxiety-related reaction and coincidental event (WHO, 2018).

Biological mechanisms of AEFI are very complex. Very often, the latency between antigen exposure and peak adaptive immune response, due to the adjuvants that help to increase the response rates to vaccines, results in prolonged exposure of the immune system cells to antigen and consequently the release of inflammatory mediators. Further on, the tissue damage or clinical disease occurrence following the immunization could be related to some of the immune-mediated mechanisms such as effector functions of T cells, effector functions of antibodies and autoantibodies, complement activation, hypersensitivity reactions etc. (Stratton, 2012).

Attenuated live viral vaccines can cause the same effects through the same mechanisms like it happens during the natural infection. Such events occur usually when the immune system is impaired or the animal immunocompromised. Some of the adverse events, like complex regional pain syndrome, syncope etc., are not directly related to the contents of the vaccine but rather to the adverse event of direct trauma from the needle (Stratton, 2012).

AEFIs can be manifested as either local or systemic reactions. Minor local reactions include pain, redness and swelling at the injection site within 48 hours of vaccination. Such reactions that extend past the nearest joint and/or persisting for 10 days or more should be reported (WHO, 2018). Arthus Reaction is a major local reaction manifested with 48 hours after the immunization as a large, localized reaction characterized by pain, swelling, induration, and edema. This reaction usually occurs when there is a large number of circulating antibodies prior to injection of the antigen. Most Arthus reactions resolve within one week. Abscess at the injection site is a swollen lump, fluid-filled, becoming very painful which usually appears within 7 days after immunization due to the bacterial contamination. The sterile abscess can persist for more than 1 month. Nodules are solid, elevated areas of tissue at the injection site with discrete or well-demarcated borders. Normally, nodules are not accom-

panied either with abscess formation, erythema or warmth. They are mainly caused by aluminum-based adjuvants and subcutaneous route of administration. Sterile nodules can take up to 1 year or more to resolve.

Cellulitis at the injection site is an acute inflammation of the subcutaneous tissue, fat, fascia or muscle, usually seen within 7 days after vaccine administration due to the bacterial contamination (WHO, 2018).

Fever is the most often seen systemic reaction. It appears within 72 hours when killed vaccines are used, while this time is prolonged with the live vaccines. A fever that begins within 24 hours after vaccination with the inactivated vaccine or persists for more than 24 hours after vaccination should not be assumed to be due to the vaccine (WHO, 2018).

The enlargement of one or more lymph nodes is usually associated with some adjuvants that produce transient chemokine and cytokine stimulation, enhanced the local activity of antigen presenting cells, and uptake by regional lymph nodes. However, the live vaccines, due to a low-grade infection, can cause the adenopathy (WHO, 2018).

Allergic reactions are an acquired hypersensitivity to a component of the vaccine. They are usually manifested as a mild form of the dermatological/mucosal and/or the respiratory systems disorders. Allergic reactions occur within 48 hours of immunization. However, anaphylaxis, a type of allergic reaction, is the potentially life-threatening adverse reaction to the immunization. It is manifested by sudden onset, rapid progression of signs and symptoms and involvement of multiple organ systems (WHO, 2018).

Many other reactions such as neurological disorders, thrombocytopenia, arthritis etc., can also be related to the immunization.

In the veterinary medicine, the most common side effects include transient swelling at the site of injection and a reaction that may change coat color in the area, transient pyrexia, respiratory distress, salivation, vomiting, diarrhea, urticaria, reduced fertility, fetal deformities and abortion (Morton, 2007). However, immune-mediated hemolytic anemia (IMHA), thrombocytopenia (IMTP), polyneuritis and polyarthritis are autoimmune disorders (primarily in the dog) that can, also, be linked to vaccination (Day, 2006). Hypersensitivity type III where the cutaneous vasculopathy occurs has been reported following rabies virus vaccination. Similarly, the formation and the deposition of immune complexes are the cause of adenoviral-related 'blue eye' in dogs. As a different category of adverse events, Day (2006) states the lack of efficacy although it is more often due to the inappropriate administration, or administration to immunosuppressed or immunodeficient individuals than to batches of subnormal efficacy. The effects that can occur due to the residual virulence

of attenuated vaccine strains, or batch contamination during manufacture are also considered as adverse events (Day, 2006).

MASS VACCINATION CAMPAIGNS IN SERBIA

In Serbia, livestock is currently being vaccinated against Lumpy Skin Disease, Bluetongue, and Classical swine fever. During the mass vaccination campaigns, when a large number of animals are vaccinated in a short period of time, adverse reactions are expected to be the most frequently reported.

BLUETONGUE DISEASE VACCINATION

Vaccination against bluetongue disease has been implemented in European countries after the incursion of the disease in 1998 (Niedbalski, 2011). At that time, modified live vaccines were commercially available and used for mass vaccination of cattle and sheep. Besides the high level of immunogenicity and protection, it has been shown that modified live vaccine caused significant side reactions. Adverse events such as fever, facial oedema, lameness, reduced milk production, and teratogenicity were reported mostly in sheep. Abortions were recorded in less than 0.5% of vaccinated animals (Savini et al., 2008b). Those reactions are commonly attributed to under-attenuation of the modified strains and their capacity of passing the placental barrier, and the spread of vaccine strain in the environment with the potential for reversion to virulence and re-assortment with field isolates (Savini et al., 2008a). It has been shown that viremia of attenuated vaccine strain lasts up to 35 days, even reaching a titer which enables infection of *Culicoides* (Savini et al., 2008b). Circulation of vaccine strains has been confirmed in the Netherlands (BTV6), Germany (BTV6), Belgium (BTV11), and BTV14 in Lithuania, Latvia, Poland, and Estonia[1]. De Leeuw et al. (2015) also reported the possible detection of RNA in circulation after vaccination against BTV8. The severity of adverse events depends also on BTV genotype. BTV16 in attenuated vaccines produced the most severe reactions (Savini et al., 2008b). Inactivated vaccines have, also, been commercialized, initially against BTV2 and BTV4, but afterward for all circulating strains in EU (Wilson and Mellor, 2009). Thus, the risk of adverse events following immunization with MLV was minimized. However, for the full protection and prevention of both clinical symptoms and viremia, two doses of inactivated vaccines are needed (Savini et al., 2008a). Many studies carried out to evaluate the safety of inactivated vaccines demonstrated well tolerance and absence

[1] https://ec.europa.eu/food/animals/animal-diseases/control-measures/bluetongue_en

of the systemic reaction (fever, weight loss, reproductive dysfunction) related to vaccination. However, some animals developed transient local reactions of variable severity with a different frequency (Savini et al., 2008a) which usually disappeared within 3 days. In a single case, a moderate local reaction persisted for 2 weeks (Hamers et al., 2006). Anaphylaxis was only reported in animals which previously received MLV. Neither side effects nor local reactions in safety studies for cattle have been reported even when five doses of the BTV-4 inactivated vaccine was administered to the same animal (Savini et al., 2008a). Inactivated BTV4 vaccine has been used in Serbia since 2015. Expected adverse events are the slight increase in body temperature and appearance of the nodule at the injection site. According to the manufacturer`s instruction, it can be expected that up to 53% of vaccinated animals display the nodules which should disappear within 35 days in sheep or even longer in cattle.

LUMPY SKIN DISEASE VACCINATION

Lumpy skin disease was confined to African continent until 1989 when occurred in Israel, for the first time outside Africa. When the disease was introduced in Turkey and Iraq in 2013, its potential for further spread was evident. By the year 2016 the virus was detected in seven European countries – Greece, Bulgaria, the Former Yugoslav Republic of Macedonia, Serbia, Albania, Montenegro, and Kosovo. For the disease control and eradication, vaccination has been immediately implemented. Live attenuated vaccines are the most commonly used. There are currently two types of them, derived either from the South-African LSDV Neethling strain or an attenuated LSDV field strain. However, even heterologous vaccines can be used but provide incomplete protection. Abutarbush et al. (2016) reported that duration between vaccine administration and appearance of adverse clinical signs ranged from 1 to 20 days. Clinical manifestations which included fever, decreased feed intake, decreased milk production and variable sized cutaneous nodules lasted from 3 to 20 days. However, no mortalities were reported due to vaccine adverse reactions. Croatia reported adverse events on 0.24% of the vaccinated farms, involving 0.09% of the total animals affected and 0.02% deaths (EFSA, 2017). Commonly, the symptoms appeared within 2 weeks after vaccination and included fever (22%), a decrease in milk production (27%), edema at injection site (21%), lumps (12%), erythema (7%), abortions (7%) and ataxia (5%) of affected animals. Katsoulos et al. (2016) described that in Greece 12% of animals had the pronounced swelling at injection-site while 10% of animals developed small-sized skin nodules. The milk production was reduced up to 16% during

the first 12 days post-vaccination. Vaccine virus was detectable in blood between 6 and 15 days post vaccination as well as in aspirates obtained from the injection-site lesions and in nodule biopsies suggesting the need for DIVA vaccine. Despite the evident adverse effects, it has been shown that vaccine based on attenuated Neethling strain was 4.3 times more effective in preventing laboratory-diagnosed LSD than the sheep pox vaccine, and 11.2 times more effective in preventing severe LSD cases (Ben-Gera et al., 2015). Though massive vaccination in many countries has been implemented, there is no evidence of the vaccine strain regaining virulence or spreading of the disease via cattle products (Tuppurainen and Galon, 2016).

CLASSICAL SWINE FEVER VACCINATION

To control and eradicate classical swine fever, the safe and highly efficacious live attenuated vaccine based on China strain (C strain) has been used since years in Serbia. Apart from the traditionally used intramuscular vaccine, the formulation for oral administration has also been confirmed as efficient and safe (Milićević et al., 2013). In order to enable differentiation between vaccine and field strain, many other vaccines were developed. However, it has been shown that the best candidate is the recombinant CP7_E2alf vaccine (Blome et al., 2017).

Blome et al. (2006) showed that vaccine based on attenuated C strain could cause no damage in vaccinated animals. The C strain was not interfered with gestation, though it passages through the placental barrier. Therefore the vaccine was safe for use in pregnant animals, even in immunosuppressed ones. The same authors reported that E2 subunit vaccines were also highly safe, apart from a possible local tissue reaction at the injection site (Blome et al., 2017).

As other, MLV for CSF is detectable for some time after vaccination. However, though the genome of the CP7_E2alf vaccine is detectable by RT-PCR in tonsils and lymph nodes for up to 63 days, the shedding of vaccine virus has not been detected. Similarly, orally vaccinated pigs did not transmit vaccine virus to susceptible contact animals. However, the genome of C strain virus after orally administered vaccine could be detected in the tonsil 21 days post-vaccination (dpv) (Kaden et al., 2010). Even more, the absence of leucopenia after vaccination and no increase of virulence were reported up to now (Koenig et al., 2007).

Adverse events following vaccination in veterinary medicine, particularly if mandatory mass vaccination is applied, are accomplished to the compensation. However, despite the reporting system is available, so far there are no official reports on adverse events in Serbia.

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DIAGNOSIS OF WEST NILE NEUROINVASIVE DISEASE IN HUMANS

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Abstract

West Nile virus (WNV) is arbovirus distributed all around the world. In humans, 80% of infection cases are asymptomatic. In 20% of infected people, a febrile self-limiting illness is reported. WNV has the potential for fatal neuroinvasive disease. In 1% of cases, the infection may result in neuroinvasive disease with permanent neurological consequences or death outcome. Neurological forms may vary presenting with encephalitis, meningitis, meningoencephalitis or acute flaccid paralysis. Outbreaks with neurological forms of WNV infection were recorded in different areas of Greece, Italy, Romania, Hungary and Serbia. During the period from 2013 to 2016, 114 samples of cerebrospinal fluid and 107 serum samples were taken from 114 patients suspected of WNV neuroinvasive disease (WNND). The presence of specific anti-WNV IgM and IgG antibodies in cerebrospinal fluid (CSF) and sera samples were tested by WNV IgM and IgG ELISA (Euroimmun, Germany). In addition, 48 samples of CSF or/and serum of people with suspected WNV infection were examined by commercial molecular tests - real time RT-PCR (WNV Real-TM, Sacace biotechnologies, Italy). The IgM antibodies against WNV were present in 25.4% (29/114) of CSF samples, and in 31.8% (34/107) of serum samples tested from 114 patients suspected of WNND. The IgG antibodies against WNV were detected in 3.5% (4/114) of CSF samples, and in 11.2% (12/107) of serum samples. The WNV RNA was detected by real time RT-PCR test in 7 out of 48 (14.6%)

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CSF or/and serum samples. In this study, detection of IgM antibodies in CSF is more frequent than detection of WNV RNA in CSF or serum samples. WNV RNA detection in CSF is confirmatory diagnostic test but has limited utility in the diagnosis of WNV neuroinvasive disease due to low viremia level at the time of clinical presentation of the disease. The limitations in the use of ELISA IgM test are linked to cross - reactivity among flaviviruses and long persistence of IgM antibodies in the serum and CSF.

Keywords: West Nile virus, ELISA IgM test, CSF, serum, real time RT-PCR, WNV genome

DIJAGNOSTIKA NEUROINVAZIVNE FORME BOLESTI ZAPADNOG NILA KOD LJUDI

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Kratak sadržaj

Virus zapadnog Nila (VZN) je rasprostranjen širom sveta. Kod ljudi u 80% slučajeva infekcija protiče asimptomatski. U 20% slučajeva infekcija se manifestuje kao blago febrilno oboljenje sa povoljnim ishodom. VZN može izazvati potencijalno fatalno neuroinvazivno oboljenje. U 1% slučajeva infekcija se može ispoljiti kao neuroinvazivna bolest sa neurološkim posledicama i smrtnim ishodom. Neurološke forme bolesti su različite i mogu se ispoljiti kao encefalitis, meningitis ili akutna flakcidna paraliza. Epidemije neuroloških formi oboljenja registrovane su u različitim područjima Grčke, u Italiji, Rumuniji, Mađarskoj i u Srbiji. U periodu od 2013. - 2016.god., 114 uzoraka likvora i 107 uzoraka krvnih seruma prikupljeno je od 114 pacijenata suspektnih na neuroinvazivnu bolest izazvanu VZN. Testovima WNV ELISA IgM i IgG (Euroimmun, Nemačka) testirani su svi uzorci seruma i likvora. Komercijalnim molekularnim testovima (WNV Real-TM,

Sacace, biotechnologies, Italija) testirano je 48 uzoraka likvora i/ili seruma pacijenata suspektnih na neuroinvazivnu bolest izazvanu VZN. Antitela IgM protiv VZN dokazana su u 25,4% (29/114) uzoraka likvora sakupljenih od 114 pacijenata suspektnih na neuroinvazivnu formu infekcije VZN uključenih u ovu studiju. Takođe, IgM antitela protiv VZN dokazana su u 31,8% (34/107) testiranih uzoraka seruma. IgG antitela protiv VZN dokazana su u 3,5% (4/114) uzoraka likvora i u 11,2% (12/107) uzoraka seruma. RNK VZN je otkrivena u 7/48 (14,6%) uzoraka likvora i/ili seruma real time RT-PCR testom. IgM antitela češće su detektovana u likvoru od genoma VZN u likvoru ili serumu. Dokazivanje genoma VZN u likvoru kod ljudi ima ulogu dijagnostičkog testa za postavljanje konačne dijagnoze neuroinvazivnog oboljenja izazvanog VZN. No, značaj detekcije genoma je ograničen zbog niskog nivoa viremije u vreme kada bolest postane manifesna. S druge strane, IgM ELISA test koji je češće pozitivan u likvoru od RNK ima ograničenja kao: ukrštenu reaktivnost s drugim flavivirusima i mogućnost duge perzistencije u likvoru i serumu.

Ključne reči: West Nile virus, ELISA IgM test, likvor, serum, real time RT-PCR, genom WNV

INTRODUCTION

The West Nile virus (WNV) is an arbovirus (arthropod-borne virus), the member of the family *Flaviviridae,* genus *Flavivirus.* WNV is widely distributed across Africa, Europe, Asia, Australia, and America among mosquitoes and vertebrates including humans and horses. Birds, as main reservoir, and mosquitoes (primarily *Culex* species) as vectors play a central role in transmission cycle of WNV in nature. Humans, equines and other animals are generally considered the "dead end" hosts because they have low viremia levels, insufficient to sustain transmission from vertebrate to feeding mosquitoes. Most WNV infections in humans remain asymptomatic. In one of four cases of WNV infection, WNV fever is developed (Petersen et al., 2013). In 1% of cases, infection may result in West Nile neuroinvasive diseases (WNND) such as meningitis, encephalitis, poliomyelitis- as a condition with flaccid paralysis, with permanent neurological consequences or lethal outcome. Estimated percentages of WNND are as following: meningitis 35 - 40%, encephalitis 55-60% and poliomyelitis 5-10% (Sejvar et al., 2007). Clinically, WNV meningitis is indistinguishable from aseptic meningitis caused by other neurotropic viruses.

WNV encephalitis is usually associated with seizures, mental status changes, focal neurologic deficits or movement disorders.

Until 1996, West Nile virus was not considered significant human pathogen because most reported infections were mild febrile disease in rural populations and a few cases of WNND were reported. WNV acquired neurotropism during the second half of the 20th century and two events occurred: outbreaks of WNND in Europe and introduction of WNV in The United States. In 1996, first explosive epidemic of WNND occurred in Europe. It was an epidemic in Romania in Bucharest, with 393 patients with chronologically confirmed or probable WNV infection, of whom 352 had acute central-nervous-system infections. Seventeen patients (5%) older than 50 years died (Tsai et al., 1998). WNV had never been detected in the Western Hemisphere of America until August 1999, when the first cases of WNV infection appeared in New York City. In the outbreak in New York City, 59 people were infected and 63% of them had clinical signs of encephalitis, and seven patients died (12 %) (Nash et al., 2001). In 1999, explosive epidemic of WNV occurred in Volgograd City, Russia, on the bank of Volga River, where WNV infection has never been detected before. 826 patients were admitted to hospitals with invasive forms of the infection (acute aseptic encephalitis, meningitis) or fever. Case fatality rate was high. Out of total 84 cases of acute aseptic meningoencephalitis, 40 (48%) were fatal (Platonov et al., 2001). At the beginning of 21st century, outbreaks of WNND continued to occur in Europe. Outbreaks of WNND were recorded in different areas of Greece, Italy, Romania, Hungary, Serbia (ECDC, 2017). Sporadic cases were recorded in Spain, Portugal, Austria and Croatia (Vilibić-Čavlek et al., 2013). In Asia and Africa where WNV has been endemic for many years, WNV encephalitis occurs in children and has benign course and good prognosis. In Europe and USA, where WNV emerged only recently, WNV encephalitis is a severe disease and many of the survivors are left with severe neurologic deficits and cognitive dysfunctions.

MATERIAL AND METHODS

During the period from 2013 - 2016, 114 samples of cerebrospinal fluid and 107 serum samples were taken from 114 patients suspected of neuroinvasive form of WNV infection (WNND). Seven serum samples were not available from the patients. All patients were hospitalized at the Clinic for Infectious Diseases, Clinical Center of Vojvodina. At the Department of Virology of the Institute of Public Health of Vojvodina, WNV IgM and IgG ELISA (Enzyme - linked immunosorbent assay) were applied for the detection of WNV-specific

IgM and IgG antibodies in cerebrospinal fluid and in sera samples. Tests were performed according to manufacturer's instructions (Euroimmun, Germany). In total, 48 samples of cerebrospinal fluid or/and serum of people suspected of WNV infection were examined using commercial molecular tests - real time RT-PCR (real time reverse transcription - polymerase chain reaction (RT-qPCR) (WNV Real-TM, Sacace biotechnologies, Italy).

RESULTS

During the 2013 season, 37 samples of cerebrospinal fluid and 33 serum samples from 37 patients suspected of neuroinvasive form of WNV infection were collected and tested using commercial WNV IgM and IgG ELISA tests. IgM antibodies against WNV were present in 43.2% (16/37) of CSF samples (Table 1). Serological study revealed that out of these 16 IgM positive CSF samples, 81.2% (13/16) had IgM antibodies in serum samples too, and 2 (12.5%) had no WNV - specific IgM response in the serum. In one case 6.2% (1/16) serum sample was not available. Out of the 16 cases of IgM positive serum samples, 3 (18.75%) were not positive for IgM antibodies in CSF. IgG antibodies against WNV were detected in 4 out of 37 (10.8%) samples of CSF and in 6 out of 33 (18.2%) tested serum samples. All cases with WNV IgG antibody positive CSF samples were also tested IgG antibody positive in serum sample. West Nile virus RNA was detected by real time RT-PCR test in 6 out of 28 (21.4%) tested CSF samples. Out of 6 cases with WNV RNA positive CSF, 4 (80.0%) cases had WNV RNA in serum sample too. In 4 out of 6 (80.0%) WNV RNA positive cases, IgM antibodies against WNV were detected simultaneously in the serum and in CSF sample. One of 6 human cases with detected WNV RNA in CSF had IgM antibodies only in serum samples and one had IgM antibodies only in sample of CSF. None of the WNV RT-PCR positive cases had IgG antibodies in the serum or in CSF.

During the 2014 season, 35 samples of CSF and 33 serum samples were collected from 35 patients and tested by ELISA test. IgM antibody positive results in CSF were obtained in 8.6% (3/35) patients. Two of three IgM antibody positive patients (66.7%) had IgM antibodies in samples, CSF and serum, and one patient (33.3%) had IgM antibodies in CSF only. Three out of 33 (9.1%) serum samples were WNV IgM antibody positive. None of the CSF samples were positive for WNV IgG antibodies. In total, WNV IgG antibodies were detected in three out of 33 (9.1%) serum samples.

Table 1. Frequency of WNV IgM positive cerebrospinal fluid/serum sample and presence of WNV RNA in patients suspected of neuroinvasive form of WNV infection

Year	ELISA IgM Liquor (CSF)		ELISA IgM Blood serum		ELISA IgG Liquor (CSF)		ELISA IgG Blood serum		Real time RT-PCR Liquor and/or serum	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
2013	16/37	43.2	16/33	48.5	4/37	10.8	6/33	18.2	6/28	21.4
2014	3/35	8.6	3/33	9.1	0/35	0	3/33	9.1	ND	ND
2015	4/21	19.0	8/21	38.1	0/21	0	2/21	9.5	1/21	4.8
2016	6/21	28.6	7/20	35.0	0/21	0	1/20	5.0	0/20	0
Total	29/114	25.4	34/107	31.8	4/114	3.5	12/107	11.2	7/48	14.6

Legend: n - number of positive samples; N - total number of samples; % - percentage of positive samples; ND not done

During the 2015 season, testing for WNV infection was performed on 21 patients with suspected WNND. In total, 21 samples of CSF and 21 serum samples were tested applying serological and molecular tests. In CSF samples testing WNV IgM ELISA was found to be positive for 4 patients out of 21 (19.0%). All these patients also reacted positive for WNV IgM antibodies in serum samples. The WNV IgM ELISA was positive for 38.1% (8/21) serum samples of the patients with suspected WNND. The WNV IgG ELISA were found negative for all CSF samples, however, two serum samples (2/21) were tested positive. The WNV RNA was detected in sample of CSF of only one patient.

During the season 2016, in total 21samples of CSF and 20 serum samples were collected from 21 patients with suspected WNND. The WNV IgM antibodies were detected in CSF of 6 patients (28.6%). Out of 20 patients' serum samples tested with WNV IgM ELISA, 7 (35%) were found to be IgM antibody positive. None of the samples of CSF tested positive for WNV IgG antibodies, but those antibodies were detected in 1 out of 20 (5.0%) tested serum samples.

DISCUSSION

Various methods are available for detecting viable WNV, WNV antigen or WNV RNA in human diagnostic samples. The viable WNV can be detected by isolation on cell culture (e.g. Vero cell) or in the brain tissue samples from the laboratory infected suckling mice. Disadvantages of virus isolation are that the method is time-consuming and it requires the use of cell cultures and handling with live, infectious virus and BSL-3 (Biosafety level 3) facility. For virus genome detection and amplification molecular techniques such as real time reverse transcription polymerase chain reactions (real time RT-PCR) and nucleic acid sequence based amplification (NASBA) methods are applied. The WNV genome RNA can be detected in human CSF, serum and other tissues. Molecular methods have great sensitivity but limited utility in diagnosing human WNV neuroinvasive disease, which is due to the low level of viremia present in most cases at the time of clinical presentation. Isolation of the virus has similarly limited significance in the diagnosis of WNND. Molecular methods and virus isolation are useful only at the beginning of the infection. The best choice at the later stages of illness are serologic methods. The serological screening method for laboratory diagnosis of human WNND is the ELISA IgM antibody assay. ELISA is the most widely used serological method because it is sensitive and easy to perform in routine practice in a diagnostic virology laboratories. In this study, IgM antibodies against WNV were detected in 31.8%

of tested serum samples from suspected WNND patients. According to the results of many studies, IgM antibodies against WNV can persist in serum for months (Papa et al., 2015). Murray detected immunoglobulin M antibodies up to 8 years after infection with WNV in a study encompassing 163 participants (Murray et al., 2013). In the study of Roehrig at al. (2003), 60% of patients with laboratory - confirmed WNV encephalitis had WNV IgM antibodies approximately 500 days after onset of the disease. Because of that, detection of WNV IgM antibodies in blood sera should not be considered a marker of acute West Nile virus infection and determination of avidity of WNV IgG is necessary to ensure the accurate and reliable diagnosis (Hrnjaković et al., 2017a). The presence of IgM antibodies in CSF establishes the diagnosis of WNND since IgM antibody does not cross the blood-brain barrier. The IgM antibodies appear in CSF within 8 days of the beginning of disease in at least 90% of patients with encephalitis or meningitis (Petersen et al., 2013). They are marker of acute CNS infection. In our study, IgM antibodies were found in CSF in 29 of 114 (25.43%) patients suspected of neuroinvasive form of WNV infection. Molecular methods are better marker of WNND but positive results can be established only at the beginning of diseases. In this study, genomic RNA of WNV was detected in 14.6% serum or/and CSF samples by real time RT-PCR. According to the study of Kapoor et al. (2004), the presence of IgM antibodies in CSF may not always reflect the acute phase of infection with WNV. These authors detected WNV IgM-specific antibodies in CSF specimens from the three patients with CNS disease persisting for 110, 141, and 199 days of post-acute phase of infection. The other problem associated with serological diagnosis of flavivirus infections is the cross-reactivity between different members of genus *Flavivirus* including Sent Louis encephalitis virus - SLEV, Japanese encephalitis virus - JEV, Yellow fever virus - YFV and Denga 1-4 viruses. According to the results of other serological studies, Tick-borne encephalitis virus - TBEV (Hrnjaković et al., 2016) and Usutu virus - USUV (Hrnjaković et al., 2017b) co-circulate in humans in Vojvodina and because of that, misinterpretation of serological results is possible. The issue of cross-reactivity may be overcome by performing the plaque reduction neutralization test (PRNT). PRNT is the gold standard for the serological diagnosis of flaviviral infections. Disadvantages of this test are that it requires facilities that are available only in specialized research institutions (BSL-3 laboratories). In this study, PRNT was not used as confirmatory assay and it was the limitation of this study.

In 2010, WNV RNA genome was detected in mosquito *Culex pipiens* collected in the city of Novi Sad applying real time RT PCR (Petrić et al., 2012). Circulation of WNV in wild birds in Vojvodina was also detected by using

molecular methods and serological tests. The WNV isolate from wild birds belonged to the lineage 2 and clustered with WNV strains isolated in Hungary 2004, Greece 2010 and Italy 2012 (Petrović et al., 2013). Circulation of WNV in pigs, boars and roe deer was also detected in Serbia using molecular methods and serological tests (Escribano-Romero et al., 2015). The first human cases of WNND on the territory of South Bačka district in Vojvodina Province of Serbia were registered in 2012. It was the first epidemic in South Bačka district with 32 patients in which neuroinvasive form of WNV infection was diagnosed. All patients were hospitalized at the Clinic for Infectious Disease of the Clinical Center of Vojvodina in Novi Sad. The largest number of patients has recovered completely. In 6.25% of patients, sequelaes were developed. Lethal outcome was registered in 3.13% cases (Sević et al, 2015). Since 2013, human WNV infections on the territory of South Bačka district have been diagnosed every year from July to October at the Institute of Public Health of Vojvodina. Complete genome sequences of the WNV isolate from a human patient with neuroinvasive disease, resident of Novi Sad, were described (Jovanović Galović et al., 2017). It was the first whole genome sequencing of the isolate from human sample in Serbia. The isolate belonged to lineage 2 and similarities with strains circulating in Greece, Hungary, Austria, and Italy were demonstrated (Jovanović Galović et al., 2017).

CONCLUSION

WNV has the ability to cause neuroinvasive disease (WNND) that can result in debilitating morbidities and long-term sequelaes. WNV RNA detection in CSF or serum is confirmatory diagnostic test but has limited utility in the diagnosis of WNV neuroinvasive disease due to low viremia level at the time of clinical presentation of disease. Diagnosis of WNND usually relies on detection of IgM antibodies in serum or cerebrospinal fluid. Special emphasis in diagnosing of WNV infection should be put on CSF analyses (IgM ELISA and RT-PCR).

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ELECTRICAL CONDUCTIVITY AND ACIDITY OF HONEY

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Abstract

In this paper, the electrical conductivity and concentration of free acids (acidity) of various types of honey collected from the area of Vojvodina was investigated. Physicochemical analyzes of parameters such as electrical conductivity and acidity of honey have an important role in defining the overall properties of honey and assessing the quality of honey. A total of 55 samples of honey were examined. Out of the total number of samples, 5 samples did not meet the requirements of the Republic of Serbia Regulations on quality of honey due to inadequate electrical conductivity. Free acid values measured in tested samples ranged from 1.5 to 30 mEq / kg, and the values of electrical conductivity were in the range between 0.08 and 1.99 mS/cm. Our assumption that there is a correlation between the tested parameters has been confirmed only in meadow and polyfloral honey. Further research on the physicochemical properties of Serbian honey is recommended and important in order to establish certification marks and criteria for assessing the quality of Serbian honey.

Keywords*:* honey, electrical conductivity, acidity

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ELEKTRIČNA PROVODLJIVOST I SLOBODNE KISELINE U MEDU

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Kratak sadržaj

U ovom radu ispitivana je električna provodljivost i koncentracija slobodnih kiselina (kiselost) različitih vrsta meda koji je prikupljena sa područja Vojvodine. Fizičko-hemijske analize parametara kao što su električna provodljivost i kiselost meda imaju značajnu ulogu u definisanju ukupnih svojstava meda i proceni kvaliteta meda. Ispitano je ukupno 55 uzoraka meda. Od ukupnog broja uzoraka 5 uzoraka nije ispunilo zahteve Pravilnika Republike Srbije o kvalitetu meda, zbog neodgovarajuće električne provodljivosti. Izmerene vrednosti za slobodne kiseline u ispitivanim uzorcima kretale su se u opsegu od 1,5 mEq/kg do 30 mEq/kg, a vrednosti električne provodljivosti u opsegu od *0,08 do 1,99* mS/cm. Naša pretpostavka da postoji korelacija između ispitivanih parametara potvrđena je se samo kod livadskog i polifloranog meda. Dalje istraživanje fizičko-hemijskih osobina srpskog meda veoma je preporučljivo i važno, kako bi se uspostavile sertifikacione oznake i poboljšali kapaciteti lokalnog pčelarstva.

Ključne reči: med, električna provodljivost, slobodne kiseline

INTRODUCTION

According to the Codex Alimentarius 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". Bees produce honey to serve as a source of food in times of scarcity or during bad weather conditions (Codex, 2001). Natural honey is one of the highly sought natural products because of its unique, highly nutritive and medicinal properties. These properties are attributed to the influence of different groups of substances that honey contains. Honey is unique sweet

natural product that can be used in human nutrition without any further processing. The quality of honey and bee products depends on its origin. Active components in plants depend on various factors and climatic conditions in different geographical locations (Vidaković et al., 2017).

The conductivity is very often used in routine honey quality control. This property of honey is considered very good criterion for assessment of botanical origin and purity of honey. Among other things, honey contains components such as organic acids and minerals, which in an aqueous solution have the ability to dissociate into the ions or to conduct an electric power. The bright colour of honey usually points to a lower conductivity than dark colour of honey (Kropf et al., 2008). The electrical conductivity of honey is defined as that of a 20% weight in volume solution in water at 20° C, where the 20% refers to honey dry matter (International Honey Commission, 2009). The measurement of electrical conductivity points indirectly to the ashes content of honey (Accorti et al., 1987). The ashes of honey give an indication of environmental pollution and hence also an indication of geographical origin. The electrical conductivity of the honey is related to the concentration of mineral salts, organic acid and proteins and proved to be useful for discriminating honeys of different floral origins (Acquarone et al., 2007). Other factors, such as floral source, amount of organic acids and proteins, and storage time can also influence the electric conductivity of honey (Karabagias et al., 2014). High electric conductivity values do not necessarily correspond to higher amounts of ash in the honey (Escuredo et al., 2014). Exact classification of honey must be carried out not only by measuring the conductivity but also in relation to optical rotation and microscopic analysis (Přidal and Vorlová, 2002).

The acidity of honey is caused by organic acids (tartaric, citric, oxalic, acetic, etc.), nectar or bees secretions (Yadata, 2014). The acidity of honey may be determined by titration with sodium hydroxide (free acidity) or directly measuring the pH value (pH value). The natural acidity of honey can be increased by the storage and ripening of honey, as well as during the fermentation of honey. Honey that is adulterated with sugar syrup has very low acidity (<1), while honey that is adulterated with invert sugar has a pronounced high acidity (Yadata, 2014). The acidity value related to the balance of organic acids naturally present in honey varies according to the floral source and the bee species (Sousa et al., 2016).

In accordance with the regulation concerning the quality of honey in the Republic of Serbia (Official Gazette, 101/2015), minimum electrical conductivity in honeydew put in the market is fixed to 0.8 mS/cm. For other types of honey, the maximum permitted value of electrical conductivity is 0.8 mS/cm.

Maximum value of free acidity in all types of honey (except in baker's honey) is 50 mEq/kg as set by regulation (Official Gazette, 101/2015)

The purpose of this study was to determine electric conductivity and free acidity of honey collected in an official monitoring in the Republic of Serbia during October 2017, in order to obtain information about the honey quality and safety. In our research, which is a part of a study of the physicochemical and sensory characteristics of Serbian unifloral and mixed honey, the behaviour of honey was examined with respect to the electric conductivity and free acidity.

MATERIAL AND METHODS

Honey samples

In total, 55 honey samples were collected from different retail chains in Vojvodina region (northern Serbia). All samples were collected as a part of official monitoring of honey and bee products quality during October 2017.

All collected samples were in their original packaging and transferred to the laboratory of Scientific Veterinary Institute "Novi Sad" for examination.

A total of 55 investigated samples included 16 samples of acacia honey, 18 samples of meadow honey, 9 samples of floral honey, 6 samples of linden honey, and 6 samples of honeydew honey.

Physicochemical analysis

Water content

Water content was determined by refractometry, measuring the refractive index (RI) according to Harmonised methods of the International Honey Commission Methods (2009), using a standard model Abbetype refractometer a 20 °C. Water content (%) was then obtained from the Chataway table.

Electrical conductivity

Electrical conductivity was measured at 20 °C in solutions of honey samples (20.0 g dry matter of honey in volume solution in 100 ml distilled water) using a conductometer Crison (Type Basic 30). Method of measuring is prescribed by International Honey Commission Methods (2009).

Free acidity

The acidity of honey was determined by volumetric method (International Honey Commission Method, 2009). Ten grams of honey were dissolved in 75

ml of distilled water and solution was titrated with 0.1 M NaOH to pH 8.30. Acidity is expressed in milliequivalents/kg honey (mEq/kg).

Statistical analysis

Statistical linear regression analysis was performed by the PAST software package, version 2.12, Oslo, Norway. Data were grouped according to the type of honey and presented as mean ± standard error, minimum and maximum values.

RESULTS AND DISCUSSION

Average values of electrical conductivity and acidity of honey obtained in this study are summarized in Table 1.

Table 1 Results of determining electrical conductivity and acidity in diverse honey samples

TYPE OF HONEY	No. of samples	Electrical conductivity (mS/cm)		Acidity (mEq/kg)	
		Range	Average value ±SD	Range	Average value ±SD
Meadow honey	18	0.08 – 1.19	0.49 ± 0.30	1.5 – 30.0	13.08 ± 8.41
Acacia honey	16	0.08 – 0.30	0.18 ± 0.07	2.5 – 9.0	5.44 ± 1.56
Linden honey	6	0.65 – 1.03	0. 82 ± 0.13	4.0 – 24.0	12.17 ± 6.73
Polyfloral honey	9	0.09 – 0.74	0.46 ± 0.18	5.0 – 26.0	13.61 ± 6.12
Forest honey	6	0.09 – 1.99	1.12 ± 0.68	5.0 – 29.0	19.33 ± 8.70

The obtained values were compared with the values that are prescribed by Regulation on the quality of honey in the Republic of Serbia (Official Gazette, 101/2015). The results were compared with the results from other authors from Serbia and other countries.

The water content values in all investigated honey samples were below 20%, which is the maximum permitted level set by local regulations for honeys (Official Gazette, 101/2015).

Free acidity in all tested samples was below 50 mEq/kg. These data indi-

cated the absence of undesirable fermentation. A wide range of acidity values was found after the analysis of all honeys (Table 1). The acidity values in the investigated samples ranged from 1.5 mEq/kg to 30 mEq/kg. *Our results also demonstrated low acidity of acacia honey and high acidity of forest honey, as compared with other examined honey types.* The composition of organic acids in honey has not yet been adequately investigated; however, some evidence suggest that acacia, chestnut and meadow honeys are characterized by particularly low contents of organic acids, while *darker honeys* in general appear to be *higher* in *acidity (Prica et al., 2014). Very similar results were obtained in our earlier research (Prica et al., 2014). Similar results of honey free acidity were reported by other authors from other Mediterranean countries (*Boussaid et al., 2018; *Chakir et al., 2016; Yadata, 2014; Kirs et al., 2011; Sousa et al., 2016).*

Electrical conductivity, closely related to the concentration of mineral and organic acids, showed a high variability within and between groups of honey. Values of electrical conductivity in the investigated honey samples *were between 0.08 and 1.99* ms/cm. Out of a total of 55 tested honey samples, 5 samples did not comply with the local regulations for honeys (Official Gazette, 101/2015). Within the group of meadow honey, electrical conductivity was above 0.8 mS/cm in one of 18 tested samples. In the group of linden honey, electrical conductivity was above 0.8 mS/cm in two from 6 tested samples. Within the group of forest honey, electrical conductivity value below 0.8 mS/cm was established in two from the total of 6 tested samples.

The data indicated lowest conductivity in acacia honey samples, while the highest conductivity is found in samples of forest and linden honey. Similar values for *electrical conductivity of* honey are reported by other authors *from our and other countries (Vranić et al., 2017; Acquarone et al., 2007;* Accorti et al., 1987; Boussaid et al., 2018; *Chakir et al., 2016; Yadata, 2014; Kirs et al., 2011; Sousa et al., 2016;* Escuredo et al., 2014; Karabagias et al., 2014).

Because both investigated properties are a function of the concentration of the ions present in the honey solution, their correlation was investigated (Figures 1 - 5).

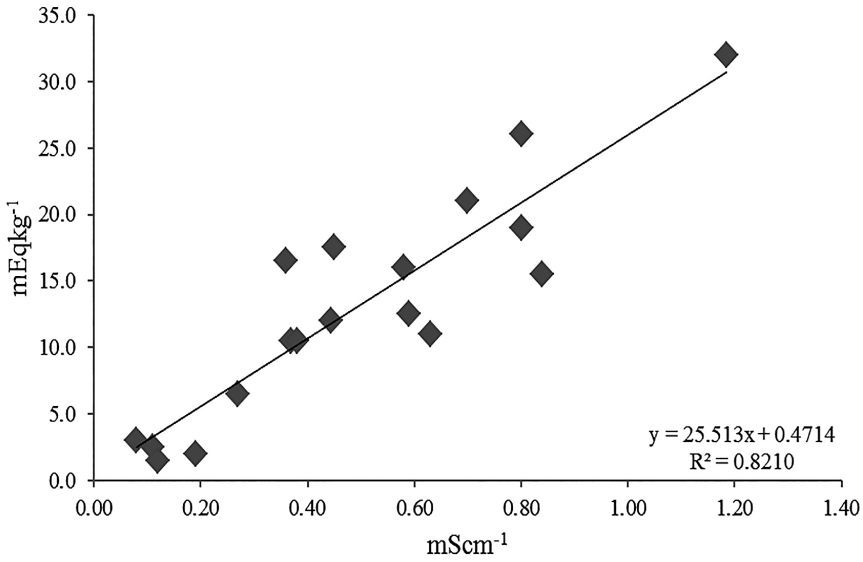

Figure 1. Correlation between acidity and electrical conductivity calculated for meadow honey samples.

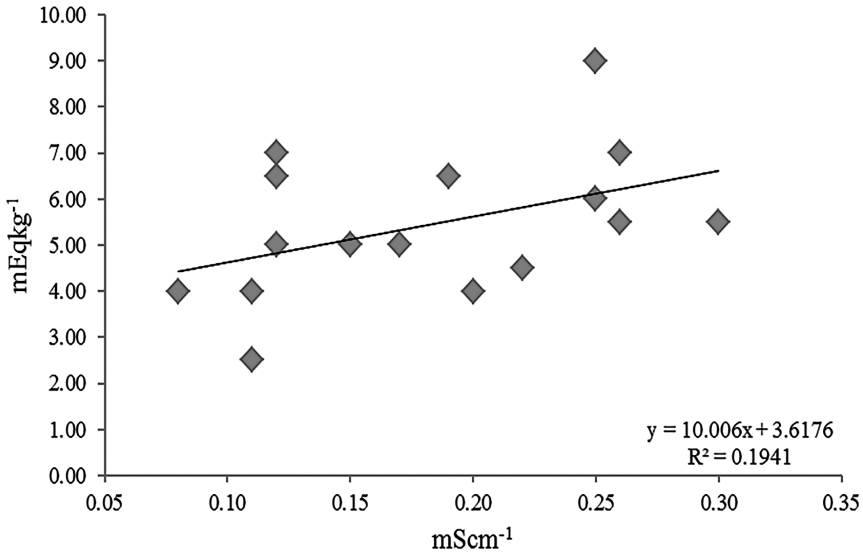

Figure 2. Correlation between acidity and electrical conductivity calculated for acacia honey samples.

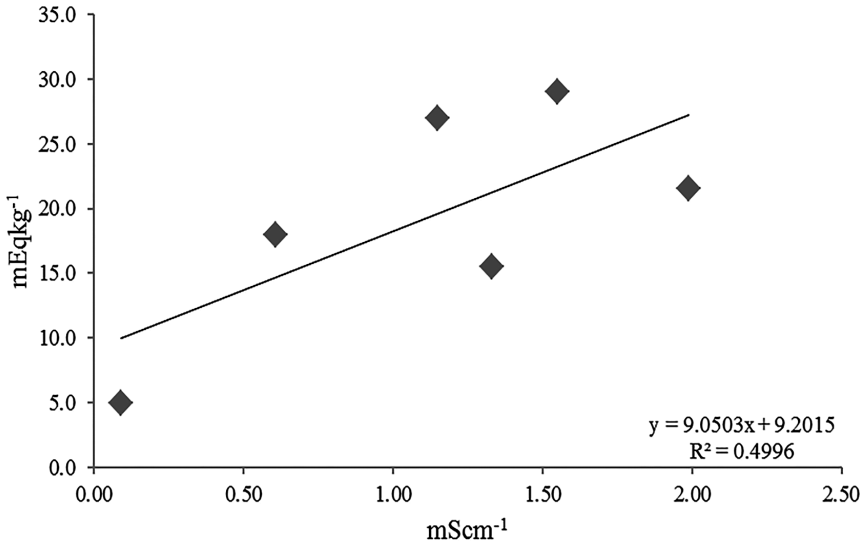

Figure 3. Correlation between acidity and electrical conductivity calculated for linden honey samples.

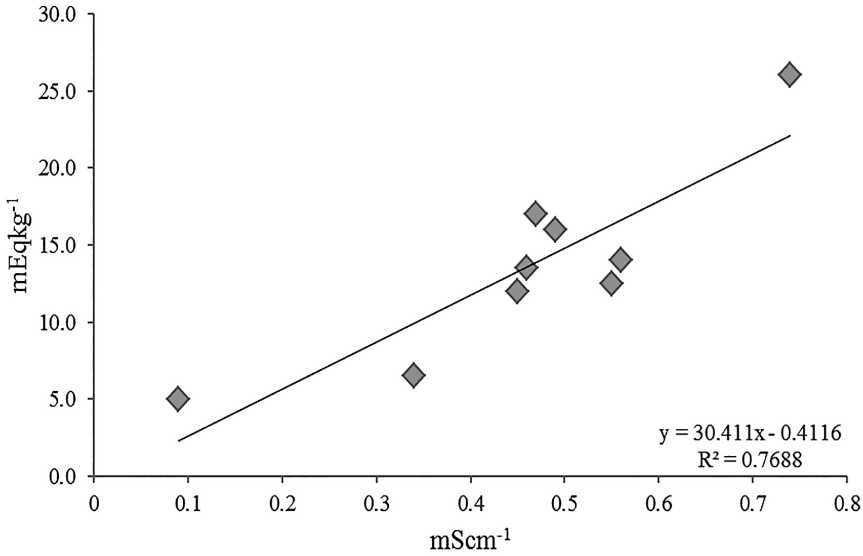

Figure 4. Correlation between acidity and electrical conductivity calculated for polyfloral honey samples.

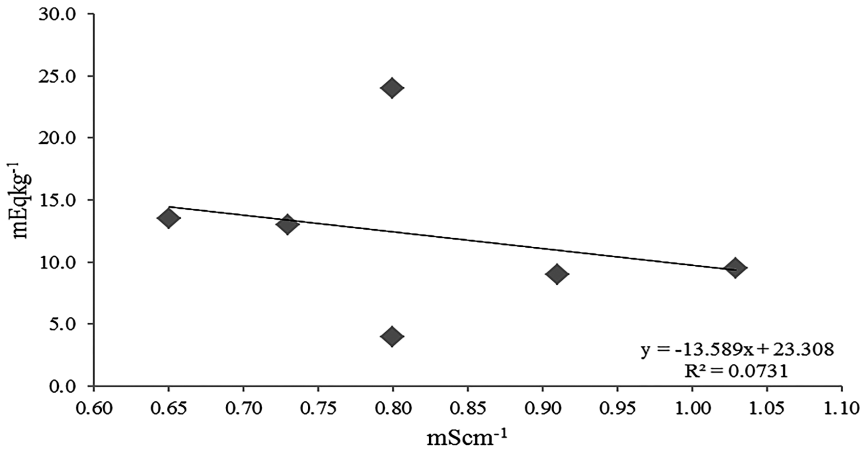

Figure 5. Correlation between acidity and electrical conductivity calculated for forest honey samples.

High correlation was established between free acidity and electrical conductivity only for meadow and polyfloral honey samples (R^2 = 0.8210 and R^2 = 0.7688).

Acquarone et al. (2007) analyzed honeys of different floral and geographical origin from Argentina. They studied the pattern of electrical conductivity and pH upon honey dilution by increasing honey concentration. The pH values decreased exponentially. Such behaviour could be attributed to the fact that, with increasing honey concentration the hydration of fructose and glucose increases and free water decreases. Therefore, the effective concentration of other ions in the solution increased. The authors also reported significant linear correlation between total acidity and free acidity. Finally, they concluded that for a given geographical region, acidity values were useful for discriminating honeys of different floral origins, but that the most adequate parameters for discriminating honeys of different geographical origin were those which described the patterns of pH and electrical conductivity with changes of honey concentration.

CONCLUSIONS

The electric conductivity and free acidity of honey collected in an official monitoring in the Republic of Serbia was determined. Free acidity in all tested samples was below 50 meq/kg indicating the absence of undesirable fermenta-

tion. *Our results demonstrated low acidity of acacia honey and high acidity of forest honey, as compared with other examined honey types. The values of electrical conductivity* in the investigated honey samples *were between 0.08 and 1.99* ms/cm. The data indicated the lowest conductivity in acacia honey samples, while the highest conductivity was found in samples of forest and linden honey. Our hypothesis that there is a correlation between the tested parameters is only confirmed for meadow and polyfloral honey samples. In our study, the data on the geographical origin of honey were not available; therefore no other conclusions were made.

The variations in the properties of honey samples illustrated the differences in the botanical origin where honeybees feed. Accordingly, further research on physicochemical properties of honey from Serbia is highly recommended and important in order to set up certification marks and improve criteria for assessing the quality of Serbian honey.

AKNOWLEDGMETS

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