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Plenary lecture

CLASSICAL SWINE FEVER: CURRENT SITUATION AND RECENT ACTIVITIES OF THE EU & OIE REFERENCE LABORATORY FOR CSF

Paul Becher

Institute for Virology, University of Veterinary Medicine, Hannover, Germany

Abstract: Classical swine fever (CSF) is considered to be one of the most important viral diseases in pigs worldwide. In many parts of the world great efforts are being undertaken to reduce economic losses caused by CSF or to eradicate the disease. Among the member states of the European Union (EU) a harmonized strategy for diagnosis, control and eradication of CSF is applied. Success of the common strategy is documented by the decreasing number of outbreaks during the last decade resulting in the absence of CSF in most EU member states. The current situation concerning CSF in Europe with special focus on the situation in the EU member states will be addressed. Moreover, recent activities of the EU & OIE Reference Laboratory for CSF will be summarized. These activities include (i) organization and quality assurance of CSF diagnosis in the EU and development of novel diagnostic tools, (ii) update of the CSFV sequence database, (iii) the CSF in wild boar surveillance database, and (iv) identification of new pestiviruses and differentiation from CSFV. Despite achieved progress towards eradication, CSF remains a continuous threat to the European pig and wild boar population.

Invited lecture

EXPERIENCE FROM AN EU REFERENCE LABORATORY FOR RABIES: MANDATE, RESPONSIBILITIES, CHALLENGES IN DISEASE DIAGNOSTIC AND NETWORKING

E. Robardet, M. Wasniewsi, A. Servat, E. Picard-Meyer and F. Cliquet

EURL for Rabies, France

Abstract: The European Commission has designated Anses, the French Agency for Food, Environmental and Occupational Health & Safety as the European Union Reference Laboratory (EURL) for Rabies and for Rabies Serology.

These mandates are accomplished under the council decision of 20 March 2000 for the EURL for rabies serology and under the commission regulation 415/2008 of 28 July 2008 (amended by the commission regulation 415/2013 of 6 May 2013) for the EURL for rabies.

While the EURL for Serology is responsible for evaluating and standardising the serological tests used in the frame of pet travel schemes, the EURL for Rabies is responsible for evaluating and standardising the rabies diagnosis tests and laboratory techniques used in the frame of oral vaccination monitoring.

Under this context, the EURL for Serology is responsible for the establishment, improvement and standardization of methods of serological titration on carnivores vaccinated against rabies. He also coordinates annual rabies serology proficiency tests. Serological test (ELISA kits) evaluation and reference material (OIE serum) have also been assessed. More recently, new tools such as filter paper blood samples, facilitating the blood sampling in the field, have been developed.

The EURL for Rabies coordinates the methods employed within EU for rabies diagnosis. Each year a proficiency test for rabies diagnosis is proposed to the National Reference Laboratory (NRL) network for their evaluation on rabies diagnosis reference techniques (Fluorescent Antibody tests (FAT) and Rabies Tissue Culture Infection Test (RTCIT) but also on molecular techniques (conventional RT-PCR and Real time PCR). Proficiency test for tetracycline detection have also been regularly organised. Techniques evaluation is also a major activity of the EURL and a deep and comprehensive comparison of Real time PCR methods is currently assessed. A workshop organised annually allows laboratories to share exeriences, to discuss the results within the network as well as to point out the needs and assessment for the future.

Invited lecture

ARBOVIRUS SURVEILLANCE IN THE MEDITERRANEAN AND BLACK SEA REGIONS: THE MEDILABSECURE PROJECT

Miguel A. Jiménez-Clavero, Elisa Pérez-Ramírez, Flavia Riccardo, Maria Grazia Dente, Silvia Declich, Marie Picard, Vincent Robert, Camille Escadafal, Lobna Gaayeb, Jean-Claude Manuguerra, Kathleen Victoir

ASF EURL, Spain

Abstract: *Objective of the study*. Emerging and re-emerging viral diseases are threatening global health. Most of them are zoonotic and/or vector-borne. West Nile virus disease and Crimean-Congo Hemorrhagic fever are examples of arthropod-borne viral (arboviral) zoonotic diseases emerging recently in the Mediterranean and Black Sea regions. Indeed, other arboviral zoonotic diseases which are now expanding their geographic range such as Rift Valley fever for instance, or even as yet unknown agents, may emerge in the future in these areas. For these emerging threats, the „One-Health" approach, consisting of a multi-disciplinary integration of human and veterinary virology, entomology and public health sciences, may support reaching better surveillance, control and preparedness.

The EU-funded MediLabSecure project (2014–2017) aims at enhancing the preparedness and response to viral threats by establishing an integrative network of virology and entomology laboratories in 19 non-EU countries of the Mediterranean and Black Sea areas. It reinforces the network established by the EpiSouth Plus project (2010–2013) by involving partners from animal virology and medical entomology laboratories additionally to partners in human virology and public health.

Methods. A total of 47 laboratories from the 19 beneficiary countries were selected to actively take part in the Medilabsecure network. After a „Heads of labs" meeting, coordinators, partners and head of laboratories established the objectives and future steps of the project according to needs and experiences. A trans-disciplinary, trans-national network of 4 fields (animal virology, human virology, entomology and public health) was established. Each field designed its own itinerary comprising training activities, external quality assessments, expert assessments, scientific visits, etc, aiming at building capacities for the involved countries to respond to emerging disease threats, under a transboundary, regional perspective.

Results. The project is now in its second half. Training programs addressing essential aspects of emerging diseases diagnostics, diagnostic harmonization, pathogen detection, vector identification and monitoring, laboratory assessments and preparedness, have been successfully launched and are currently ongoing with promising results.

Conclusion. By enhancing diagnostic capacities and regional multidisciplinary cooperation, the Medilabsecure network could represent the cornerstone of a corporate preparedness and response to vector-borne and respiratory viral threats in the Mediterranean and Black Sea regions.

Key words: *arbovirus, surveillance, biosafety, laboratory, network.*

WEST NILE AND OTHER FLAVIVIRUSES EMERGING IN THE MEDITERRANEAN

Elisa Pérez-Ramírez and Miguel Ángel Jiménez-Clavero

ASF EURL, Spain

Abstract: Mosquito-borne flaviviruses provide some of the most significant examples of emerging and reemerging diseases of global impact. In the recent years, several mosquito-borne epornitic flaviviruses have emerged in different parts of the world. In the particular case of Europe and the Mediterranean area, several countries are currently facing clinical outbreaks or silent circulation of a wide diversity of flaviviruses. In particular, West Nile virus (WNV) and Usutu virus (USUV) have spread in an unprecedented way, illustrating the complexity of the different factors that contribute to their emergence, resurgence and spread. This situation is of concern for animal health because these viruses can cause disease in birds and some of them also in certain mammals like horses. In addition, as zoonotic agents, these viruses pose a significant threat for human health.

Apart from WNV and USUV, other mosquito borne flaviviruses are present in the Mediterranean region. As an example, in Southern Spain, a third flavivirus (Bagaza virus, BAGV, synonymous with Israel turkey meningoencephalomyelitis virus, ITMV) is co-circulating together with WNV and USUV, affecting the same wild bird species and sharing the same mosquito vectors and a common natural life cycle. This new epidemiological situation represents a relevant challenge that calls for improvements in the detection techniques and surveillance plans. WNV diagnosis clearly needs to be enhanced in Europe and worldwide. The specificity of the detection tests should be improved and new tools for the differential diagnosis of closely related flaviviruses need to be developed. In addition, other areas of research should be promoted in order to assess the risk that the co-ocurrence of these viruses poses for human and animal health: new knowledge is needed on the pathogenicity of the strains found in an area for the different hosts, including humans. Moreover, data on host range and vector competence for the diversity of species of hosts and vectors found in a given area, as well as in areas where these viruses could spread, would be crucial to design adequate surveillance plans adapted to particular epidemiological situations.

Key words: *flavivirus, West Nile virus, Usutu virus, Bagaza virus, Mediterranean, Europe.*

Invited lecture

VIRAL VECTOR BORNE DISEASES: EARLY DETECTION AND RISK ANALYSIS

Vesna Milićević[1*], Jelena Maksimović-Zorić[1], Ljubiša Veljović[1], Vladimir Radosavljević[1], Ivan Pavlović[1]

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INTRODUCTION

Vector borne diseases are diseases that are transmitted by the vector and which, due to climate changes and progressive trend of globalization, are (re)emerging in many regions of the world. Though animals are mostly reservoirs and not heavily affected, 17 million human deaths per year [1], due to highly infectious diseases, are enough for global veterinary public health concern. Overpopulated and economically poor countries are particularly at risk.

Additionally, there are (re)emerging infectious diseases transmitted by vectors which makes epidemiological situation even more complicated and unpredictable. Diseases transmitted by Arthropods (mosquitoes, ticks, midges etc.) are known as Arboviral diseases. These include three virus genera, Flavivirus, Alphavirus and Bunyavirus, causing West Nile fever (WNF), Yellow fever (YF), Dengue, Murray Valley fever (MV), Japanese encephalitis, Equine encephalitis, Chikungunya (CHIK) Fever, Rift Valley fever (RFV), tick-borne encephalitis (TBE), Crimean-Congo haemorrhagic fever (CCHF) and other less common diseases [2].

So far, it has been seen that global environmental and socio-economic changes created favourable conditions for emergence and transmission of vector-borne diseases.

International movements enable import of vectors and consequently pathogens into new regions. Not all imported vectors and pathogens are able to adapt, but this is how malaria and WNF have been established in Canada. It is anticipated that such scenario could happen with Rift Valley fever, Japanese encephalitis, and chikungunya [3].

Spread of vector-borne pathogens is also conditioned by global climate changes. Therefore, the northward spread from endemic areas is not surprising. Since the 1980s, tick vectors have increased in density and spread into higher latitudes and altitudes in Europe. Ticks are now found in abundance along the Baltic Sea coastline up to latitude 65 °N in Sweden [4, 5].

While some diseases emerge, there is a list of diseases that have disappeared and now re-emerge. Changes to their geographic range and ecology driven by environmental changes such as climate change explain this phenomenon.

Animals including wildlife, as mentioned, are not heavily affected, but play important role as reservoir maintaining causal agents. Wildlife is also influenced by environmental and socio-economic changes (e.g. climate change, and land use changes due to urbanization and agricultural expansion) which alter the dynamics of transmission amongst natural reservoir hosts, changing the geographic footprint of endemic areas and the likelihood of animal–human transmission.

Having the country close to the regions where vector borne diseases are endemic and on crossroad between east and west, led us to investigate the risks of their emergence in Serbia. The aim of this study is to present the capacity and readiness of the Institute of Veterinary Medicine of Serbia (IVMS) to diagnose and to early detect viral diseases that have not been diagnosed before in this region and that are transmitted by vectors.

MATERIAL AND METHODS

For the risk analysis, we used qualitative approach based on data related to diseases and vectors distribution, and dominant traffic flows in our region.

Selection of priority diseases presented in this paper was based on the results of (i) vectors known to be present in Serbia, (ii) diseases known to be present in the region, (iii) diseases with unknown status in Serbia and (iv) diseases which could be related to dominant traffic routes.

We systematically searched for relevant publications in repositories of the World Health Organization Library (WHOLIS), U.S Centers for Disease Control and Prevention (CDC), Food and Agricultural Organization (FAO), European Centre for Disease Prevention and Control (ECDC). According to these findings, diagnostic procedures have been established in IVMS [6, 7, 8, 9]. These procedures do not require virus propagation. Mosquitoes collected for West Nile fever and Bluetongue disease monitoring were tested for the presence of USUTU and Rift Velley fever virus by RT-PCR. Insects were collected in Belgrade region during the summer 2015, and were initially screened for the presence of vector species (*Aedes spp, Anopheles spp, Culex spp*). After these species were confirmed, they were tested for USUTU and RFV. Following this procedure, we tested 200 samples.

Ticks, collected from the south-west Serbia, where the densest sheep population is located, were used for TBE and CCHF testing. Screening procedure was the same for 20 samples of ticks, after *Ixodes ricinus* and *Hyalomma marginatum* had been detected in the samples.

RESULTS

Investigating incidence and risk factors for the disease occurrence, we found several viral vector diseases of high importance for our country. They are Crimean–Congo haemorrhagic fever, tick borne encephalitis, USUTU viral infection and Rift Valley fever.

As a major risk factor for emerging, vector arthropod species for these four diseases are known to be present in Serbia. *Hyalomma marginatum*, CCHF virus vector, apart from Serbia is also present in Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, France, Greece, Italy, Kosovo, the Former Yugoslav Republic of Macedonia, Moldova, Montenegro, Portugal, Romania, Russia, Spain and Ukraine [10, 11]. *Ixodes ricinus*, vector of TBE, covers a wide geographic region including the Balkans. Despite wide range, there are still changes in its distribution, conditioned by climate changes which resulted in higher altitude settlement [12]. Beside *I. ricinus*, *I. persulcatus* is also present in Serbia [13, 14]. As across Europe, *Culex pipiens*, transmitting USUTU virus among other viruses, is indigenous species in Serbia [15]. So far Rift valley fever has been restricted to Africa and partially middle east, but vector species cover much wider region including Serbia where *Aedes albopictus* has, also, been reported [16].

Epidemiological data about these vector diseases in Serbia are very limited.

According to literature data, it is assumed that CCHF is endemic in Kosovo based on serology and PCR on the samples from 2005 and 2009 [17]. However, the infection has not been confirmed in Serbia so far [18].

Routine use of suitable diagnostic tests for TBE is uncommon in Serbia. Therefore, both the prevalence and the incidence of tick-borne encephalitis remain unknown [19]. However, TBE is, across large regions of Europe and Asia, endemic [20]. Similarly, USUTU virus monitoring in Serbia is patchy indicating circulation of the virus in particular regions by serological tests [21]. So far, Rift valley fever has not been introduced in either Europe or the region. But specifically for the Balkan region, intensive traffic flow to and from middle east countries can play important role in epidemiology and occurrence of this disease in Serbia.

Laboratory diagnosis in IVMS for these diseases is based on virus genome detection by molecular methods from variety of samples. There is also capacity for diagnosis of other vector borne diseases that, under certain circumstances, could emerge in Serbia such as equine encephalomyelitises, Japanese encephalitis, Dengue fever, Yellow fever and Zika virus.

By molecular methods, in 200 mosquito samples containing vector species for RFV and USUTU, genomes of these viruses were not detected. Similarly, CCHF and TBE viral genome were (not) detected in 20 tick samples.

DISCUSSION

Nowadays we are the witnesses of climate changes and its side effects. Those changes basically include wetter winters and drier summers. Over the last 100 years Europe has been warmed by 0.8 °C [22] leading to reduction of overwintering mortality of vectors and consequently new areas for the diseases occurrence. Changes are not uniform and are more evident to the north and during the last decades.

In Serbia, for the last 30 years the temperature has been increased to the level 4.54 °C/100 years [23]. However, this value is lower for the wider period of time: for the last 100 years, it is 1.5 °C which is, anyhow, higher than average in Europe. There are, also, in Serbia specific areas where positive trend of temperature increment is more evident such as north of the country, Loznica, Belgrade and Negotinska krajina regions.

Precipitation trend in Serbia has been negative since 1982, and reaches maximum for the last 30 years. However, there are regions with extreme negative trend (–30% for the last 50 years), such as east of the country and Negotinska krajina. On the contrary, in the Zlatibor area, Pester and west of the country, precipitation trend is positive, 40% for the last 50 years. It has been observed that the periods with temperature positive trend overlap with precipitation negative trend.

With new environmental conditions, many vectors expand their ranges and new vector species may be introduced from the tropics. For example, a major vector of RFV, *Aedes albopictus*, has spread to 22 northern provinces in Italy since being introduced [22]. Such scenario could be predictable for dengue though the last occurrence in Europe was recorded in Greece 1927/28.

Tick distributions are also closely linked with climate. While milder winters will reduce tick and host mortality and extend the period when ticks are active, drier summers will increase tick mortality. There is recent evidence that the northwards movement of the tick *Ixodes ricinus* in Sweden was related to the milder climate experienced in the 1990s [22].

Recent events in Serbia related to vector borne disease were West Nile fever introduction in 2012 and Bluetongue disease outbreak in 2014, while the diseases which are the subject of this paper have not been confirmed so far.

Crimean-Congo haemorrhagic fever was reported in Kosovo in 2002, when out of 12, 2 patients died. Due to very severe clinical symptoms and along with the history of having been bitten by the ticks, it is assumed that CCHF cases have not been misdiagnosed or undetected in Serbia [23]. *Hylomma marginatim* ticks, a species present in Serbia, have a great capacity to support a wide range of temperature and humidity conditions [9, 24, 25]. Already inhabited with competent ticks, if the virus would be also present, major conditions for the disease occurrence would have been fulfilled in Serbia [27]. The very intensive trade between Balkan countries

favors CCHV introduction in Serbia. Even legal trade does not prevent the disease due to the lack of tick control on imported livestock. It is often seen that on one animal up to 100 Hyalomma ticks can be found. Our results support the hypothesis that CCHF is not common disease in Serbia, though the bias, due to small number of samples, has to be taken into consideration.

Tick-borne encephalitis (TBE) is a human viral infectious disease but approximately two-thirds of infections are asymptomatic. *Ixodes ricinus*, feeding on the small rodents, transmit the virus to the humans. *Ixodes spp.* is widely distributed in Serbia, mostly in woodland habitats. TBE is common in Austria, Estonia, Latvia, the Czech Republic, Slovakia, Germany, Hungary, Poland, Switzerland, Russia, Ukraine, Belarus, and northern regions of the former Yugoslavia. It occurs at a lower frequency in Bulgaria, Romania, Denmark, France, the Aland archipelago between Sweden and Finland and the neighbouring Finnish coastline, as well as along the coastline of Southern Sweden, from Uppsala to Karlshamn. Serologic evidence for TBE infection, as well as sporadic cases, has been reported from Albania, Greece, Northern Italy, Norway, and Turkey [28]. Due to mostly asymptomatic manifestation and lack of surveillance but widely distribution in the region, the disease possibly have been present in Serbia for a longer period of time but undiagnosed. However, we did not confirm the virus by molecular methods: for the more accurate TBE assessment, more samples need to be tested for a wider region.

The first emergence of USUV was reported in Austria in 2001. However, it has been shown that the visus had been present much earlier in Tuskany: retrospective analysis of archived tissue samples from bird deaths in 1996 confirmed USUTU virus [29].

After in Austria, the virus has been detected in several countries, including Hungary, Italy, Spain and Switzerland. Serological evidence has bead demonstrated the Czech Republic, England, Germany, Italy, Poland, Spain and Switzerland. Though competent vector species in Serbia is present, and despite earlier reported seroprevalence in humans at certain level, our results were not confirmed the virus, at least in Belgrade region.

Rift Valley fever is transmitted by mosquitoes. So far, the only occurrence out of Africa was in 2000–2001 in Saudi Arabia and Yemen. The most common form of the disease is a self-limiting, flu-like illness. Sheep, goats and cattle are thought to be the primary amplifying hosts among domesticated animals, although other species such as camels could also be involved [16]. *Aedes albopictus,* vector of RFV, was first reported in Europe in 1979 in Albania. Only just in 2001 it was detected in Montenegro. In Serbia, monitoring of invasive and vector mosquitoes and vector borne diseases started in 2009 confirming the *A.albopictus* [29]. Having the competent vector species in Serbia and with the potential introduction of the virus in different routes, Rift Valley fever could be, evidently, established in the country. As a major risk factor for the virus

introduction, we recognized very intensive trade and traffic flows, across Balkans to the east and south. Due to unspecific symptoms, the disease could be unrecognized and/or misdiagnosed, surveillance system enabling early detection and reaction should be developed. For this purpose, in the case that diseases have not been present earlier as it is assumed for Serbia, serology could be, also, applied. Our results, based only on mosquito testing, support the previous assumption that the diseses was not present in Serbia.

The presented work is our initial investigation of few viral vector borne diseases presence in Serbia. Though our results did not confirm the causal viral agents, we cannot exclude the possibility that the diseases have been present in Serbia earlier. This is due to the small number of samples, not representing the whole country. The samples, also, were collected only during the summer 2015.

As a major risk factor for the diseases incursion in Serbia, we found the intensive traffic and trade. The mitigating circumstance is that this risk factor can be minimized by following strict procedures and quarantine measures. Climate change is also an important risk factor which we cannot, on short term, influence significantly but we can prevent its effects by desinsection and deratisation.

For more accurate data on diseases distribution in Serbia, due to the complexity of vector borne diseases, all professions, human doctors, veterinarians, entomologists etc, need to be included in surveillance system development which will serve for effective risk analysis and disease management in the future.

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BIODIVERSITY AND SEASONAL DISTRIBUTION OF TICKS ON GREEN AREAS OF BELGRADE

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Abstract: Ticks are obligate haematophagous ectoparasites which have multiple adverse effects on the host organism. A particular problem is that they spread diseases to humans, domestic and wild animals, which can be reservoirs, vectors and/or transient hosts for the tick-borne pathogens.

In the period of March–November 2010–2012, ticks were collected at various localities in the Belgrade area by flagging vegetation (in the context of ticks control funded by the Secretariat for Environmental Protection). Tick species and sex/gender were identified by morphometric characteristics.

Ixodes ricinus (51.01%) was absolute dominance, followed by *Rhipicephalus sanguineus* (39.74%), *Dermacentor marginatus* (8.24%) and *Dermacentor reticulatus* (1.01%). Overall, the female-male ratio during the course of our study was 61.02%: 38.98% in favor of females, with a higher percentage of females established in all three years of research. Of the all species we established a larger number of females. For the two most commonly found species *Rhipicephalus sanguineus* and *Ixodes ricinus*, this ratio was 69.50%: 30.50% and 63.42% : 36.58%, respectively in favor of females. Population dynamics of the established species of ticks during the all years of examination was next: in March were found: *I.ricinus, R.sanguineus* and *D.marginatus*. In April, we observed the occurrence of the D. recticulatus, and species that reached maximum numbers were *D.marginatus. I.ricinus* reached maximum abundance in May. In June, the population peak is observed for *R. sanguineus* which is the most common types in July and August. In September, we saw an increase in the population of *I.ricinus* and *D.marginatus*, while in October we observed the emergence of the *R.sanguineus.*

There are differences in the type of vegetation at the investigated localities. While Zvezadara and Miljakovac Forest represent typical wooded localities, Ada Ciganlija, Topčider and Košutnjak are a mixture of parklands and small wooded complexes and city parks are represented primary grassy area. Logically, the abundance of ticks was significantly greater at the wooded localities. In the parklands, and especially at city parks, the anthropogenic factors affect the abundance of ticks, both directly

(by grass cutting) and indirectly (trough alteration of microclimatic conditions).

Key words: *ticks, Belgrade, green area.*

INTRODUCTION

Ticks are relatve small group of hematphag arthropods (896 described species) of the phylum *Arthropoda*, subtypes *Chelicerata*, class Arachnidae, subclass Acari, order *Parasitoformes,* suborder *Ixodia* and superfamily *Ixodidea* [4, 5, 6]. They are divided into three families: *Argasidae* soft shell ticks; *Ixodidae* hard shell ticks and Nuttalliellidae which has the properties and each other tick [16]. Ticks have a rich way of life. Like all arthropods began its rise in Devon, and its continued success through the next 136 million years. Tick fossils are relatively rare. They were found in coprolites in North America and fossilized in amber originating from the Dominican Republic and the Baltic. Based on them, we can see that the Paleozoic never experienced a greater morphological transformation, especially argasidne kind, and very reminiscent of today known species [6, 11, 19].

Ticks are obligate haematophagous ectoparasites which have multiple adverse effects on the host organism. A particular problem is that they spread diseases to humans, domestic and wild animals, which can be reservoirs, vectors and/or transient hosts for the tick-borne pathogens (*Borelia burgdoferi,* Kyasanur forest disease, CCHF, *Ehrlichia sp., Anaplasma sp., Babesia sp., Thaileria spp,* Tick-born encephalitis, Q fever, arboviroses and etc.) [2, 3, 4, 12, 15].

In Belgrade area the most frequent Ixod ticks are *Ixodes ricinus, Rhipicephalus sanguineus, Dermacentor marginatus* and others [13, 16]. In order to control ticks in the area of central municipalities of the City of Belgrade was formed in the Secretariat for Environmental Protection Expert Group on protection of the population against infectious diseases in the area of pest control (mosquitoes, ticks and rodents). This paper presents the results of tick population established in main green area in Belgrade in period 2010–2012.

MATERIAL AND METHODS

Ticks were collected by flagging vegetation at numerous localities in the Belgrade. We presented examination at several different ecological types' wooded localities, parklands and parks of Belgrade (Ada Ciganlija, Košutnjak, Topčider, Miljakovac Forest, Zvezdara forest and central city parks – Kalemegdan, Ušće and Hajd park) in the period of March–November 2010–2012.

All specimens were placed into glass specimen bottles which had a piece of hard paper inserted bearing the name of locality, name of host and date and hour of collection. The tick species and sex/gender were identified by morphometric characteristics [4, 5].

RESULTS

During examination a total of 967 ticks were collected. The greatest number of ticks were recorded at the Topčider (338), followed by Miljakovac Forest (271), Košutnjak (199), and Ada Ciganlija (159). The number of ticks per 100 m^2 slightly varies between localities.

Relative abundance analysis revealed that the species *Ixodes ricinus* was absolutely dominant (942/97.41%) in relation to *Dermacentor reticulatus* (25/2.59%). All specimens of the species *D.reticulatus* were collected at the Ada Ciganlija locality. These results are in correlation with examination by Milutinović et al. (1996) and Pavlović et al. (2009).

Overall, the female-male ratio during the course of our study was 61.02% : 38.98% in favor of females, with a higher percentage of females established in all three years of research. Of the all species we established a larger number of females. For the two most commonly found species *Rhipicephalus sanguineus* and *Ixodes ricinus*, this ratio was 69.50% : 30.50% and 63.42% : 36.58%, respectively in favor of females.

The population dynamics of ticks was monitored from March to November 2004. Maximal abundance of ticks was recorded at the Ada Ciganlija locality in mid April, but at other localities during May (Košutnjak and Topčider in the first decade, Miljakovac and Zvezdara forest in the second, and city parks in the third). Unfavorable environmental conditions (temperature, relative air humidity, and precipitation) caused a decrease in abundance of the tick population in autumn.

*Table 1.*The monthly value of ticks by the flag-hour

LOCALITY	month											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Ixodes ricinus	+	+	++	+++	++++	+++	++++	+++	++	+	+	+
Rhipicephalus sanguineus	-	-	+	+	+++	++	+	+++	+	+	-	-
Dermacentor marginaus	-	-	-	++	-	+	-	++	-	-	-	-
Dermacentor recticulatus			-	++	-	+	-	+	+	-	-	-

Legend: - negative, + 1–5 ticks, ++ 6–10 ticks, +++ 11–15 ticks, ++++ more then 15 ticks

DISCUSSION & CONCLUSION

Influence of microclimate in different parts of Serbia and West Balkan pointed by Milutinović et al. [7, 8, 9, 10] and Pavlović et al. [16, 17, 18] They are aimed at establishing faunistic research with the occurrence, frequency, sex ratio and seasonal dynamics of individual species ixodid ticks. These findings have been fully demonstrated that the seasonal dynamics of the most important types of ticks encountered in the area of Belgrade – *Ixodes ricinus*, *Rhipicephalus sanguineus* and *Dermacentor marginatus* are subject to seasonal variation, which has two peaks – the vernal and autumnal [8, 9, 13]. It is spring dots per significant number of individuals, but it is depending on the winter hibernation and lows that have ticks in this period [1, 2, 3]. Finally, this was confirmed during the study seasonal dynamics of occurrence and incidence of babesiosis of dogs in the Belgrade-made by Pavlovic et al. [14] and Terzina (2005) [20].

From number of ticks great influence had differences in the type of vegetation at the investigated localities. While Miljakovac and Zvezdara Forest represent typical wooded localities, Ada Ciganlija, Topčider and Košutnjak are a mixture of parklands and small wooded complexes and city parks are represented primary grassy area. Logically, the abundance of ticks was significantly greater at the wooded localities than at the parkland localities or parks. In the parklands of Ada Ciganlija, Topčider and Košutnjak, and especially at city parks, the anthropogenic factor affects the abundance of ticks, both directly (by grass cutting) and indirectly (trough alteration of microclimatic conditions). Similar findings were reported by Milutinović et al. [8, 9] and Pavlović et al. [13, 16].

Ticks are most often found in places where large fluctuations in animals and humans. The most common hosts are mammals, or in the absence of them can be found on birds and reptiles. For the first host to the serpent after the presentation of the eggs in the larval stage in which there are three pairs of legs. After the second metamorphosis occurs self extraction and transition to the third – the final host where they will be transformed into an adult and physically fully developed, become sexually mature individual and the reproduced.

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SEROEPIZOOTIOLOGICAL SITUATION OF BRUCELLOSIS IN REPUBLIC OF SERBIA

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Abstract: In Serbia, brucellosis in domestic animals is controlled in keeping with the Programme of measures of health protection of animals which are adopted at an annual level, by Ministry of Agriculture, Forestry and Waters.The diagnostic examination of samples of blood sera of cattle, sheep, goats, breeding boar, rams and bulls, as well as animals that had aborted, using rapid methods Rose Bengal, and in the case of a positive result also using the implementation of iELISA and confirmed using the cELISA or CFT. In the course of 2011, 98 serologically positive animals were registered, 16 cattle in 6 municipalities and 82 sheep and goats in 8 municipalities. One year later, there were 36 positive animals, 7 cattle in 2 municipalities and 29 sheep and goats in 1 municipality. During 2013, the total number of positive animals was 110, of which 4 cattle in 3 municipalities and 106 sheep and goats in 4 municipalities. The following year saw the highest number of registered positive animals, 227, specifically 27 cattle in 6 municipalities and 200 sheep and goats in 13 municipalities. During 2015, there were 28 registered serologically positive animals, 15 cattle in 11 municipalities and 13 sheep and goats in 9 municipalities. The number of serologically positive pigs ranged from 7 (5 municipalities) in 2015, to 40 (2 municipalities) positive animals in 2013 year.

Key words: *Brucellosis, animals, serological, diagnosis*.

INTRODUCTION

Brucellosis is a chronic contagious disease in many species of domestic and wild animals. Moreover, brucellosis is one of the most important bacterial zooanthroponoses on a global scale (Lopes et al. 2010). The causes belong to facultatively intracellular bacteria of the genus

Brucella. The genus *Brucella* covers nine species, including six classic members (*B.melitensis*, *B.abortus*, *B.suis*, *B.canis*, *B.ovis* and *B.neotomae*) and there are three new species (*B.ceti*, *B.pinnipedialis* and *B.microti*) and one proposed specie (*B.inopinata*) (Hubálek et al. 2007, Foster et al. 2007, Scholz et al. 2008, Scholz et al. 2010). A recently isolated strain from a baboon has been marked as *Brucella* sp.NVLS 07-0026 (taxonomy database identifier: 520448). *Brucellae* can cause disease in domestic animals such as cattle, sheep, goats, pigs, horses and dogs (Megid et al. 2010).

The proven presence of *Brucella* in wild boar, hares and foxes indicates the possibility that wild animals, as natural reservoirs of infection, can play a significant role in the spreading of the disease among domestic animals, in particular pigs (Cvetnic et al. 2009, Hofer et al. 2010, Kreizinger et al. 2014). This is precisely how it is interpreted that an infection of cattle with *B.suis* biovar 2 broke out in Poland, indicating that the cause originated in wild boar or hare in the area in which this *B.suis* biovar 2 infection had previously been established (Szulowski et al. 2013).

The main clinical signs of brucellosis in animals are abortion, retained placenta, stillbirth, orchitis and arthritis (Poester et al. 2010). *Brucellae* enters the organism most frequently through the digestive, genital or respiratory tract. *Brucellae*, protected by their intracellular position, persist in the organism for quite a long time, even life-long, without clear symptoms of the disease and with the possibility of spreading the cause in their environment (Ficht 2003).

The disease is most often introduced into a healthy herd through an infected animal. The main pathways for the spread of the disease are through food, water, and objects and surfaces that are contaminated with the contents of the uterus of an infected animal (Radojičić 2005).

Brucellosis is also one of the most significant zoonoses that has spread throughout the world. In humans, this is the most frequent professional disease among cattle farmers, butchers and veterinarians. It is transferred through direct contact with an infected animal, its excretions or by consuming products made from infected animals, such as non-pasteurised milk or cheese (Lopes et al. 2010). There have also been reports of infections among persons working in laboratories (Yagupsky and Baron, 2005).

Brucellosis is diagnosed using a combination of several assays. The most frequently used are serological examinations, then the isolation and identification of the bacteria, and mollecular methods (Poester et al. 2010, Winchell et al. 2010). Isolation and identification, as the basic bacteriological methods, still remains as reliable diagnostic tests. However, as their implementation requires highly qualified personnel, equipment and level 3 biosecurity, which, in addition to the present risk to the health of laboratory staff, this method being used infrequently and only in laboratories equipped for this purpose. The basis of the diagnosing and

eradication of brucellosis is made up of numerous serological assays (Nielsen and Yu, 2010, Christopher et al. 2010). There are certain difficulties in serological diagnostics because of the antigenic similarity between Brucella and other bacteria, the dynamics of the forming of antibodies that belong to different classes and subclasses of immunoglobulins, as well as their different levels during the course of infection (Corbe et al. 1984, Staak et al. 2000, Christopher et al. 2010). In spite of this, serological diagnostics remains the most user manner of detecting infected animals today. Serological diagnostics of brucellosis has advanced considerably lately, with the development of very sensitive and specific tests, easily performed in routine work.

MATERIAL AND METHODS

In keeping with the Programme of measures of health protection of animals which are adopted at an annual level, by Ministry of Agriculture, Forestry and Waters, cattle older that 12 months are diagnostically examined for brucellosis, sheep and goats older that 6 months, breeding boar, rams and bulls, as well as animals that had aborted. Breeding rams and billy goats which are used for natural breeding and the production of semen in centers for artificial insemination are also examined for infection with *B.ovis.*

In the Republic of Serbia, around 660.000 blood samples from cattle and 1.250.000 blood samples from sheep and goats are examined serologically every year. The authorized scientific and specialistic veterinary institutes perform the diagnostic examination of samples of blood sera of cattle, sheep and goats using rapid methods Rose Bengal or fluorescence polarisation assay (RB, FPA), and in the case of a positive result also using the implementation of indirect enzyme-linked immunosorbent assay (iELISA). In the case when a positive result is obtained, blood is sampled again and examinations are repeated again with the serological method, competitive enzyme-linked immunosorbent assay or complement fixation test (cELISA, CFT) in order to detect specific antibodies against the *Brucellae* species. The examination methods are implemented in the way prescribed in the latest edition of the OIE Manual of Standards for Diagnostics Test and Vaccines.

RESULTS

In the course of 2011, 98 serologically positive animals were registered, 16 cattle in 6 municipalities and 82 sheep and goats in 8 municipalities. One year later, there were 36 positive animals, 7 cattle in 2 municipalities and 29 sheep and goats in 1 municipality. During 2013, the total number of positive animals was 110, of which 4 cattle in 3 municipalities and 106 sheep and goats in 4 municipalities. The following

year saw the highest number of registered positive animals, 227, specifically 27 cattle in 6 municipalities and 200 sheep and goats in 13 municipalities. During 2015, there were 28 registered serologically positive animals, 15 cattle in 11 municipalities and 13 sheep and goats in 9 municipalities. During 2011, there were registered 10 serologically positive pigs in 1 municipality, then in 2013, in 2 municipalities 40 positive animals, in 2014, 31 positive in 3 municipalities and during 2015, 7 serologically positive pigs in 4 municipalities. The results of investigation are shown in table 1.

Table 1. Results of the serological investigations of brucellosis in Serbia

Year	Cattle		Sheeps and Goats		Pigs	
	No. positive animals	No. municipalities	No. positive animals	No. municipalities	No. positive animals	No. municipalities
2011	16	6	82	8	10	1
2012	7	2	29	1	0	0
2013	4	3	106	4	40	2
2014	27	6	200	13	31	3
2015	15	11	13	9	7	4

DISCUSSION

The reduction or the maintenance of the incidence of brucellosis in animals in the Republic of Serbia is the result of several years' long serological monitoring and the implementation of the prescribed measures.

The increased number of serologically positive animals in 2014 ocurred during and after the extensive floods that hit Serbia that year. This took place in the spring and summer, when, in addition to the considerable presence of surface and underground waters, the temperature of the outer environment favoured the survival and spread of the bacteria.

Brucellosis has also been registered in neighboring countries, in Bosnia and Herzegovina (source: BiH State Veterinary Office), Macedonia (Food and veterinary Agency) Greece (HCDCP), Croatia (Cvetnic et al. 2014) and Bulgaria (Arnaudov 2014).

The programme for brucellosis control must cover a well-organized veterinary service with a team of experts for the realization of speedy and correct diagnostics, a massive education of the population, and support from state institutions. It is possible to achieve the eradication of brucellosis or the reduction of its frequency to the lowest levels through the implementation of systematic serological examinations of large numbers of animals. The removal of infected animals reduces the possibility of the spread of the infection within and between herds.

The detecting, suppression and eradication of brucellosis is a long-term process which demands the strict implementation of control measures which will result in the suppression and eradication of this important zooanthroponosis.

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REQUIREMENTS AND PROCEDURES IN MICROBIOLOGICAL LABORATORIES FOR WORK WITH CONTAGIOUS INFECTIOUS AGENTS

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Abstract: Requirements, necessary technical and technological conditions and procedures that should be established in diagnostic and research laboratories highly depend on the infectious agents that are, or plan to be detected or tested in them. Laboratories are as well as pathogens divided into four levels of biosafety: Biosafety Level 1 (BSL1) or the basic level of the safety, Biosafety Level 2 (BSL2) or the second basic level of biosafety, Biosafety Level 3 (BSL3) or „containment“ and the Biosafety Level 4 (BSL4) or maximal „containment“. Most frequently the laboratory level of biosafety corresponds to the level of the risk group of the pathogens that are to be work with in the laboratory. However, this is not always the rule. Frequently, and from the safety reasons, the work with the pathogen from the lower risk group is done in the higher biosafety level laboratory. In this paper, propositions related to biosafety levels in the microbiological laboratories, prescribed by the World Health Organization (WHO), are presented. A detailed explanation of the demands and procedures are given, as well as the description of necessary technical and technological conditions for different biosafety levels in laboratories, starting with Biosafety Level 1 up to Biosafety Level 4. A special attention is given to the demands regarding access to the laboratory, the protection of personnel, rule book on behaviour, working procedures, characteristics of laboratory equipment, laboratory area and its design, and handling with laboratory waste.

Keywords: *biosafety levels, requirements, procedures, design.*

INTRODUCTION

Published reports of laboratory associated infections (LAIs) first appeared around the start of thetwentieth century. According to the literature data 4079 LAIs resulting in 168 deaths occurring between 1930 and 1978 have been described (Sulkin and Pike, 1951; Pike and Sulkin,

1965; Pike, 1978, 1979). The ten most common causative agents of overt infections among laboratory workers found in these studies were Brucella spp., *Coxiella burnetii*, hepatitis B virus (HBV), *Salmonella typhi*, *Francisella tularensis*, *Mycobacterium tuberculosis*, *Blastomyces dermatitidis*, Venezuelan equine encephalitis virus, *Chlamydia psittaci* and *Coccidioides immitis*. During the following 20 years, 1267 overt infections with 22 deaths were described. The diagnostic and research laboratories accounted for respectively 45% and 51% of the total LAIsreported (Harding and Byers, 2000). *Mycobacterium tuberculosis*, *Coxiella burnetii*, hantavirus, arboviruses, HBV, *Brucella* spp., *Salmonella* spp., *Shigella* spp., hepatitis C virus, and *Cryptosporidium* spp. accounted for 1074 out of the total of 1267 infections. The authors also identified an additional 663 cases that presented as sub-clinical infections.

There are many published classifications, requirements and instructions from number of international organizations (WHO, OIE, FAO etc.) and national organizations and legislative bodies as well as organizations from experts in the field of biosafety (International Veterinary Biosafety Group etc.) that are responsible for the field of biosafety.

To understand this technique, it is necessary to explain the basic terminology. The term biosafety levels applies to the level of biosafety of the work process in diagnostic and research laboratories, and means safety of laboratory staff during work and safety of the environment i.e. entire surroundings from accidents that might occur in the laboratory. Biosecurity is considered as the safety of laboratory staff, especially the safety of environment i.e. live world outside the laboratory from the accidents that might occur in the laboratory with the purpose. Term biosecurity is recent and connected to various form of bioterrorism. Term „containment‟ means to preserve the content i.e. all that is in the laboratory stays inside the space, meaning that environment will be protected from the conditions in the lab and term isolation means that all that is outside the laboratory stays out from the laboratory i.e. the laboratory is protected from the outside world (this apply for instance to the laboratory for cell culture and sterile conditions). The fundamentals of „containment‟ include the microbiological practices, safety equipment and facility safeguards that protect laboratory workers, the environment and the public from exposure to infectious microorganisms that are handled and stored in the laboratory.

In this paper, propositions connected with biosafety levels in the microbiological laboratories given by World Health Organization (WHO) and by International Veterinary Biosafety Working Group are described.

CLASSIFICATION OF MICROORGANISMS INTO THE RISK GROUPS

Classification of microorganisms, given by WHO, considered only to the biosafety levels in laboratory work. There are four levels of risk groups such as:

- Risk Group 1 (*no or low individual and community risk*)

A microorganism that is unlikely to cause human or animal disease.

- Risk Group 2 (*moderate individual risk, low community risk*)

A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.

- Risk Group 3 (*high individual risk, low community risk*)

A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

- Risk Group 4 (*high individual and community risk*)

A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

THE CLASSIFICATION OF THE LABORATORIES ACCORDING TO THE BIOSAFETY LEVEL

Laboratories are as pathogens divided to four levels of biosafety: Biosafety Level 1 (BSL1) or the basic level of the safety, Biosafety Level 2 (BSL2) or the second basic level of biosafety, Biosafety Level 3 (BSL3) or "containment" and the Biosafety Level 4 (BSL4) or "maximal containment". The most frequently, level of biosafety corresponds to the level of the risk group of the pathogen that are to be work with in the laboratory. However, this is not always the rule. Often and from the safety reasons, the work with the pathogen from the lower risk group is done in the higher biosafety level laboratory. As for an example, if the production of the high quantities of certain pathogen (vaccine production or excretion from the infected animals) from the risk group 2 and if at that occasion much of the aerosol particles are produced or if such pathogen is exotic in certain area, the work with it is done in the Biosafety Level 3 laboratory.

Besides international classification it is very important that each country or its region establish the classification of the microorganisms by risk groups, taking care of:

1. Pathogenicity of the organism;

2. Mode of transmission and host range of the organism. These may be influenced by existing levels of immunity in the local population, density and movement of the host population, presence of appropriate vectors, and standards of environmental hygiene;
3. Local availability of effective preventive measures. These may include: prophylaxis by immunization or administration of antisera (passive immunization), sanitary measures, e.g. food and water hygiene and control of animal reservoirs or arthropod vectors;
4. Local availability of effective treatment. This includes passive immunization, post exposure vaccination and use of antimicrobials, antivirals and chemotherapeutic agents, and should take into consideration the possibility of the emergence of drug-resistant strains.

The classification of certain pathogens in different groups must be done according to the risk analysis for which, except at some points, possible outcome after exposure must be taken into consideration, none natural infection pathway and transfer that occur during laboratory work, stability of the pathogen in the environment, concentration of the pathogen and the quantity of the concentrated pathogen to be manipulated with, as well as genetic manipulations that might lead to the susceptibility of the other (none natural) hosts or if sensibility is changeable to until then effective antimicrobial drugs. Therefore to establish the laboratory biosafety level, the pathogen itself that will be used for the work is taken in to account, the available specific equipment and houses, as well as indispensable procedures and availability of the measures taken to secure the work in the laboratory.

REQUIREMENTS PROCEDURES AND MINIMAL TECHNOLOGICAL CONDITIONS FOR BIOSAFETY LEVELS 1 AND 2 LABORATORIES

Laboratories of the Biosafety Level 1 (BSL1) are laboratories of the lowest level of biosafety. These laboratories are mostly foreseen to conduct lectures and studding (schools, University laboratory used for training etc.), as well as for certain research. In these laboratories the work is done with known microorganisms and they by the rule do not accept samples for examination and their basic concept is to apply good laboratory practice. Diagnostic and health-care laboratories (public health, clinical or hospital-based) must all be designed for Biosafety Level 2 or above. As no laboratory has complete control over the specimens it receives, laboratory workers may be exposed to organisms in higher risk groups than anticipated. This possibility must be recognized in the development of safety plans and policies. In some countries, accreditation of clinical

laboratories is required. Globally, national and international standard precautions should always be adopted and practised.

Code of practice:

Each laboratory, according to national and international standards must build and apply the „How to behave Manuel" or „Code of good practice" as safety operational procedures that identify known and potential hazards and define procedures for the prevention, elimination and minimization of these hazards. Based on this „Code" of minimal requirements that must be full field by such laboratories are:

Access to laboratory

1. The international biohazard warning symbol and sign must be displayed on the doors of the rooms where microorganisms of risk group 2 or higher risk groups are handled;
2. Only authorized persons should be allowed to enter the laboratory working areas;
3. All persons entering the laboratory must be advised of the potential hazards and meet specific entry/exit requirements;
4. Laboratory personnel must be provided medical surveillance, as appropriate, and offered available immunizations for agents handled or potentially present in the laboratory;
5. Laboratory doors should be kept closed;
6. Children should not be allowed to enter laboratory working areas;
7. Access to animal houses should be specially authorized;
8. No animals should be admitted other than those involved in the work of the laboratory.

Personal protection

1. Laboratory coveralls, gowns or uniforms must be worn at all times for work in the laboratory;
2. Appropriate gloves must be worn for all procedures that may involve direct or accidental contact with blood, body fluids and other potentially infectious materials or infected animals. After use, gloves should be removed aseptically and hands must then be washed;
3. Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas;
4. Safety glasses, face shields (visors) or other protective devices must be worn when it is necessary to protect the eyes and face from splashes, impacting objects and sources of artificial ultraviolet radiation;
5. It is prohibited to wear protective laboratory clothing outside the laboratory;

6. Open-toed footwear must not be worn in laboratories;
7. Eating, drinking, smoking, applying cosmetics and handling contact lenses is prohibited in the laboratory working areas;
8. Storing human foods or drinks anywhere in the laboratory working areas is prohibited;
9. Protective laboratory clothing that has been used in the laboratory must not be stored in the same lockers or cupboards as street clothing.

Procedures of work

1. Pipetting by mouth must be strictly forbidden;
2. All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets;
3. All procedures involving the manipulation of infectious materials that may generate an aerosol should be conducted within a BSC or other physical containment devices;
4. The use of hypodermic needles and syringes should be limited. They must not be used as substitutes for pipetting devices or for any purpose other than parenteral injection or aspiration of fluids from laboratory animals. Used disposable needles and syringes must be carefully placed in conveniently located puncture-resistant containers;
5. All spills, accidents and overt or potential exposures to infectious materials must be reported to the laboratory supervisor. A written record of such accidents and incidents should be maintained. A written procedure for the clean-up of all spills must be developed and followed;
6. Contaminated liquids must be decontaminated (chemically or physically) before discharge to the sanitary sewer;
7. Broken glassware must not be handled directly. Instead it must be removed using a brush and dustpan, or forceps. Plastic ware should be substituted for glassware whenever possible;
8. Incidents that may result in exposure to infectious materials must be immediately evaluated and treated according to procedures described in the laboratory biosafety manual. All such incidents must be reported to the laboratory supervisor;
9. Written documents that are expected to be removed from the laboratory need to be protected from contamination while in the laboratory.

Laboratory working areas

1. The laboratory should be designed so that it can be easily cleaned. Carpets and rugs in laboratories are not appropriate;
2. Animal and plants not associated with the work being performed must not be permitted in the laboratory;

3. The laboratory should be kept neat, clean and free of materials that are not pertinent to the work;
4. Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day;
5. All contaminated materials, specimens and cultures, and other potentially infectious materials must be decontaminated before disposal or cleaning for reuse. Materials to be removed from the facility for decontamination must be packed in accordance with applicable local, state, and federal regulations;
6. Equipment must be decontaminated before repair, maintenance, or removal from the laboratory;
7. When windows can be opened, they should be fitted with arthropod-proof screens.

Biosafety management

1. It is the responsibility of the laboratory director (the person who has immediate responsibility for the laboratory) to ensure the development and adoption of a biosafety management plan and a safety or operations manual;
1. The laboratory supervisor (reporting to the laboratory director) should ensure that regular training in laboratory safety is provided;
2. Personnel should be advised of special hazards, and required to read the safety or operations manual and follow standard practices and procedures. The laboratory supervisor should make sure that all personnel understand these. A copy of the safety or operations manual should be available in the laboratory;
3. There should be an arthropod and rodent control program;
4. Appropriate medical evaluation, surveillance and treatment should be provided for all personnel in case of need, and adequate medical records should be maintained.

Laboratory design

1. Ample space must be provided for the safe conduct of laboratory work and for cleaning and maintenance;
2. Walls, ceilings and floors should be smooth, easy to clean, impermeable to liquids and resistant to the chemicals and disinfectants normally used in the laboratory. Floors should be slip-resistant;
3. Illumination should be adequate for all activities. Undesirable reflections and glare should be avoided;
4. Laboratory furniture should be sturdy and must be capable of supporting anticipated loads and uses. Open spaces between and under benches, cabinets and equipment should be accessible for cleaning. Bench tops must be impervious to water and resistant

to heat, organic solvents, acids, alkalis, and other chemicals and chairs used in laboratory work must be covered with a non-porous material that can be easily cleaned and decontaminated;

5. Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on bench tops and in aisles. Additional long-term storage space, conveniently located outside the laboratory working areas, should also be provided;
6. Space and facilities should be provided for the safe handling and storage of solvents, radioactive materials, and compressed and liquefied gases;
7. Facilities for storing outer garments and personal items should be provided outside the laboratory working areas;
8. Facilities for eating and drinking and for rest should be provided outside the laboratory working areas;
9. Hand-washing basins, with running water if possible, should be provided in each laboratory room, preferably near the exit door;
10. An eyewash station must be readily available;
11. Doors should have vision panels, appropriate fire ratings, and preferably be self-closing;
12. At Biosafety Level 2, an autoclave or other means of decontamination should be available in appropriate proximity to the laboratory;
13. Safety systems should cover fire, electrical emergencies, emergency shower and eyewash facilities;
14. First-aid areas or rooms suitably equipped and readily accessible should be available;
15. The provision of mechanical ventilation systems that provide an inward flow of air without recirculation instead windows should be considered. If there is no mechanical ventilation, windows should be able to be opened and should be fitted with arthropod-proof screens;
16. There should be no cross connections between sources of laboratory and drinking-water supplies. An anti-backflow device should be fitted to protect the public water system;
17. There should be a reliable and adequate electricity supply and emergency lighting to permit safe exit. A stand-by generator is desirable for the support of essential equipment, such as incubators, biological safety cabinets, freezers, etc., and for the ventilation of animal cages;
18. There should be a reliable and adequate supply of gas. Good maintenance of the installation is mandatory;
19. Laboratories and animal houses are occasionally the targets of vandals. Physical and fire security must be considered. Strong doors, screened windows and restricted issue of keys are

compulsory. Other measures should be considered and applied, as appropriate, to augment security.

Laboratory equipment

Together with good procedures and practices, the use of safety equipment will help to reduce risks when dealing with biosafety hazards. Equipment should be selected to take account of certain general principles, i.e. it should be:

1. Designed to prevent or limit contact between the operator and the infectious material;
2. Constructed of materials that are impermeable to liquids, resistant to corrosion and meet structural requirements;
3. Fabricated to be free of burrs, sharp edges and unguarded moving parts;
4. Designed, constructed and installed to facilitate simple operation and provide for ease of maintenance, cleaning, decontamination and certification testing, glassware and other breakable materials should be avoided, whenever possible.

Essential biosafety equipment

1. Pipetting aids – to avoid mouth pipetting;
2. Biological safety cabinets, to be used whenever: infectious materials are handled, such materials may be centrifuged in the open laboratory if sealed centrifuge safety cups are used and if they are loaded and unloaded in a biological safety cabinet; when there is an increased risk of airborne infection and when procedures with a high potential for producing aerosols are used (these may include centrifugation, grinding, blending, vigorous shaking or mixing, sonic disruption, opening of containers of infectious materials whose internal pressure may be different from the ambient pressure, intranasal inoculation of animals, and harvesting of infectious tissues from animals and eggs);
3. Plastic disposable transfer loops. Alternatively, electric transfer loop incinerators may be used inside the biological safety cabinet to reduce aerosol production;
4. Screw-capped tubes and bottles;
5. Autoclaves or other appropriate means to decontaminate infectious materials;
6. Plastic disposable Pasteur pipettes, whenever available, to avoid glass.

Waste handling

Waste is anything that is to be discarded. In laboratories, decontamination of wastes and their ultimate disposal are closely interrelated. In terms of daily use, few if any contaminated materials will

require actual removal from the laboratory or destruction. Most glassware, instruments and laboratory clothing will be reused or recycled. The overriding principle is that all infectious materials should be decontaminated, autoclaved or incinerated within the laboratory. Steam autoclaving is the preferred method for all decontamination processes. Materials for decontamination and disposal should be placed in containers, e.g. autoclavable plastic bags that are color-coded according to whether the contents are to be autoclaved and/or incinerated.

Identification and separation system for infectious materials and their containers should be adopted. National and international regulations must be followed. Categories should include:

1. Non-contaminated (non-infectious) waste that can be reused or recycled or disposed of as general, „household“ waste;
2. Contaminated (infectious) „sharps“ – hypodermic needles, scalpels, knives and broken glass; these should always be collected in puncture-proof containers fitted with covers and treated as infectious;
3. Contaminated material for decontamination by autoclaving and thereafter washing and reuse or recycling;
4. Contaminated material for autoclaving and disposal;
5. Contaminated material for direct incineration.

After use, hypodermic needles should not be recapped, clipped or removed from disposable syringes. The complete assembly should be placed in a sharps disposal container. Disposable syringes, used alone or with needles, should be placed in sharps disposal containers and incinerated, with prior autoclaving if required. Sharps disposal containers must be puncture-proof/-resistant and must not be filled to capacity. When they are three-quarters full they should be placed in „infectiouswaste“ containers and incinerated, with prior autoclaving if laboratory practice requires it. Sharps disposal containers must not be discarded in landfills. No pre-cleaning should be attempted of any contaminated (potentially infectious) materials to be autoclaved and reused. Any necessary cleaning or repair must be done only after autoclaving or disinfection. Apart from sharps, which are dealt with above, all contaminated (potentially infectious) materials should be autoclaved in leak proof containers, e.g. autoclavable, color-coded plastic bags, before disposal. After autoclaving, the material may be placed in transfer containers for transport to the incinerator. If possible, materials deriving from healthcare activities should not be discarded in landfills even after decontamination. Reusable transfer containers should be leak proof and have tight-fitting covers. They should be disinfected and cleaned before they are returned to the laboratory for further use. Discard containers, pans or jars, preferably unbreakable (e.g. plastic), should be placed at every work station. Incineration of contaminated waste must meet with the approval of the public health and air pollution authorities, as well as that of the laboratory biosafety officer.

REQUIREMENTS, PROCEDURES AND MINIMAL TECHNOLOGICAL CONDITIONS FOR BIOSAFETY LEVEL 3 LABORATORIES

The containment laboratory – Biosafety Level 3 is designed and provided for work with risk group 3 microorganisms and with large volumes or high concentrations of risk group 2 microorganisms that pose an increased risk of aerosol spread, as well as work with microorganisms that is considered as exotic for region/country. Laboratories in this category should be registered or listed with the national or other appropriate health authorities.

Code of practice

The code of practice for basic laboratories – Biosafety Levels 1 and 2 applies except where modified as follows:

1. The laboratory supervisor must enforce the institutional policies that control access to the laboratory;
2. A laboratory-specific biosafety manual must be prepared and adopted as policy, and must be available and accessible;
3. All persons entering the laboratory must be advised of the potential hazards and meet specific entry/exit requirements;
4. The international biohazard warning symbol and sign displayed on laboratory access doors must identify the biosafety level and the name of the laboratory supervisor who controls access, and indicate any special conditions for entry into the area, e.g. immunization;
5. Laboratory protective clothing must be of the type with solid-front or wrap-around gowns, scrub suits, coveralls, head covering and, where appropriate, shoe covers or dedicated shoes. Front-buttoned standard laboratory coats are unsuitable, as are sleeves that do not fully cover the forearms. Laboratory protective clothing must not be worn outside the laboratory, and it must be decontaminated before it is laundered;
6. Open manipulations of all potentially infectious material must be conducted within a biological safety cabinet or other primary containment device;
7. Respiratory protective equipment may be necessary for some laboratory procedures or working with animals infected with certain pathogens;
8. Equipment must be decontaminated before repair, maintenance, or removal from the laboratory;
9. Laboratory personnel should receive appropriate training regarding their duties, the necessary precautions to prevent exposures, and exposure evaluation procedures. Personnel must

receive annual updates or additional training when procedural or policy changes occur;

10. Medical examination of all laboratory personnel who work in containment laboratories – Biosafety Level 3 is mandatory. This should include recording of a detailed medical history and an occupationally-targeted physical examination;
11. Incidents that may result in exposure to infectious materials must be immediately evaluated and treated according to procedures described in the laboratory biosafety manual. All such incidents must be reported to the laboratory supervisor.

Laboratory design

The laboratory design and facilities for basic laboratories – Biosafety Levels 1 and 2 apply, except where modified as follows:

1. The laboratory must be separated from the areas that are open to unrestricted traffic flow within the building. Additional separation may be achieved by placing the laboratory at the blind end of a corridor, or constructing a partition and door or access through an anteroom (e.g. a double-door entry or basic laboratory – Biosafety Level 2), describing a specific area designed to maintain the pressure differential between the laboratory and its adjacent space. The anteroom should have facilities for separating clean and dirty clothing and a shower may also be necessary;
2. Anteroom doors may be self-closing and interlocking so that only one door is open at a time;
3. Surfaces of walls, floors and ceilings should be water-resistant and easy to clean. Openings through these surfaces (e.g. for service pipes) should be sealed to facilitate decontamination of the rooms;
4. The laboratory room must be sealable for decontamination. Air-ducting systems must be constructed to permit gaseous decontamination;
5. Windows must be closed, sealed and break-resistant;
6. A hand-washing station with hands-free controls should be provided near each exit door;
7. There must be a controlled ventilation system that maintains a directional airflow into the laboratory room. A visual monitoring device with or without alarm should be installed;
8. The building ventilation system must be so constructed that air from the containment laboratory – Biosafety Level 3 is not recirculated to other areas within the building. When exhaust air from the laboratory (other than from biological safety cabinets) is discharged to the outside of the building, it must be dispersed away from occupied buildings and air intakes. Depending on the

agents in use, this air may be discharged through HEPA filters. A heating, ventilation and air-conditioning (HVAC) control system may be installed to prevent sustained positive pressurization of the laboratory. Consideration should be given to the installation of audible or clearly visible alarms to notify personnel of HVAC system failure;

9. All HEPA filters must be installed in a manner that permits gaseous decontamination and testing;
10. Biological safety cabinets should be sited away from walking areas and out of crosscurrents from doors and ventilation systems;
11. The exhaust air from Class I or Class II biological safety cabinets, which will have been passed through HEPA filters, must be discharged in such a way as to avoid interference with the air balance of the cabinet or the building exhaust system;
12. An autoclave for the decontamination of contaminated waste material should be available in the containment laboratory. If infectious waste has to be removed from the containment laboratory for decontamination and disposal, it must be transported in sealed, unbreakable and leak proof containers according to national or international regulations, as appropriate;
13. Backflow-precaution devices must be fitted to the water supply. Vacuum lines should be protected with liquid disinfectant traps and HEPA filters, or their equivalent. Alternative vacuum pumps should also be properly protected with traps and filters.

Laboratory equipment

The principles for the selection of laboratory equipment, including biological safety cabinets are the same as for the basic laboratory – Biosafety Level 2. However, at Biosafety Level 3, manipulation of all potentially infectious material must be conducted within a biological safety cabinet or other primary containment device. Consideration should be given to equipment such as centrifuges, which will need additional containment accessories, for example, safety buckets or containment rotors. Some centrifuges and other equipment, such as cell-sorting instruments for use with infected cells, may need additional local exhaust ventilation with HEPA filtration for efficient containment.

REQUIREMENTS, PROCEDURES AND MINIMAL TECHNOLOGICAL CONDITIONS FOR BIOSAFETY LEVEL 4 LABORATORIES

The maximum containment laboratory – Biosafety Level 4 is designed for work with risk group 4 and exotic microorganisms. Operational “maximum containment laboratories” – Biosafety Level 4

should be under the control of national or international appropriate health authorities.

Code of practice

The code of practice for Biosafety Level 3 applies except where modified as follows:

1. The two-person rule should apply, whereby no individual ever works alone. This is particularly important if working in a Biosafety Level 4 suit facility;
2. A complete change of clothing and shoes is required prior to entering and upon exiting the laboratory;
3. All manipulations of infectious agents must be performed within a BSC or other primary barrier system;
4. Appropriate communication systems must be provided between the laboratory and the outside (e.g., voice, fax, and computer);
5. A visual and camera monitoring system of the work done in the laboratory must be installed;
6. Practical and effective protocols for emergency situations must be established. These protocols must include plans for medical emergencies, facility malfunctions, fires, escape of animals within the laboratory, and other potential emergencies;
7. Personnel must be trained in emergency extraction procedures in the event of personnel injury or illness;
8. A method of communication for routine and emergency contacts must be established between personnel working within the Biosafety Level 4 and support personnel outside the laboratory.

Laboratory design

The features of a containment laboratory – Biosafety Level 3 also apply to a maximum containment laboratory – Biosafety Level 4 with the addition of the following:

Primary containment. An efficient primary containment system must be in place, consisting of one or a combination of the following:

— Class III cabinet laboratory

Passage through a minimum of two doors prior to entering the rooms containing the Class III biological safety cabinets (cabinet room) is required. In this laboratory configuration the Class III biological safety cabinet provides the primary containment. A personnel shower with inner and outer changing rooms is necessary. Supplies and materials that are not brought into the cabinet room through the changing area are introduced through a double-door autoclave or fumigation chamber. Once the outer door is securely closed, staff inside the laboratory can open the inner door to retrieve the materials. The doors of the autoclave or fumigation chamber

are interlocked in such a way that the outer door cannot open unless the autoclave has been operated through a sterilization cycle or the fumigation chamber has been decontaminated.

— *Suit laboratory*

A protective suit laboratory with self-contained breathing apparatus differs significantly in design and facility requirements from a Biosafety Level 4 laboratory with Class III biological safety cabinets. The rooms in the protective suit laboratory are arranged so as to direct personnel through the changing and decontamination areas prior to entering areas where infectious materials are manipulated. A suit decontamination shower must be provided and used by personnel leaving the containment laboratory area. A separate personnel shower with inner and outer changing rooms is also provided. Personnel who enter the suit area are required to don a one-piece, positively pressurized, HEPA-filtered, supplied-air suit. Air to the suit must be provided by a system that has a 100% redundant capability with an independent source of air, for use in the event of an emergency. Entry into the suit laboratory is through an airlock fitted with airtight doors. An appropriate warning system for personnel working in the suit laboratory must be provided for use in the event of mechanical system or air failure.

The BSL-4 facility design parameters and operational procedures must be documented. The facility must be tested to verify that the design and operational parameters have been met prior to operation. Facilities must also be re-verified annually.

Controlled access

The maximum containment laboratory – Biosafety Level 4 must be located in a separate building or in a clearly delineated zone within a secure building. Entry and exit of personnel and supplies must be through an airlock or pass-through system. On entering, personnel must put on a complete change of clothing; before leaving, they should shower before putting on their street clothing.

Controlled air system

Negative pressure must be maintained in the facility. Both supply and exhaust air must be HEPA-filtered. The ventilation system must be monitored and alarmed to indicate malfunction or deviation from design parameters. There are significant differences in the ventilating systems of the Class III cabinet laboratory and suit laboratory:

— *Class III cabinet laboratory*

The supply air to the Class III biological safety cabinets may be drawn from within the room through a HEPA filter mounted on the cabinet or supplied directly through the supply air system. Exhaust air from the Class III biological safety cabinet must pass through two HEPA filters prior

to release outdoors. The cabinet must be operated at negative pressure to the surrounding laboratory at all times. A dedicated non-recirculating ventilating system for the cabinet laboratory is required.

— *Suit laboratory*

Dedicated room air supply and exhaust systems are required. The supply and exhaust components of the ventilating system are balanced to provide directional airflow within the suit area from the area of least hazard to the areas of greatest potential hazard. Redundant exhaust fans are required to ensure that the facility remains under negative pressure at all times. HEPA-filtered supply air must be provided to the suit area, decontamination shower and decontamination airlocks or chambers. Exhaust air from the suit laboratory must be passed through a series of two HEPA filters prior to release outdoors. Alternatively, after double HEPA filtration, exhaust air may be recirculated, but only within the suit laboratory and under no circumstances shall the exhaust air from the Biosafety Level 4 suit laboratory be recirculated to other areas. All HEPA filters need to be tested and certified annually. The HEPA filter housings are designed to allow for *in situ* decontamination of the filter prior to removal. Alternatively, the filter can be removed in a sealed, gas-tight primary container for subsequent decontamination and/or destruction by incineration.

Decontamination of effluents

All effluents from the suit area, decontamination chamber, decontamination shower, or Class III biological safety cabinet must be decontaminated before final discharge. Heat treatment is the preferred method. There is a possibilities for chemical decontamination by acids and alkaline. Decontamination of all liquid wastes must be documented. The decontamination process for liquid wastes must be validated physically and biologically. Biological validation must be performed annually or more often if required by institutional policy.Water from the personnel shower and toilet may be discharged directly to the sanitary sewer without treatment.

Sterilization of waste and materials

A double-door, pass-through autoclave must be available in the laboratory area. Other methods of decontamination must be available for equipment and items that cannot withstand steam sterilization (like with formaldehyde).

Airlock entry ports for specimens, materials and animals must be provided, and ***Emergency power*** and dedicated power supply lines must be provided. In table 1 is the summary of the requirements for different biosafety levels.

Table 1: Summary of biosafety level requirements

BIOSAFETY LEVEL REQUIREMENTS	BIOSAFETY LEVEL			
	1	2	3	4
Isolation[a] of laboratory	No	No	Yes	Yes
Room sealable for decontamination	No	No	Yes	Yes
Ventilation:				
— inward airflow	No	Desirable	Yes	Yes
— controlled ventilating system	No	Desirable	Yes	Yes
— HEPA-filtered exhaust air	No	No	Yes/No[b]	Yes
Double-door entry	No	No	Da	Yes
Airlock	No	No	No	Yes
Airlock with shower	No	No	No	Yes
Anteroom	No	No	Da	—
Anteroom with shower	No	No	Yes/No[c]	No
Effluent treatment	No	No	Yes/No[c]	Yes
Autoclave:				
— on site	No	Desirable	Yes	Yes
— in laboratory room	No	No	Desirable	Yes
— double-ended	No	No	Desirable	Yes
Biological safety cabinets	No	Desirable	Yes	Yes
Personnel safety monitoring capability[d]	No	No	Desirable	Yes
[a] Environmental and functional isolation from general traffic.				
[b] Dependent on location of exhaust.				
[c] Dependent on agents used in the laboratory.				
[d] For example, window, closed-circuit television, two-way communication.				

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EQUINE VIRAL ARTERITIS: OUR EXPERIENCE AND REQUIRMENTS FOR DISEASE CONTROL IN THE REPUBLIC OF SERBIA

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Equine Viral Arteritis (EVA) is contagious disease of viral etiology that can infect all breeds and categories of equides, especially horses. The disease causes various health disorders, and consequently, large economic losses. Equine arteritis virus (EAV) was detected as a causative agent of infection and isolated for the first time in 1953. Since then until today, great efforts were made in order to find adequate measures for the timely diagnosis and to prevent the spread and outbreak of new cases. Many countries worldwide, especially those with developed horse breeding, adopted various laws and by-laws to prevent the occurrence of infection and created programs for control, prevention and eradication of EAV.

The disease is usually spread via venereal route, by mating or artificial insemination with the semen of persistently infected stallions. According to literature data, infected stallions can become long-term carriers that shed virus with the each ejaculate during the whole life. The majority of infections is subclinical but sometimes can occure clinical signs of the disease with fever, conjunctivitis, oedema of prepuce and scrotum in stallions and abortions of mares.

First examinations on the presence of EVA in the Republic of Serbia started relatively late, in 2002. These testings encompass only imported horses in quarantine. Additionally, the International Agricultural Fair, that is held each year in Novi Sad, introduced mandatory laboratory testing of EVA, in parallel with other diseases that are regularly carried on in equines. Many sport horse keepers are acquainted with the significance of this disease, and started individually to analyze their animals. According to results from a ten-year period of investigation of EVA infection at the Virology Department in the Scientific Veterinary Institute „Novi Sad“, the infection exists in our country and is also detected in persistently infected stallions. So far, in Serbia haven’t been created any regulations and legislations for the control of the disease, while in other nearby countries were already completed, and it should be the intention in the forthcoming period.

The aim of this article is to give the overview of our experience with Equine Viral Arteritis and recommendation for prevention of further spreading of the disease among Serbian horse population.

Key words: *equine viral arteritis, epizootilogy, diagnosis, control, elimination*

INTRODUCTION

Equine viral arteritis (EVA) is contagious disease that can infect all equides (donkeys, mules, zebras), especially horses of different breeds and categories, such are: foals, colts, mares, stallions and geldings. The causative agent of the disease is virus (*Equine arteritis virus* – EAV) that belongs to the family *Arteriviridae*. Vira particle is enveloped and spherical with the diameter between 50 and 70 nm. Viral genome represents single-stranded RNA of 12.7 kb size (Balasuriya, U.B. et al. 1999). Virus was isolated in 1953 in the USA (Doll, E.R. et al. 1957) and since then the disease is registered in many countries and almost on all continents. It is considered that only Japan, Island and New Zealand are free of EVA (McFadden, A.M. et al. 2013). Spreading of the disease between horses is usually via respiratory and venereal route during the acute phase of infection which lasts 7–14 days. Equine arteritis virus is excreted from the respiratory system and is transferred to other horses by aerosol, but for the occurrence of infection is also necessary close contact between infected and susceptible animals (McCollum, W.H et al. 1971, Collins, J.K. et al. 1987). The excretion of EAV is also possible through the urine, aborted foetuses and other secretions of acutely infected horses. Especially important route of viral transmission is via the genital tract of persistently infected stallion, since EAV is often detected in semen. The typical clinical signs of the disease are bronchopneumonia and enteritis in young animals, and limb oedema and oedema of the scrotum and prepuce in stallins. Abortions caused by EAV are also very frequently detected in pregnant mares. Final diagnosis encompasses detection of specific antibodies against EAV in blood sera or virus isolation from the infected specimens. The prevention of infection is based on vaccination, implementation of biosecurity measures and legislations which determ general strategy for control of the EVA and its spreading.

ETIOLOGY OF EVA

Equine arteritis virus was isolated in 1953 from the lung of an aborted foetus from a stud farm of „Standarbred" near the town Bucyrus in Ohio State (USA). On that studfarm, before the virus isolation, were described a large number of abortions and respiratory disorders.

Newly detected virus was classified into the family *Arteriviridae* and genus *Arterivirus*, together with the virus that causes respiratory-reproductive syndrome of swine (PRRSV), Simian hemorrhagic fever virus of monkeys (SHFV) and lactate dehydrogenise elevating virus in mice (LDV) (Balasuriya, U.B. et al. 2013). The name of the family and genus is connected to the pathogenicity of the virus. The typical representative of the family *Arteriviridae* and *Arterivirus* is EAV. The virus replicates in macrophages, endothelial cells and muscle layers of small arteries with the development of degenerative process and inflammation. Additionally, one of the main characteristics of this family of viruses is to form asymptomatic (persistent) infections. Under the certain circumstances these viruses can influence the development of severe symptoms of the disease, such are heavy bleeding and exudations, caused by bacterial superinfection and reactivation of latent infection (for exp. EHV) with consequently fatal outcome.

Equine arteritis virus has a positive-stranded RNA with a genome of about 12.7 kb, but in some strains the size of RNA is between 12.704 and 12.731 bp, which includes 50 sequences of 224 nucleotides and 10 open reading frames (ORFs) for reading of information. According to its dimensions, EAV belongs to the smaller viruses with the average diameter of 50–70 nm, and nucleocapsid of about 35 nm. Nucleocapsid has spherical shape and is surrounded with a lipid envelope with the integrated surface antigens. These antigens are responsible for the entrance of virus into host cells with the process of endocytosis, through cell receptors. In horses, the main role in virus replication has the macrophages. In the cytoplasma of macrophages occurs intensive replication and formation of the large number of genomes, which will be incorporated into the nuclecapsid of the future virion. Virions obtain nucleocapsid by budding through the endoplasmatic reticulum and Golgi complex, and further move to the cell surface and are released by exocytosis.

EPIZOOTIOLOGICAL CHARACTERISTICS OF EVA

According to literature data (Holyoak, R.G. et al. 2008), EVA is registered in the horse population on all continents: North and South America, Europe, Asia, Australia and Africa. Only Island, Japan and New Zealand declared evidence for absence of equine viral arteritis. Many examinations conducted during the 60' and 70' in the last century revealed the following seroprevalence: Swiss 11,3%, England 2,3%, the Netherlands 14% and Germany 1,8%. However, the analyses performed in 1998 in Germany showed that the established seroprevalence was approximately 20%, while in the USA was about 2% among non-vaccinated horses (Holyoak, R.G. et al. 2008). Oppositely, Timony and McCollum (1993) reported the seroprevalence of 70–90% in trotter horses in the USA, while in English fullblood horses the seroprevalence was only 2–3%. Equine

arteritis virus is also widespread in Sweden, where were detected 33% and 15% seropositive animals among trotter horses and half-blood horses, respectively (Albihn, A., Klingeborn, B. 1996). In Austria EVA seroprevalence quite vary, from 10–12% to near 100% in particular herds. In central region of Turkey, in Anatolia, 23,4% horses have specific antibodies against EAV (Bulut, O. et al. 2012).

Examinations regarding EVA seroprevalence in Serbia are still mostly limited to one epizootiological region, smaller number of stud farms or individual animals. Officially, the testing started in 2002 and encompassed only imported horses in quarantine (Lazić, S. et al. 2013). The first evidence on the presence of EVA in SR Yugoslavia, based on serological investigation in 2001, was described by Trailović et al. (2001). Furthermore, Petrović et al. (2002) conducted serological examinations in South Bačka and Srem regions and concluded that EVA is present in the horse population in these epizootiological districts. Also, 3 out of 13 tested stallions of different breeds in 2003 were seropositive with detected high titar of specific antibodies against EAV (Urošević, et al. 2003). In the following years, probably because of the growing interest and importance of the disease, many private owners demanded serological examinations, primarily for breeding animals.

In that way, the owners by themselves controlled the infection among their horses with the aim to prevent further spreading of EVA.

Many investigations of the prevalence of equine viral arteritis were focused on the way of viral excretion and transfer of the disease from the infected to naive animals. It is known that infected horses excrete the virus through the nasal and ocular discharge as well as by urine and aborted foetuses, while stallions excrete EAV through the semen. Infected stallions serve as natural reservoir of the virus. It is assumed that if the virus attacks the accessory glands, stallions remain infected for the whole life and often excrete virus through the semen (Del Piero, F. 2000, Nowotny, N. 2001, Holyoak, R.G. et al. 2008). The carrier status of stallions and possibility of virus excretion through the sperm has been the subject of investigation of many researchers. In parallel, with the development of up-to-date laboratory diagnostic tools such are molecular techniques, was established the increased number of seropositive stallions that shed the virus through the sperm (Ramina, A. et al. 1999, Balasuriya, U.B. et al. 2002, S. et al. 2002, Guthrie, A.J. 2003, Chenchev, I. et al. 2010, Aymeric, H. et al. 2015).

Since 2012, at the Scientific Veterinary Institute „Novi Sad" has been conducted more extensive research on the prevalence and transmission of EVA in stud farms. There were carried out serological survey on the larger number of sera samples by virus-neutralization test and detection of virus in semen samples by molecular method RT-PCR. During 2012 and 2013 were tested 6 stables on the territory of Vojvodina Province. In total, 150 horses out of 375 animals situated on these horse farms were examined. Eqine arteritis virus was detected only in one stable with the seroprevalence of

33% (51/62). It was tested: 9 stallions, 20 mares, 4 geldings, 8 colts and 10 foals and seropositive result was established in: 4 stallions, 12 mares and 1 foal (Lazić, S. et al. 2013a, Lazić, S. et al. 2013b). During the breeding season 2013–2014 were analyzed by RT-PCR 14 semen samples from 2 stallions which sperm were used for the artificial insemination. From the first stallion were tested 11 semen samples and from the second one 3. Equine arteritis virus was detected in all samples from the first stallion, while in the semen samples from the second stallion EAV was not detected. However, mares that were inseminated with the sperm of the second stallion also seroconverted, which is the evidence that this stallion also excreted periodically virus in the semen (Lazić, S. et al. 2015). Despite these resuts, clinical signs of EVA never have been registered on this horse farm. There also were no evidence of increased number of abortions, oedema in horses or pneumonia and enteritis in foals. The conclusion is that the disease is present in subclinical and chronic form, which mostly depends on immunological status of the horses (Balasuriya, U.B. et al. 2013, Szeredi, L. et al. 2005, Del Piero, F. 2000).

Further, the total number of 156 horses coming from 10 different stud-farms in the Vojvodina region has been tested. The tested population consists of 86 stallions and 70 mares. The age of horses vary from 1 to 26 years (the mean age was 9,9 years). Our preliminary results showed that121 sera were negative (77,60%), thirty three were positive (21,15%) and two sera were cytotoxic (1,25%). Among the positive sera fifteen (45,45%) exhibit an antibody titer range from 4 to 16, ten (30,30%) sera exhibited a titer ranged from 24 to 96 and eight (24,24%) sera had a titer above 128 (*Lupulović, et al. 2015*).

CLINICAL FEATURES OF THE DISEASE

Typical clinical sign of the disease is fever with the temperate up to 41,8° C, which can last 2–9 days (Balasuriya, U.B. et al. 2013). In young animals (colts and older foals) usually occur broncho-pneumonia and different types of enteritis, while in very young foals, from 1 to 3 months, can develop symptoms of broncho-pneumonia and enteritis simultaneously, which further progress into „pneumo-eneteritic“ syndrome (Vaala, W.E. et al. 1992). On the other side, in older horses were described, as a consequence of panvasculatis: periorbital oedema, oedema of the limbs and abdomen, oedema of scrotum and prepucium in stallions and various hemorhagges. Typical signs of the disease are abortions in pregnant mares, which can be individual or widespread, when abort almost 70% of animals on the horse farm (Balasuriya, U.B. et al. 2013, Szeredi, L. et al. 2005). Abortions mostly occur during the 3- to 10-month stage of gestation as a result of viral infection of foetus and placenta. However, the presence of antibodies against EAV in newborn foals, before consumption of colostrum,

shows that infection of foetuses not always have fatal outcome with abortions and deaths (Szeredi, L. et al. 2005). In stallions is usually present chronic form of the disease without the development of clinical symptoms. Equine arteritis virus infects the cells of ductus deferens and accessory sex glands and thus infected stallions excrete and spread the virus through the ejaculate (Balasuriya, U.B. et al. 2013). Almost 70% of stallions remain persistently infected after the first contact with the infection and became long-term carriers (Aymeric, H. et al. 2015).

DIAGNOSIS

Diagnosis of EVA is based on clinical features, and above all, on the results of laboratory testing. As previously mentioned, the disease may exist without the development of clinical symptoms, and in that case, the finding of specific antibodies against EAV is the first evidence of infection. For the detection of anti-EAV antibodies is used virus-neutralisation test (VNT) or enzyme linked immunosorbent assay (ELISA). First immunological response and specific antibodies are possible to detect 7–14 days after the infection. The highest level antibodies reach in the period from 2 to 4 months after the infection and may persist 3 and more years (Balasuriya, U.B. et al. 2013, Balasuriya, U.B. et al. 1999). Each value of antibody titar of 2 $\log_2$ and higher established by VNT or positive finding detected by ELISA is consider positive result (OIE Terrestrial Manual, Equine Viral Arteritis, Chapter 2.5.10, 2008). In various tissue samples EAV can be detected by: isolation on RK-13 cell lines, molecular technique (RT-PCR) or immunocytochemical method (OIE Terrestrial Manual, Equine Viral Arteritis, Chapter 2.5.10, 2013). Special interest refers to the examination of stallion's semen. In the last twenty years have been developed several molecular techniques based on using the reverse transcriptase-polymerase chain reaction (RT-PCR) for the fast identification of EAV in sperm samples *(*Balasuriya, U.B. et al. 2013, Szeredi, L. et al. 2005, Aymeric, H. 2015, Echeverria, M. G. 2003). Virus isolation on cell culture from the semen of carrier stallions is often unsuccessful, which was described by several authors (Szeredi, L. et al. 2005, Lazić, et al. 2015). Seroconversion of seronegative mares 4 weeks after mating is also reliable sign of stallion´s infection and can be used in laboratory diagnosis of the semen (OIE Terrestrial Manual − Chapter 2.5.10, 2013; Belasurya, et al. 2013).

CONTROL AND PREVENTION

In order to prevent EVA spreading is important to control stallions and pregnant mares. Animal mating should be strictly controlled, as the venereal route of EAV transmission is crucial for the spreading of infection. Control of mating is based on the analysis of the presence of specific antibodies against EVA, both in stallions and mares, and the detection of

seroconversion. A major risk in the dissemination of EAV represents mating of seropositive and/or seropositive mares.

It is very important to prevent direct contact between horses of unknown health status. Introducing of seropositive animals into the stable with naive horse population may cause outbreak of EVA with the possible health and economic losses.

Sport horses are often in close contact with each other during the transport and races, so special attention should be given to this problem. Private owners and organizers of equestrian events should be aware of the risk of EAV spreading.

Vaccination is a good way to prevent outbreak of EVA and clinical signs of the disease, but it cannot stop viral excretion when the animal is already infected. Better results could be achieved by regular vaccination and re-vaccination from the first months through the whole life of the horse. Very satisfactory results were obtained with the *„Arivac"* vaccine (*Fort Dodge Animal Health*) by the permanent vaccination of foals, stallions, mares before mating and other horse categories (Holyoak, G.R., Balasuriya, U.B., Broaddus, C.C, Timoney, P.J. 2008). However, so far have been detected many different EAV genotypes which open the question of vaccination efficiency. Existing vaccines are not able to protect horses form all kind of EAV genotypes (Balasuriya, U.B. et al. 2013).

Equine viral arteritis is not very often detected on the stud farms, but EAV can be a significant cause of stagnation in horse breeding in some stables. The consequences of the disease vary in their severity, from the reduced ability for working to death. Laboratory testing by serological and virology methods are the most important for the timely detection of the disease. Molecular techniques also should be implemented into routine diagnostic protocols. A major risk for the emergence and spread of the disease represents a direct contact, and especially mating of horses of the unknown health status.

In the Republic of Serbia is necessary to determine the seroprevalence on the whole territory of the country and to continue with EAV identification and molecular genotypisation. Experts and professional authorities should initiate the creation of laws and bylaws according to data and experience in the neighbouring countries. The implementation of legislation will help to decrease the losses and to eradicate the disease.

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SURVEILLANCE AND CONTROL OF VIRAL DISEASES IN FRESHWATER FISH AND THE EUROPEAN NETWORK OF FISH DISEASES LABORATORIES

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Abstract: The most important viral diseases in European fresh water fish are viral haemorrhagic septicaemia (VHS), infectious hematopoietic necrosis (IHN), infectious pancreatic necrosis, sleeping disease in salmonids and koi herpes virus infection (KHV), carp edema virus infection (CEV), and spring viremia of carp (SVC) in cyprinids. VHS, IHN and KHV are notifiable diseases and are contained in many European countries.

The diseases are still controlled by avoidance through surveillance and biosecurity measures and by traditionally eradication programs in case of outbreaks, but vaccination and breeding programs for resistance are in progress as supplementary tools to contain them. In addition molecular tracing of disease outbreaks have improved our understanding of the diseases and how they are spreading.

Through the EU Reference Laboratory a strong network of National Reference Laboratories for Fish Diseases has been established. The primary goal is to harmonize detection and surveillance procedures for the listed fish diseases but also to keep vigilance on new emerging diseases and to enhance the capacities on fish health management. This is done by organization of inter-laboratory proficiency tests, workshops, training courses, implementation of quality control, diseases surveys, and pathogen characterization by in-vivo and in-vitro studies.

AQUACULTURE AND FISH HEALTH IN SERBIA

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Abstract*:* Aquaculture in Serbia is mainly based on common carp (*Cyprinus carpio*) and rainbow trout *(Oncorhynchus mykiss).* Total fish production in Serbia varied from 12 to 14 thousand tonnes per year. Due to the increasement of disease problems in the fish farming industry and the impact disease may have on both feral and farmed fish, monitoring and surveillance on diseases have for many years been considered to be of great importance. Serbia has a national legislation as basis for their surveillance and disease control in aquatic animals, as well as regulations listing notifiable diseases of concern. In addition to national legislation, the principles laid down in the Council Directive 2006/88/EC as regards animal health requirements for aquaculture animals and products thereof are accepted. Serbia runs a surveillance programme for viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) based on EU regulations and a monitoring programme for infectious pancreatic necrosis (IPN), spring viraemia of carp (SVC) and bacterial kidney disease (BKD) takes place in Serbia on a national level. Serbia have established appropriate surveillance and monitoring for fish diseases of concern to the fish farming industry.

Key words: *aquaculture, fish health, surveillance*

INTRODUCTION

On a global scale, fish and fishery products are the main food supply for human beings. It is widely known that the supplies of fish from traditional fisheries are more or less constant and that the shortage in fish and fish products has to be met by aquaculture. The intensive aquaculture is often characterized by high density of fish, poor water quality, accumulation of pathogens in the production systems and in the environment. As a result, most populations of fish from intensive rearing systems are characterized by chronic stress. Stress leads to increased susceptibility to disease, and prevalence of disease depends on the

interaction between fish pathogens and the environment (Jeremic 2003). The appearance and development of fish diseases is a consequence of the interaction of pathogen, host and environment. Also, international trade of live fish and their products is a major hidden cause of many outbreaks. Damages caused by the disease significantly delay the development of fisheries and prevent its transition to modern forms of intensive aquaculture. Many diseases affecting farmed fish also represent a threat to natural fish populations (Thoesen 1994). The relatively small number of pathogenic bacteria is responsible for major economic losses in cultured fish (Toranzo et al. 2005). In addition to the release of active substances in aquatic ecosystems, the usual therapeutic interventions in aquaculture can lead to antibiotic resistance in bacterial pathogens of fish, but also in other bacteria present in the environment (Alderman and Hastings 1998).

Aquaculture in the Serbia

Aquaculture is based on cold water species, rainbow trout (*Onchorhynchus mykiss*), brown trout (*Salmo trutta* m. *fario*) and warm water species, common carp (*Cyprinus carpio*), grass carp (*Ctenopharingodon idella*), silver carp (*Aristichthys nobilis*), bighead carp (*Hypophthalmichtys molitrix*), catfish (*Silurus glanis*), pike (*Esox lucius*), pike perch (*Stizostedion lucioperca*). Fish are produced in carp and trout fish farms (over 95%), to a smaller extent in cages, enclosed or partitioned natural or man made aquatic ecosystems. There is 13.500–14.000 ha of fish farms in Serbia, with 99,9% of carp farms and 0,1% of trout farms. The total fish production in recent years is between 10.000 and 15.000 tons with 70 to 75% of consumable fish.

Three types of production systems are present: extensive, semi-intensive and intensive. Extensive production is sporadic and is present only at a few carp production units, mostly not economic for semi-intensive production due to remoteness from other production units or neglect. The principal type of production (75–80%) is semi-intensive production of cyprinids, with common carp as the main species. Common carp is present with more than 80% of the total production in warmwater fish farms. The traditional type of feeding is slowly changing. Cereals are more often, at over 50% of production surfaces, totally of partially replaced by complete, peletted and even more extruded feed. This has resulted in an increase of production per surface unit in recent years. Intensive production systems in carp culture are less present, at a small number of earthen ponds with aeration systems, mainly for fish fry production, and in cages.

However, rainbow trout, the only salmonid species cultured for consummation, is exclusively produced in intensive systems at trout farms in Serbia.

Over the last decade, several emerging or serious diseases in fish have been diagnosed in farmed and feral populations, creating large problems in the fish farming industry and thus being the subject of

surveillance and monitoring programmes. The differences between infectious diseases in fish and those of terrestrial animals mean that the approach to the problems and the eradication efforts differ as the diseases may spread effectively through flowing water (Håstein et al. 1999).

Fish health control in the Serbia

Serbia has a national legislation as basis for their surveillance and disease control in aquatic animals, as well as regulations listing notifiable diseases of concern. In addition to national legislation, the principles laid down in the Council Directive 2006/88/EC as regards animal health requirements for aquaculture animals and products thereof are accepted. The monitoring and surveillance for fish viral diseases has mainly been based on the testing procedures given in the Commission Decision 2001/183/EC while for the bacterial diseases, standard diagnostic procedures has been used for screening purposes.

Serbia runs a surveillance programme for viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) based on EU regulations and a monitoring programme for infectious pancreatic necrosis (IPN), spring viraemia of carp (SVC) and bacterial kidney disease (BKD) takes place in Serbia on a national level. The aim of the monitoring and surveillance programmes for fish diseases in question is either to document and maintain freedom of disease, to eradicate a disease or to keep a disease under control within certain bonds. The main target population for the monitoring and surveillance programmes is rainbow trout and common carp.

The basis for the surveillance and monitoring programmes is partly based on EU regulations, OIE criteria or criteria derived from national legislation. The participation is compulsory as regards approval and maintenance of disease free status for the diseases. For the sampling of fish for surveillance the responsible authorithy is the district veterinary inspector in coordination with local veterinary institute and national reference laboratory.

Organisation of the surveillance in the Serbia

Fish farms are inspected clinically biannually and samples for virological examinations are collected from the fish farms each year to document freedom for KHV, BKD, SVC in cyprinids, and IHN, IPN VHS in salmonid fish. The examination procedures given in the OIE Diagnostic Manual for Aquatic Animal Diseases are the basis for examinations as regards KHV, BKD and SVC.

The Veterinary Authorities is responsible for the implementation of measures that will be used in order to control a given notifiable disease. The implementation involves both central and regional veterinary officers. If disease is diagnosed, stamping out procedures followed by cleaning, disinfection and fallowing will be carried out. Prevention may be achieved

by avoiding introduction of disease free eggs and/or fishinto disease free farms as well as using protected water supply (e.g. spring-, borehole water). An infected farm may restock after fallowing if no signs of infection appear after a sanitation programme has been carried out. In Serbia affected farm(s) will have to pay themselves for any measures imposed by the authorities for the time being, because no compensation is granted. Health certificates and/or transportation documents is needed in connection with deliveriesof live fish for stocking into grow out farms and restocking into rivers. The record of findings is kept by the responsible authorities, both regionally and centrally. The diagnostic laboratories also keep the necessary documentation on the examinations carried out. Furthermore all farms have to keep records on events in the farms that can be requested by Competent Authority.

CONCLUSIONS

Serbia have established appropriate surveillance and monitoring for fish diseases of concern to the fish farming industry. Due to these systems and good management practises, the fish disease situation is generally good compared to other countries.

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OXIDATIVE STATUS IN DAIRY COWS AND EWES DURING LACTATION PERIOD

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Abstract: Oxidative stress, defined as the imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is known to be involved in a number of processes and disease states in domestic animals. To investigate the effects of the antioxidant mechanisms during different phases of milking and during dry period 5 Frisona cows and 5 Comisana ewes were enrolled in this study. Blood samples were taken on days 4 and 200 of milking and during dry period. Serum samples were assessed for reactive oxygen species (dROMs), antioxidant barrier (Oxy-adsorbent), and thiol antioxidant barrier (SHp). A statistically significant effect of lactation on all studied parameters was found both in cows and ewes ($P<0.05$). Oxidative processes increased at the end of milking period, together with a compensative response of the organism to this stress and suggest the important role of oxidative status in lactating cows and ewes.

Keywords: *oxidants, antioxidants, cow, lactation, ewe*

INTRODUCTION

Oxidative stress occurs when oxidative substances and, particularly, reactive oxygen species overwhelm antioxidant defenses (Pourova et al. 2010).

The balance between antioxidants and prooxidants compound at cellular level represents an important determinant of various physiological processes and its maintenance is the main aim of so called an integrated antioxidant system built in the animal body.

In farm animals, oxidative stress is involved in a number of pathological conditions, including those associated with animal production, reproduction and welfare (Lykkesfeldt and Svendsen 2007). The alteration of oxidative balance, if not adequately restored by the antioxidant barrier, induces an oxidative stress with degenerative damage of the cellular structures (Trevisan et al. 2001, Piccione et al., 2010), that makes the

organism sensitive to degenerative diseases (McCord 2000). In the last years, the evaluation of oxidative stress has become a field of research in veterinary medicine and particularly in ruminant health (Celi 2010, 2011).

Given the remarkable zooeconomic impact of metabolic stress, it is interesting to establish the effects of the different lactating phases on the efficiency of the antioxidant mechanisms. It has been demonstrated that increased energetic requirements during milking period stimulate oxidative metabolism, which increases the electron flow into the mitochondria1 electron transport chain and induces the subsequent production of free radicals which have a great impact on the normal function of molecules like nucleic acid, proteins and cell membrane phospholipids (Gabai et al. 2004). The combined assessment of free radicals' concentration and antioxidant power allows knowing the real status of physiological defenses. It is thus important to consider oxidative stress as a potential index of animal welfare to be able to detect, preventively, excessive animal exploitation, thus protecting health status. In view of such considerations, the aim of the present study was to evaluate the oxidative status of cows and ewes during milking period.

MATERIAL AND METHODS

Five Frisona cows, (5 years old, mean body weight of 640±30 kg) and five Comisana ewes (4 years old, mean body weight of 46±2 kg), clinically healthy, with the same level of production and during the same milking period were enrolled in the study. They were taken to graze at 09:30 in the morning and to shelter at 16:30. All animals were milked in the morning from 07:00 to 09:00 and in the afternoon from 16:00 to 17:30 and were fed once a day a supplementary diet according to their physiological phases and water was available *ad libitum*. From each animal blood samples were taken by jugular venipuncture during early lactation (day 4), end lactation (day 240) and during dry period, using vacutainer tubes (Terumo Corporation, Japan) without anticoagulant agent at the same time in the morning (09:00). Blood samples were centrifuged at 2325 x *g* for 20 min and the obtained sera were immediately analyzed by means of a UV spectrophotometer (model Slim, Seac Florence, Italy), for the assessment of the following parameters: d-ROMs, Oxy-Adsorbent (Oxy-Ads) and SHp. The assessment of the free radicals (d-ROMs) and the anti-oxidant power (Oxy-Ads and SHp) was made with the so-called „spin traps" system that is molecules reacting with free radicals from complexes visible with a spectrophotometer. The d-ROMs test assesses the concentration of hydroperoxides (R-OOH), a class of reactive metabolites of the oxygen, in a biological sample (serum, plasma, tissues and cells). The Oxy-Ads assess the anti-oxidant power of the plasmatic barrier by measuring its ability to contrast the oxidative action of hypochlorous acid. The SHp test assesses the thiol anti-oxidant plasma barrier, which contrasts the propagation of the

perioxydative processes by inactivating both the alkoxyl and the hydroxide radicals.

All the results were expressed as mean ± standard error of the mean (SEM). Data were normally distributed ($P>0.05$, Kolmogorov-Smirnov test) and one-way repeated measures analysis of variance (ANOVA) was used to determine the significant effect of lactation on d-ROMs, Oxy-Ads and SHp levels. Bonferroni's multiple comparison test was applied. P values<0.05 were considered statistically significant. Data were analyzed using statistical software program Prism v. 7.00 (Graphpad Software Ldt., CA, USA).

RESULTS AND DISCUSSION

The Figure 1 shows the mean values (± SEM) of the d-ROMs, Oxy-Ads and SHp obtained in cows and ewes during the monitoring period, together with the relative statistical differences. A statistically significant effect of lactation on all studied parameters was found both in cows and ewes ($P<0.05$). In particular, d-ROMs showed higher levels at the end of lactation and during dry period compared to early lactation in both species. Higher Oxy-Ads levels were found during dry period respect to early lactation in cows, whereas lower Oxy-Ads values were measured during dry period compared to early and end lactation in ewes. SHp obtained in cows showed lower values during end lactation and dry period compared to early lactation; whereas SHp obtained in ewes showed higher levels during end lactation and dry period compared to early lactation.

The assessment of oxidative stress implies a comparative analysis of the concentration of free radicals and antioxidant power, two strictly interdependent factors whose interaction allows discrimination of the different forms of stress, acute and chronic, and distress (Piccione et al. 2010). Low dROMs' values at the start of experimental period could be due to the energetic deficit which occurs in cows and ewes during the last period of pregnancy (Piccione et al. 2006).

In lactating cows and ewes oxidative stress physiologically acts at mammary gland level as a cellular turnover modulator. However, the excess of free radicals drastically depresses milk production (Stefanon et al. 2002). The most critical time, in cows, appears to be the beginning of the lactation when the condition of negative energy balance favours lipomobilization and gluconeogenesis that intensify the basal metabolism, increasing O_2 consumption, with subsequent excess of free radicals (Alicjuzel et al. 2001). The pattern of dROMs characterized by low values at the beginning of lactation and a significant increase at the mid-point of lactation, was previously observed in lactating cows and ewes (Piccione et al. 2006, 2007). The high values of Oxy-adsorbent and SHp at the end of the lactation period testify to the compensatory response of the organism to oxidative stress. In our study, the course of free radicals together with trend

of Oxy-Ads and SHp levels allows us to affirm that during the whole lactation, despite the increment of oxidative phenomena under the hormonal and metabolic influence, oxidative homeostasis was maintained by the efficiency of the antioxidant defences.

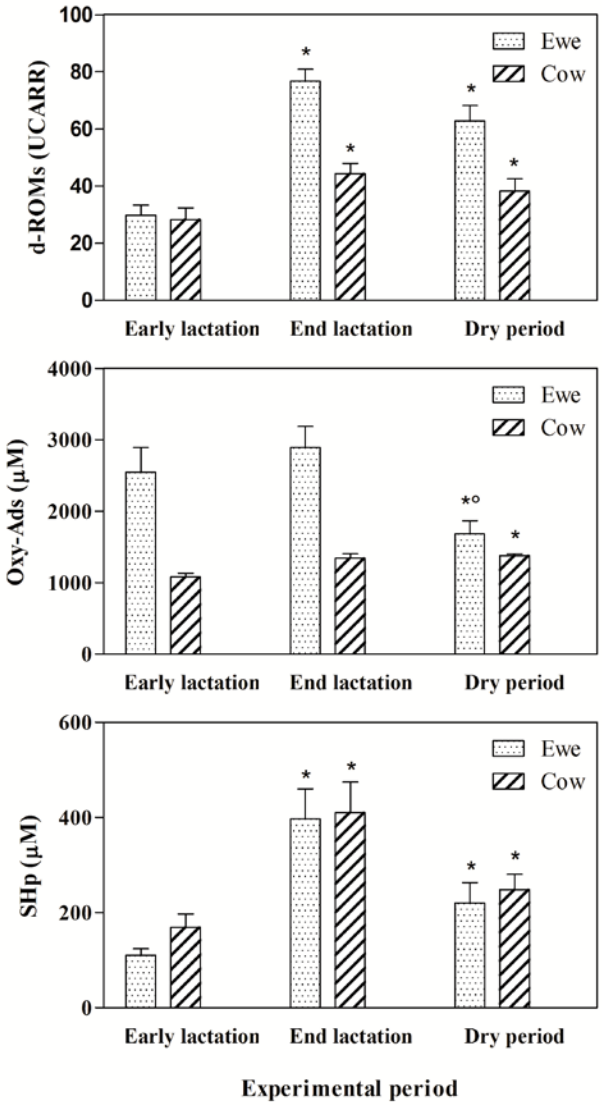

Figure 1. Mean values (±SEM) of reactive oxygen species (dROMs), antioxidant barrier (Oxy-ads) and thiol antioxidant barrier (SHp), expressed in their convectional unit of measurement, obtained during early lactation (day 4), end lactation (day 200) and dry period in dairy cows and ewe

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Invited presentation

MANNAN OLIGOSACCHARIDES – MODES OF ACTION AND POSSIBILITIES FOR USAGE

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Abstract: Mannan-oligosaccharides (MOS) are prebiotics that may prevent bacterial adhesion to mucosal surfaces and neutralize their toxins without causing tissue damage. For that reason, they have been used as feed additives for more than 20 years. They exert positive influence on animals by selective stimulation of growth and/or activity of one or limited number of non pathogenic bacteria in the digestive tract. Systemic positive effects of MOS may be attributed to absorption of bacterial fermentation products and by improving hosts health status.

Addition of mannan-oligosaccharides (MOS) to cow's colostrum (5 g/L) resulted in significant enhancement of IgG concentration in neonatal calf's sera. Calves were fed colostrum, supplemented with Bio-Mos (5g/L), three times during the first 24 hrs of life and Ig G concentration was determined at 6, 12, 24 and 48h following birth.

Same phenomenon was documented in numerous experiments on piglets which were allowed to suckle free, followingper oral application of MOS suspension. Piglets were orally dosed with 10 mL of a 75g/L mannan-oligosaccharide suspension in saline water at parturition and 12 hrs after birth. In the piglet's trial, blood samples were collected only 48h after birth.

In our trials, IgG concentration was measured by radial immuno-diffusion (anti-bovine and anti-pig IgG RID plates, INEP, Zemun). In treated, calves and piglets, a significant improvement in IgG sera concentration was achieved. Titersof the antiBHV-1 antibodies in the calvessera 24 hrs after birth and after 7 days, were higher in the Bio-Mosfed group and differences were statistically significant when compared to the control group. Improvement of passive immunological transfer significantly reduced calves mortality rate on the farm.

We have also investigated possibilities for therapy of sows, suffering from puerperal endometritis by intra uterine application of sterile MOS suspension. We have used MOS-based product (Yeast Call Wall, Alltech, Fermin, Senta) in the amounts of 5, 10 and 20g (I, II and III group) suspended in 100 mL of saline. In groups II and III we noted significant

clinical improvement in treated sows and moreover higher body weight gain in piglets.

Key words: *calves, endometritis, IgG, mannan-oligosaccharides, piglets, sows, passive immunity*

INTRODUCTION

Over the past decade we have conducted several studies aimed to explore possibilities for usage of mannan-oligosaccharides in veterinary medicine and animals breeding. Mannan-oligosacharides (MOS) are prebiotcs that exert positive influence on animals by selective stimulation of growth and/or activity of one or limited number of bacteria in the digestive tract. Recently they are named gut active carbohydrates (GAC) and they are derived from the cell wall of yeast which can absorb pathogens expressing type-1-fymbrae, reducing their ability to colonize gastrointestnal tract (Spring et al. 2000). There are published data confirming constant improvements in piglet, sow, broiler and turkey performances where GAC has been fed over cotinuous period of time (Miguel et al. 2002, Hooge at al. 2003, 2004a, 2004b, Sims et al. 2004, Rozeboom et al. 2005). In many trials, the ability of GAC to bind and eliminate pathogeniic bacteria like *Salmonella spp* and *Clostridiim perfrigens* was documented (Spring et al. 2000, Sims et al. 2004). It was postulated that by action of binding and pottentially altering the bacterial populations in the gut, (Fioramonti et al. 2003) and their activity as receptor analogues, oligosaccharides may be involved in the immune cross-talk interactions (Kelly 2004). They can also exert positive systemic effects following absorption of bacterial fermentation products and by improving hosts health status.

Investigations of mannan-oligosaccharides influence on colostral IgG absorption in calves

In the first study we investigated the influence of mannan-oligosacharides on the colostral immunoglobuline absorption in Holstein-Friesiancalves. The experiment was conducted on the total of 36 cows and thier off spring devided in three equal groups according to their sex and bodymass. Calves from the control group (K) were nipple fed with 1,5 L of colostrum 2, 12 and 24 hours after birth. Second group of calves (M) were fed in the same way and with the same amount of colostrum with addition of clinoptillolite (5g/L) and third group was fed colostrum that contained 5g/L of mannan-oligosacharide suspension (Bio-Mos, Alltech, USA).

Our results indicated that mean values of the Ig G concentration in blood sera of cows from all three groups were similar and with in physiological range. The mean values for Ig G concentration in primary,

secondary and tertiary colostrum were also similar when compared between different groups. Within the same group of cows, differences in Ig G concentration between primary, secondary and tertiary colostrum were significant. The highest Ig G concentration was measured in primary and the lowest in tertiary colostrum. In all three groups of calves, the total colostral Ig G intake was nearly the same during the first 24 hours and no statistical differences were documented between them. Ig G concentration, estimated from the 6th hour of life till the day 21. was higher in the group of calves fed colostrum with Bio-Mos addition when compared to other two groups. Addition of Min-A-Zelal so enhanced colostral Ig G uptake, but these differences were statistically significant only 6 hours after partitution ($p<0,01$). After 48 hours, Ig G concentrations in groups M and K were nearly the same while in the group fed colostrum with Bio-Mos, elevated values were recorded till the day 21. Efficiency of colostral Ig G was highest in the gruop fed Bio-MOS supplemented colostrum. The mean BHV-1 antibody titar values in the primary colostrum of cows were similar in all three groups and no statistically significant differences were documented. The titers of anti BHV-1 antibodies in the calvessera 24 hrs after birth and after 7 days were highest in the Bio-Mos fed group and these differences were statistically significant when compared to the other two groups. In the clinoptilolite fed group, the similar effect was noted but differences were not statistically significant. Finally, the improvement of passive immunological transfer significantly reduced the calves mortality rate on the farm (Shabanovic 2005, Lazarevic et al. 2010).

The influence of mannan-oligosaccharides on piglets blood biochemical profile and body weight gain up to weaning

The main goal of this study was to explore effects of Bio Mos (Alltech, SAD) administered orally twice within the first 12 hrs of piglets life on their body weight and values of the following biochemical parameters in piglets sera: total protein, albumin, triglyceride, cholesterol, Ig G, glucose andIGF concentrations and activity of enzymes AST and ALT during the first 30 days of their life. Ten litters origating from the Yorkshire sows was included in the trial with total of 94 piglets (50 in treated and 44 in control group). Very shortly after farrowing and once again after 12 hrs. half of the litter was adminstred 10 mL of Bio Mos suspension (75 g/L). The second half of litter was treated in the same manner and at the same intervals with 10 mL of saline (Tokic 2012, Lazarevic *et al.* 2012).

Glucose level was estimated from the whole blood samples on days 2, 5, 10 i 30 after partitution. Blood sampling and sera collection was performed on days 2, 5, 10 and 30 after partitution in order to measure (a) concentration of total proteins, albumine, triglyceride, cholesterol, Ig G and

IGF I and (b) activity of ALT i AST. Body weights of piglets from the treated and control groups was measured on the days 2, 5, 10 and 30 after partitution. Thereafter, mean body weight, total and daily body weights were calculated for the monitored intervals.

We were able to conclude that peroral application of Bio Mos (twice within the first 12 hrs of piglets life, 0,75g per piglet) resulted in statistically significant rise in total protein (+12,6%) and Ig G concentrations after 48 hrs (+19,4%). This treatment did not affect albumine concentration showing continous elevation up to the age of 30 days. The same treatment did not alter concentrations of triglycerides, cholesterol and IGF I in the piglets sera. Glucose level in the sera was highest on days 2 and 5 and decreased to basic levels thereafter. Treatment with MOS did not influence this parameter either. Moreover, activity of ALT and AST was not changed during the trial. Treated piglets were in average of 505g hewier at weaning when compared to controles and BWDG (body weight daily gain) was +21,8g MOS also reduced the number of piglets died and incidence of diarrhea.

Similar results in piglets regarding Ig G absorption enhancement were reported by Hengartner et al. 2005 and in piglets and calves (Lazarevic 2003, 2003a, 2005).

Mannan-oligosaccharides in therapy of sow´s with puerperal uterine infection

In this study, we investigated effects of intra uterine application of sterile mannan-oligosaccharide (MOS) suspension to sows suffering from puerperal endometritis (Miljas, N. 2014, Lazarevic et al. 2012). As mannan-oligosaccharides may prevent bacterial adhesion to mucosal surfaces and neutralize their toxins without causing tissue damage, it was postulated that this approach may result in successful curing, lower percent of recidivism and positive effects on piglet's growth due to improved milk production.

A trial was conducted in four experimental and one control group of sows consisting of 10 animals each. Experimental groups were formed of sows with purulent vaginal discharge, 2−3 days post farrowing along with reduced apetite. Animals were fed standard food mixtures (AOC Tables, 1993). Clinical examination was performed on the day of therapy and 2−5 days later.

Uterus content samples for bacteriological and cytological examination were collected on the day of first clinical examination and immediately after that, sterile MOS suspension or Lotagen were administered by means of catheter. After 2−5 days, a second sampling was performed. We have used MOS-based product (Yeast Call Wall, Batch No 6.9.175, Alltech, Fermin, Senta) in the amounts of 5, 10 and 20g (I, II and III group) suspended in 100 mL of saline. Sows from the group IV were treated in the same way by 100 mL of 2% Lotagen solution.

Smears for cytological analyses were stained by May-Grunwald Giemsa method and analyzed by means of direct light microscopy (Olympus BH-2, Japan), using immersion objective and at total magnification of 1000 X. We have determined presence of neutrophil and eosinophil granulocytes, lymphocytes, monocytes, epithelial cells and bacteria as well as their structure.

Bacteriological examination was performed by standard plating procedures on Columbia agar (CM331, Oxoid, Basingstoke, UK) with addition of 5% ovine blood and MacConkey agar (CM115, Oxoid). Plates were incubated in aerobic conditions at 37 ^{0}C over 24−48 hrs. Grown colonies were counted in plates containing 30−300 colonies to estimate number of CFU (Colony Forming Unites).

Piglets from each litter were weighed individually on the day of sow´s therapy, 2−5 days later at control examination and at the moment of weaning in order to calculate total and daily body weight gains. Body weight was measured by electronic scale with sensitivity of 10g. In the last trial phase, reproductive results of sows were determined in their next breeding cycle. We have recorded a total number of piglets born, number of alive and stillborn and percent of piglet's loss after 3 days post farrowing.

Treatment of sows with puerperal dysgalactia by intrauterine application of MOS suspension resulted in significant clinical improvement and percent of recidivism was the smallest in groups treated with 10 and 20g.

On the stained smears of uterine content a presence of neutrophil and eosinophilgranulocytes, lymphocytes, monocytes, epithelial cells and bacteria was noted. In all experimental groups, very highly significant differences were present between mean number of neutrophilgranulocytes, at the moment of therapy and 2−5 days later. The best effects in therapy were achieved by 10 and 20g of MOS suspended in 100 mL of saline. Following treatment, highly significant differences were noted between groups treated with 5 g and 10g and 20g. Differences between groups treated with Lotagen and those treated with 10 and 20g of MOS were very highly significant.

Degree of bacterial colony number reduction was the highest in groups treated by intrauterine application of 10 and 20g of MOS ranging from 1361 to 1444 times. In the sows treated with Lotagen solution this value was 32.

The most abundant bacterial species isolated from sow's uterine content were: *E. coli, Streptococcus dysgalactiae subspecies equisimilis, Staphylococcus aureus, Arcanobacterium pyogenes* andcoagulase negative *Staphylococcus*.

At the moment of weaning, piglets from the groups of sows treated with 10 and 20g of MOS were heavier then piglets from the control and Lotagen treated group of sows.

Treatment of sows with PDS by intra uterine application of MOS suspension and Lotagen did not significantly influenced number of piglets born in the next reproductive cycle and between experimental groups no statistically significant differences were noted. A total piglet´s loss during first days of life was the highest in the control group.

Treatment of sows with PDS by intra uterine application of MOS suspension resulted in significant clinical improvement with small percent of recidivism and exerted positive effects on piglets body weight gain up to the moment of weaning.

CONCLUSION REMARKS

Based on the numerous literature data and our personal experiences, from three different field trials, we are able to conclude that there are numerous possibilities for application of MOS based feed additives in animal breeding. Apart from the well-known and well documented benefits of MOS as growth promoters, and gut health regulators, improvement in IgG absorption and enhancement of the passive immunity in new born piglets and calves is of substantial importance. In addition, surprisingly constant recovery of sows suffering from puerperal dysgalactia, for sure deserves more attention.

Possible mechanisms of MOS action includes: prevention of bacterial adherence to gut mucosa, adsorption of bacterial and other toxins in the intestinal lumen, stimulation of beneficial bacterial growth and absorption of their products. Finally, improvement of IgG absorption may be attributed to MOS capability to stimulate phagocytosis, but this hypothesis still needs experimental confirmation. These findings are of special interest because, in some cases, this approach may decrease needs for antibiotic treatments.

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PATOMORPHOLOGICAL MANIFESTATIONS OF *TRUEPERELLA PYOGENES* INFECTION IN DAIRY CATTLE

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Abstract: *T. pyogenes* is an opportunistic pathogen of economically important livestock such as dairy and beef cattle. Formerly, *Arcanobacterium pyogenes* was recently renamed *Trueperella pyogenes*. *T. pyogenes* is being able to cause disease in a different animal species including horses, dogs, cats, chickens, turkeys, bisons, camels and deer. *T. pyogenes* is a rare cause of infections in humans and these infections are often a result of occupational exposure, as *T. pyogenes* is not a part of the human normal microflora.

T. pyogenes expresses several known and putative virulence factors. These factors are required for adherence, subsequent colonization, and to cause the host tissue damage associated with infection by *T. pyogenes*. The main virulence factor of *T. pyogenes* is the potent extracellular toxin, pyolysin.

T. pyogenes could be isolated from the rumen, and also from the udder and proximal portion of respiratory tract of healthy cows. Although *T. pyogenes* has ability to be a primary pathogen, infection can be happen after physical injury or influence of other pathogenic microorganisms, followed by dissemination of bacteria within organism.

This bacterium is related to different pyogenic infections and sporadic abortions in cattle. Bronchopneumonia, mastitis, endometritis, and cellulitis/myositis are the most common patomorphological manifestations. In addition, arthritis, endocarditis, osteomyelitis and vesiculitis could also be observed. The organism could also be isolated from other cases of septicemia, nephritis, pleural and myocardial abscesses.

Histologically, apostematous bronchopneumonia and apostematous mastitis are the common finding in cases with *T. pyogenes* infection. In cases with co-infection fibrinous and fibrinopurulent bronchopneumonia could be observed. Purulent and necrotic endometritis are the most common finding in cases with *T. pyogenes*. In cases of cellulitis suppurative process in subcutaneous tissue can be observed.

Although opportunistic pathogen, *T. pyogenes* can cause significant pathological manifestations and economic losses in modern cattle husbandry.

Keyword: *Trueperella pyogenes*, dairy cattle, pathomorphological manifestations

INTRODUCTION

Trueperella pyogenes (*T. pyogenes*) is commensal and opportunistic microorganism, which can cause mastitits, apostematous hepatitis and pneumonia, purulent cellulitis in cattle and swine. It is often inhabitant on the mucosal surfaces in cattle and swine, and could be isolated from the udder, urogenital tract and upper part of respiratory system of healthy animals. It is part of normal ruminal flora, and sometimes could be found in pig stomach. Since the causative agent is commensal infection is usually autogenous. Even though *T. pyogenes* has ability to be primary pathogen, infection usually could be happen after physical injury or influence of other microorganisms on mucosal surfaces, which is later followed by dissemination of the agent and infection. *T. pyogenes* could cause abortus, abscesses on parenchymatous organs, arthritis, endocarditis, mastitis, pneumonia, osteomyelitis, endometritis and vesiculitis (Lewis 1997). *T. pyogenes* is a rare cause of infections in humans and these infections are often a result of occupational exposure, as *T. pyogenes* is not a part of the human normal microflora (Jost and Billington 2005).

T. pyogenes is Gram positive, non-motile asporogenous rod, which was firstly reclassified from the genus *Actinomyces* to the genus *Arcanobacterium*, and recently in the genus *Trueperella*. It is grown in aerobic and anaerobic conditions, but the optimal growth is achieved in the atmosphere with CO_2. Haemolysis on the agar with added sheep or bovine blood could be observed. *T. pyogenes* has different virulence factor which are important in causing infection. One of the most important is cholesterol dependent cytolysin designated as pyolysin. Pyolysin is haemolysin which has cytotoxic activity against immune cells including macrophages. There are also neuraminidases which are important for adherence of *T. pyogenes* on epithelial cells and components of extracellular matrix (Jost and Billington 2005).

This bacterium is related to different pyogenic infections and sporadic abortions in cattle. To our investigations (unpublished data) bronchopneumonia, mastitis, endometritis, and cellulitis/myositis are the most common patomorphological manifestations. The organism could also be isolated from other cases of septicemia, nephritis, pleural and myocardial abscesses.

BRONCHOPNEUMONIA

Complex of bovine respiratory disease (Bovine respiratory disease complex – BRDC) is a multifactorial clinical syndrome resulting from the

interaction of microorganisms, environment and animal immune response. One of infectious agents which plays an important role in the onset of BRDC is *T. pyogenes*. A post-mortem finding in the lungs infected with *T. pyogenes* is reflected in the cranio-ventral consolidation of lung parenchyma with purulent and necrotic foci of different size. In the affected parenchyma characteristic lesions in the form of raised nodules, white, clearly limited by the parenchyma, with caseous necrotic content, size from 2 to 10 mm or larger can be observed. In addition to these, larger white nodules, some sizes of up to 3 cm which instead caseous exudate contain liquid content white-yellow in color as a result of competitive bacterial infection can be observed (Caswell and Williams 2007). Sometimes, less fibrin deposits covered the surface of the pleura while in the trachea and bronchi could find sparkling content. The most common histological lesions are characterized with large number of alveoli and bronchi which are filled with polymorphonuclear leukocytes and usually lung parenchyma with fields of caseous necrosis can be observed. Co-infection with *Mycoplasma bovis* and *Pasteurella multocida* can be also observed (Cvetojević et al. 2016). Pulmonary abscessation can occur as the pneumonia becomes chronic. Abscesses develop in about 3 weeks but do not become encapsulated until 4 week. *T pyogenes* is frequently cultured from these abscesses (Campbell 2015)

Stress is an important risk factor for disease in all animals. Specific stress factors may include capture, transport, weaning, changes in social structure, adverse climate, and poor nutrition. Decreased resistance may have allowed *T. pyogenes*, which is generally an opportunistic bacterium and a commensal on mucosal surfaces of domestic ruminants, to proliferate and cause disease (Palmer and Whipple 1999).

MASTITIS

Trueperella pyogenes has a role in etiology of „sommer mastitis‟ in dairy cows. Additionally, *T. pyogenes* is occasionally seen in mastitis of lactating udders after teat injury, and it may be a secondary invader. It can also be diagnosed in heifers and dry cows. The inflammation is typified by the formation of profuse, foul-smelling, purulent exudate. Mastitis due to *T. pyogenes* is common among dry cows and heifers that are pastured during the summer months on fields. The vector for animal-to-animal spread is the fly *Hydrotaea irritans*. Summer mastitis is diagnosed in 39–54% dairy herds, and the incidence is on average 2.1 to 4.1 cases per herd (Milanov et al. 2011).

T. pyogenes has a good invasive properties and penetrates deep into the glandular parenchyma of the udder, similar to *S. aureus* and *Streptococcus uberis.* The penetration of the pathogen into the tissue of the mammary gland is accompanied by a strong inflammatory reaction,

purulent exudation and abscess formation, tissue accumulation of debris, leukocytes and fibrin (Milanov and Stojanović 2010).

Preventive treatment of heifers and dry cows in susceptible areas with long-acting penicillin preparations has effectively reduced infections. Therapy is rarely successful, and the infected quarter is usually lost to production. Infected cows may be systemically ill, and cows with abscesses usually should be slaughtered.

ENDOMETRITIS

Inflammation of the uterus in cows, recently classified as puerperal metritis, clinical endometritis, subclinical endometritis, and pyometra represents one of the most important causes of (sub)infertility in dairy herds. Therefore intrauterine antimicrobial treatment represents a common and frequent procedure in dairy farms even though the results of the treatment are variable. Nevertheless, bacterial contamination of the uterus in early postpartum cows is common and the development of uterine inflammation depends on local immunity and on the intensity of contamination and the spectrum of contaminants. Thus an effective control of postpartum contamination of the uterus provides the chance to improve both fertility and general health condition of dairy herds (Dolezel et al. 2010).

At necropsy, in cases of puerperal endometritis uterus is enlarged while palpation feels fluctuations and can assume that larger quantity of liquid is contained intraluminal. After opening the uterus, usually finding is presence of smelly, suppurative/fibrinous liquid content of red-brown color. In the most cases the entire endometrial surface is covered with diphtheritic necrotic debris – yellow to gray pseudomembrane. Sometimes, the whole mucosa can be covered. In more acute cases, this finding usually lack, and just hyperaemia can be observed on the mucosa of the uterus.

Isolation of *T. pyogenes* from the contents of the uterus in these cases is not surprising considering that microorganisms can easily invade the ascending uterus via the vagina and cervix during and after partus. Although *T. pyogenes* considered equally ubiquitous bacteria present on the mucous membranes of cattle, it is important to emphasize that belongs to a group-specific pathogens, which usually lead to the infection of the uterus.

Beside *T. pyogenes* other bacteria can be isolated as well. In our experience usually *T. pyogenes* is detected in co-infection with *E. coli*. Sometimes, some viruses can also be detected, such as BHV4. In such cases uterus is the source of toxins and microorganisms, and there is possibility to penetrate into the circulation, leading to severe septicemia and toxemia which usually lead to the death.

CELLULITIS/MYOSITIS

Purulent cellulitis/myositis are very important findings in pathology of farm animals. It can be observed as a local circumscript pyogenic inflammation (abscesses) or diffuse purulent inflammation as phlegmona. In cattle one of the most frequent causative agents is *T. pyogenes*.

Infection of muscles can be happen directly or by haemetogenous dissemination of causative agent. There are defense mechanisms such as skin, subcutaneous tissue, fascia, collateral vascularization, specific and non specific immune response which are very important and can prevent infection. Directly, infection can be happen in cases of penetrating wounds, intramuscular injection, bone fracture, directly from the infected joints, tendon and lymph nodes and in cases of „crush" injuries.

In the early inflammatory phase, infectious agent cause local cellulitis. If the body cope with the infection by preventing its spread then restitution occurs with minimal formation of connective tissue. However if there is a progression of infection the possibility of abscess formation is increased. In this case abscess formation means existance of pyogenic connective tissue membrane and external membrane, and in the center purulent content. Abscesses in cattle can be observed often on the hind legs. Clinically, oedema and lameness can be observed because necrotic process in subcutaneous tissue and muscles can be comprehensive.

T. pyogenes as ubiquitous microrganism can colonize muscles by direct contamination of wounds or hematogeneous dissemination. This bacterium is normal inhabitant of ruminal wall as well as uterus, and after parturation transitionally bacteriaemia can be happen, and *T. Pyogenes* can colonize ijuried muscles. Severity of lesions depends on the virulence of causative agent and can vary from intramuscular abscesses near the injection site to diffuse purulent cellulitis which can spread through the surrounding connective tissue and fascia. For example, non-sterile intramuscular injection in the gluteal muscles of cattle can cause an infection that spreads on the femoral muscles fascia to the tibia. Macroscopically, abscesses are filled with yellow-green pus with unpleasent odor. If there is cellulitis then purulent exudate on the surface of the fascia and intermuscular connective tissue can be observed. Inflammation can gradually spreads to adjacent muscular fibers leading to myonecrosis and consequential organization by connective tissue. Histologicaly, small pleomorphic gram-positive bacteria are often visible within the tissue sections or cytological preparations (McGavin and Zachary 2007).

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ANTIMICROBIAL RESISTANCE IN BACTERIA: WHY IS IT IMPORTANT?

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Abstract: Bacteria use a number of drug-resistant mechanisms for all classes of antibiotics which are used in clinical practice or for prevention purposes. They have developed strategies of expelling antibiotics out of the cell, or changing the antibiotic molecule by using enzymes or provoking mutations on target genes in order to prevent the attachment of antibiotics to the cell. Bacteria have the ability to exchange their genetic material and disseminate mobile genetic elements carrying the resistance genes. Hence, the horizontal gene transfer is an important way of sharing resistance determinants among bacteria.

Nowadays, the following antibiotic and corresponding resistance patterns are of great concern: meticillin resistant *Stapyhloccocus aureus*, vancomycin resistant *Enterococcus* spp., resistance to fluoroquinolones in *Salmonella* spp. and *Campylobacter* spp. and resistance to cephalosporins of the third and fourth generation and carbapenems in Gram negative bacteria. Since most of these bacteria cause infections in humans after they have consumed food of animal origin, it is the subject of veterinary sciences to contribute in breaking the chain of food contamination during production, processing, storage and transportation. Besides the good hygiene and management practice, prudent use of antibiotics is an important strategy both in human and veterinary medicine. It is also widely accepted that therapy of animals with antibiotics which are used in human medicine has to be minimized or completely avoided.

A comprehensive campaign under the National Monitoring System is needed to evaluate the resistance to key antibiotics in the livestock industry in Serbia. Such programs are performed annually for *Salmonella*, *Campylobacter jejuni* and *C. coli*, *Enterocccus faecalis* and *E. faecium* and *Escherichia coli*. The ultimate goals are to analyze the risks of consuming food contaminated with the resistant bacteria and to restrict or even completely forbid the use of some target antibiotics in agriculture.

Key words: *bacteria, food, animals, resistance, genes*

INTRODUCTION

Bacteria acquire a number of genetic mechanisms to develop resistance against antibiotics. The mobility of plasmids, transposons and integrons facilitates the exchange of genetic elements and the development of multi drug resistant phenotypes. Unfortunately, even new generations of antibiotics could be inhibited by bacteria and because of that, therapeutic options in human and veterinary medicine, are becoming more and more problematic. In fact, if hygiene and management practice as well as public awareness of safe food handling and proper use of antimicrobial agents are not taking place as they already are, it would be impossible to combat the resistant microorganisms at present and in the future (Bennett 2008).

Plasmids carry an array of resistance and virulence genes and they could be exchanged between bacteria by the conjugation mechanism. Gen cassette encoding resistances to antibiotics are often integrated in integrons which provide a proper genetic environment for them. Integrons have an *attI* integration site, an integrase enzyme which mediates recombination and they also have the promoter, which is necessary for the activation and regulation of the gene expression systems (Benett 1999). The efflux pump is also an important mechanism of resistance in bacteria. They activate the efflux pump not only to expel antibiotics but also disinfectants and toxic substances out of the cell. When bacteria develop resistance for target antibiotics simultaneously with the efflux pump mechanisms, the resistance increases and it becomes difficult to implement a proper therapy. Scientists are therefore considering the possibility of combining efflux inhibitors with the antibiotics for the new generation of drugs (Weber and Piddock 2003).

Of the highest public health importance are bacteria causing nosocomial infections. For example, when a tourist from the USA got infected in a hospital in the Philippines with *Salmonella* Swarcengrund, which was resistant to ciprofloxacin, he transferred S. Swarcengrund to the USA upon his return. After a while, this *Salmonella* was isolated from two nursing homes in the State of Oregon (in one of the nursing homes the former tourist was a resident) as well as in a local hospital. The nosocomial contamination with *S*. Swarcengrund took place over several years. The origin of this isolate was identified based on pulsed field gel electrophoresis profiles and based on the mutations on target topoisomerase genes. This was the first report on an isolation of the ciprofloxacin resistant *Salmonella* in the USA (Olsen et al. 2001). However, it is not only the „hospital environment" which may present a place of origin for such bacteria. Many increasingly resistant microorganisms are actually livestock or food associated, indicating the important role of veterinary medicine in establishing the practice of prudent use of antibiotics in preventing the occurrence of resistance. It is documented that withdrawal of some antibiotics in animal breeding has a significant impact in decreasing the resistance rate in bacteria which takes place soon after the removal of the

antibiotic (Aarestrup et al. 2001, Marshall and Levy 2011). Antibiotics that are prescribed in human medicine should not be used in the livestock industry. Such is the case with fluoroqinolones and cephalosporins of the third and fourth generation. The same applies to those antibiotics that have been used as growth promoters and yet were structurally related to important antibiotics for humans (Marshall and Levy 2011). The epidemiological aspect of the infection with multi-drug resistant (MDR) bacteria has to be taken into account also. Multi resistant strains may perpetuate in a herd and infect other animals and contaminate the environment. They could spread to farm workers and their home members or be further disseminated in the food chain (Marshall and Levy 2011). The possibility of keeping animals treated with highly important antibiotics in isolation for some period of time remains a dilemma. Prevention and control has become the most important strategy in human and veterinary medicine since therapy has become more and more difficult due to the emergency of highly resistant microorganisms.

MULTI DRUG RESISTANT BACTERIA OF THE HIGH IMPORTANCE

Methicillin-resistant *Staphylococcus aureus* (MRSA) has developed a resistance to penicillin and cephalosporin antibiotics. There are three large groups defined by genetic elements of the MRSA and the host organisms. The health-care associated MRSA (HA-MRSA) and the community acquired (CA-MRSA) or livestock associated MRSA (LA-MRSA) are presented in these major groups. The resistance to penicillin and the beta-lactam antibiotic develops over the *mecA* gene expression. This gene is part of the mobile genomic island staphylococcal cassette chromosome *mec* (SCC*mec*). The large SCC*mec* locus is represented by three types (I, II and III) in HA-MRSA. The CA-MRSA possesses the smaller SCC*mec* called type IV and V (Chao et al. 2013). The predominant clonal complex CC398 is widespread in the livestock in the EU countries. The LA-MRSA is lacking many virulence factors such as PVL toxins and other enterotoxins comparing to HA and CA-MRSA strains but it can still cause human diseases, having a strong capacity of host adaptation and transmission and presenting a serious occupational hazard for farm workers, their close family members and people residing near animal farms (Cuny et al. 2013).

Vancomycin is a glycopeptide antibiotic which is used in human medicine for the therapy of patients infected with Gram positive bacteria which are resistant to beta lactam antibiotics. Enterococci had developed a resistance to vankomycin soon after its discovery in 1988 and since then vancomycin resistant *Enterococcus* (VRE) represents a serious threat to public health. The VRE is a typical nosocomial pathogen, but it is also isolated from feces of food animals and foods (McDonald et al. 1997). In the livestock industry the use of avoparcin as a feed additive has led to the

occurrence of VRE in farm animals. The resistance to vancomycin in *Enterococcus faecium* in Denmark was very high (72.7% in chicken flocks in 1995), following the immediate decrease of resistance after the drug withdrawal to 5.8% in 2000. The ban of avoparcin in 1996 in Germany has also led to a decrease in resistance to glycopeptides-resistant enterococci in healthy humans from 13% in 1994 to 4% in 1997 (Aarestrup et al. 2001). Similar trends in the decrease of resistance were noticed after the removal of the growth promoter virginiamycin in poultry flocks. The resistance rate of *Enterococcus* was 76.3% in 1997 and it decreased to 12.7% in 2000 in Denmark. Resistance to virginiamycin has promoted resistance to macrolide antibiotics. Namely, both antibiotics have similar mechanisms of action which probably contributed to the development of resistance to macrolides as well. The decrease of resistance to tylosin in *Entercocci* was also evident since tylosin was not used any more for growth promotion. However, tylosin is still in use for the therapy of pig herds, and the resistance rate fluctuates having a tendency to steadily decrease. Resistance to glycopeptides antibiotics and macrolides tylosin in clonal strains from pigs may evolve because the genes encoding resistance to these classes of antibiotics are located nearby on the same plasmid. Hence, careful use of tylosin in pig production has led to a clear decrease of resistance to glycopeptides from 1999 and thereafter. Drugs used as growth promoters may generate resistance to chemically similar antibiotics, some of which are used in human medicine for therapy. It is evident that the ban of growth promoters has had a positive influence on resistance development which occurred soon after the withdrawal of the therapeutic agent (Aarestrup et al. 2001).

Resistance to fluoroquinolones in *Salmonella* spp. and *Campylobacter* spp. is important from the public health aspects. Both bacteria usually do not cause clinical disease in animals while in humans they may cause serious health disorders. In cases when therapy of patients is required, fluoroquinolones are often prescribed for infections caused by these bacteria. Mutations on target genes encoding Gyrase A and topoisomerase IV enzymes i.e. *gyrA*, *gyrB* and *parC* and *parE* genes respectively, are responsible for the phenotype resistance to ciprofloxacin. However, *Campylobacter* species acquired a high resistance rate to ciprofloxacin (CIP) (MIC $\geq$32 mg/L) after a single point mutation in the *gyrA* gene. The most frequent mutation occurs at the codon 86 where threonine is replaced with isoleucine (Thr86→Ile). In *Salmonella*, a single point mutation in the *gyrA* gene will cause resistance to nalidixic acid and an increased MIC to CIP, but for high resistance to CIP, two point mutations in the *gyrA* gene are required. In increasingly resistant mutants, amino acid exchanges usually occur at the *gyrB*, *parC* and/or *parE* genes as well. The most frequent double mutant have the following amino acid transitions in the *gyrA* gene in *Salmonella*: Ser83→Ala, Phe or Tyr and

Asp87→Asn. The second target for (fluoro)quinolones in *Salmonella* is the *parC* gene where the Ser80→Ile amino acid transition is often found (Velhner and Stojanović 2012). Because both bacteria have a tendency to develop resistance over time, epidemiological cut-off values (ECOFFs) which are much lower comparing to clinical breakpoints, need to be tested and reported annually from EU member states to the European Food Safety Authority (EFSA), which coordinates data from the framework program and annually records the emerging risks in food safety. This program includes resistance monitoring of *Salmonella* spp. in fowl (Galus gallus), turkeys, pigs and *Campylobacter coli* and *C. jejuni* from broilers (EFSA Journal 2015). Resistance to ciprofloxacin and cephalosporins are of highest concern due to the frequent use of these antibiotics in human medicine.

HOW TO REACT TO THE OCCURENCE OF THE ANTIMICROBIAL RESISTANCE IN PATHOGENIC AND COMMENSAL BACTERIA

In developed countries a ban of antimicrobial agents used for growth promotions has led to an immediate decrease of the resistance rate in bacteria isolated from food producing animals (Aarestrup et al. 2001). However, trade and traveling still pose a serious risk due to the frequent „importation" of highly resistant bacteria. The first step in control programs is the monitoring systems, which will provide the basic information of the levels of resistance in pathogenic *Salmonella* and *Campylobacter* species and indictor bacteria like *Escherichia coli* and *Enterococcus faecium* and *E. faecalis*. From the research works it is evident that resistance to ciprofloxacin in *Salmonella* Infantis and *Salmonella* Kentucky isolates from poultry and turkey flocks has taken place in Serbia (Rašeta et al. 2014, Velhner et al. 2014a, Velhner et al. 2014b). Therefore, the enrofloxacin antibiotic should be prescribed only in urgent situations for susceptible isolates. It is important to note that therapy for poultry infected with *Salmonella* does not provide any benefit to animals since it eliminates *Salmonella* from the gut only temporarily. Moreover, *Salmonella* control programs are closely related to the Framework Programs on Antimicrobial Resistance. The awareness of the public sector, particularly the farmers, of the risks from acquiring and transmitting bacterial disease from food producing animals to humans, is important. Food producing animals have to be protected by introducing good management practice and by providing alternative treatments such as probiotics and similar feed additives. Vaccination against *Salmonella* has to take place in order to increase the herd/flock immunity for better performances and to obtain the safety of the final products. Farmer's initiative regarding good management practice is often crucial for the benefits in the animal breeding industry and has

indirect implications on human health through consumption of the safe food. In Serbia such programs and initiatives are either in the developing phase or completely absent.

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EXTENDED SPECTRUM β-LACTAMASE-PRODUCING (ESBL) ENTEROBACTERIACEAE

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Abstract: Beta lactam antibiotics are used to treat bacterial infections in humans and animals. They exhibit the bactericidal effect by inhibiting the bacterial cell wall synthesis. The uncontrolled use of these antimicrobial agents leads to the development of resistance, by producing extended spectrum β-lactamase enzymes (ESBL). Bacteria from the family *Enterobacteriacae* are sometimes ESBL producers. The occurrence of ESBL producing bacteria in food producing animals has a significant impact on public health. Most commonly detected enzymes are bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$. The ampC producing bacteria are also of high importance. Bacteria producing AmpC enzymes are not inhibited by clavulanic acid. Such resistance phenotype may occur in strains with the mutation in the promoter region of the chromosomal gene, or it exerts the resistance effect over plasmid encoded enzymes. The most common gene encoding the AmpC enzyme from the plasmid is bla_{CMY}. ESBL resistance genes are found on plasmids and could be disseminated in nature by means of horizontal gene transfer. These genes are often found in multi drug resistant bacteria and because of that, ESBL producing bacteria present a worldwide problem in human and veterinary medicine. In this work, a distribution of some important ESBL producing bacteria in food animals is briefly presented. The major reservoirs of ESBL producing bacteria in the livestock industry are pigs and cattle. However, the occurrence of resistance varies in countries from the EU and non member states. In each case, the practice of using antibiotics reflects the presence of resistant genes in the environment. A prudent use of antibiotics is required in order to lower, as much as possible, the occurrence of resistance mechanisms that have a high range of transferability among bacteria species, especially for antibiotics that are used in human medicine.

Key words: *Enterobacteriaceae, resistance, ESBL, food, animals*

INTRODUCTION

Cephalosporins are antimicrobial agents which are in addition to fluoroquinolones often used to treat different bacterial infections. They represent medication which is used for treating respiratory infections in humans (cephalothin, cephalexin, cefuroxime), while in animals, apart from respiratory infections, they are used for treating mastitis-metritis-agalactia syndrome in sows, exudative epidermidis as well as different types of meningitis (ceftiofur) (Cavaco et al. 2008). So far, four generations of cephalosporins have been described. The first generation was used exclusively for the treatment of infections caused by Gram positive bacteria, while the II generation of cephalosporins was used to treat infections of Gram negative bacteria. Cephalosporins III and IV generation have a wide range of treatments (Xian-Zhi Li et al. 2007). Their uncontrolled use has led to the creation of ESBL (extended-spectrum β-lactamases) strains, or bacteria which produce β-lactamases (Cavaco et al. 2008).

The bacterial resistance from the Enterobacteriacae family, to cephalosporins III and IV generation represents a serious problem in the world (Pfeifer et al. 2010). Carbapenems are used for the treatment of infections caused by these bacteria. However, there are strains which are also resistant to carbapenems (Rupp and Mark Paul Fey 2003). A major threat for mankind is the existence of bacteria strains that carry genes for the resistance to cephalosporins because the presence of these bacteria in animals can be found in the human food chain (Cavaco et al. 2008). ESBL producers are distributed worldwide, and its prevalence has been increasing significantly. It is believed that 10–40% of *E. coli* strains and *Klebisella pneumoniae* are producing ESBL (Paul Mark Rupp and Fey 2003).

THE BIOLOGICAL CHARACTERISTICS OF ESBL PRODUCERS

Cephalosporins belong to the β-lactam antibiotic group, and its basic structure is composed of 7-aminocephalosporanic acids. The aminocephalosporanic acid base is composed of the β-lactam ring, which is essential for the antibacterial effect of cephalosporins. Its bactericidal activity inhibits the synthesis of the cell wall building element – peptidoglycan (Jezdimirović 2005).

Eextended-spectrum β-lactamases (ESBL) are bacteria which produce the enzyme β-lactamase. This enzyme has the ability to hydrolyze the β-lactam ring and thereby prevent the effect of cephalosporins (Pitout and Laupland 2008). The largest numbers of ESBL producers are found in clinical isolates of human origin, and that of patients who were treated with cephalosporins (Xian-Zhi Li et al. 2007). Recently, more ESBL producers have been found in animals. In addition to their therapeutic use in animals, cephalosporins are also being used for prophylactic purposes (Cavaco et al.

2008, Jorgensen et al. 2007). This way, animals become reservoirs of ESBL producing bacteria allowing their entry into the food chain for humans. The most common bacteria that have the ESBL producer role in humans and animals are from the Enterobacteriacae family, and they include *Escherichia coli*, *Klebsiella* spp. (Pitout Laupland and 2008), and *Salmonella* spp. (Xian-Zhi Li et al. 2007).

The ESBL producers, depending on the enzymes they develop, are divided into three major groups: TEM, SHV and CTX-M (Cavaco et al. 2008). There are two types of TEM enzymes encoded by chromosomes that are responsible for the resistance of *Escherichia coli* to penicillins and I generation cephalosporins, the TEM-1 β-lactamase and TEM-2 β-lactamase. TEM-2 β-lactamase, differs from TEM-1 in the substitution of lysine with glutamine at position 39. The SHV β-lactamase, as well as the TEM is responsible for the resistance to penicillins and I generation cephalosporins, but in contrast to TEM, it can be encoded from the plasmid. *Klebsiella pneumoniae* is one of the producers of this enzyme (Rupp and Mark Paul Fey 2003). Until now, the most described were the ESBL producers, with the CTX-M enzymes, because they are the most numerous in the world (Cavaco et al. 2008).

Bacteria from the Enterobacteriacae family (*Escherichia coli*, *Citrobacter* spp., *Enterobacter* spp.), in addition to ESBL, can also create AmpC β-lactamase, which is usually encoded from the chromosome or may be encoded from plasmids. AmpC β-lactamase leads to the hydrolysis of the β-lactam ring, but unlike ESBL, it is not inhibited by clavulanic acid (Mark Rupp and Paul Fey 2003). Such resistance occurs in strains with mutations on the gene promoter, on a chromosome, or it can occur with a plasmid encoded gene. The most frequent gene encoding the enzyme AmpC β-lactamase production from plasmid is bla_{CMY}.

THE GEOGRAPHICAL DISTRIBUTION OF THE ESBL PRODUCER (extended spectrum beta-lactam producing bacteria)

The geographical distribution of ESBL producers in the world has changed significantly over the last decades. The results obtained by Kanton and Coque from 2006 show that the *Klebsiella pneumoniae* was the most common ESBL producer during the 1990s, and TEM and SHV enzymes were synthesizes most frequently. After the year 2000, *E. coli* became the most important ESBL producer, and the group of enzymes CTX-M – the most common. There are many types of CTX-M enzymes. The most frequent among them are CTX-M1, CTX-M2, CTX-M8, CTX-M9, CTX-14 and CTX-M25 (Cavaco et al. 2008).

The distribution of CTX-M enzymes is different in various parts of the world. The enzyme CTX-M1 is the most common in Italy (Canton Coque and 2006, Pitout and Laupland 2008), CTX-M2 in most South

American countries, Japan and Israel, while CTX and CTX-M9-M14 are most common in Spain, the UK and China (Canton Coque and 2006).

ESBL PRODUCER RESERVOIRS IN THE LIVESTOCK INDUSTRY

An uncontrolled application of cephalosporin has led to the emergence of ESBL strains in the livestock production. Pigs and cattle are the main reservoirs of ESBL producers among the livestock (Tamang et al. 2013, Meunier et al. 2006). The big problem is that products of these animals may be found on peoples plates. In addition to this, farmers are directly exposed to ESBL producers due to close contact with animals. Some researchers state that there are also ESBL producers in pets (Dierikx et al. 2012).

Tang et al. 2008 conducted a study on 22 cow farms in South Korea. Sampling was done on 1536 samples of cow milk, cow feces, farm environmental swabs, nasal and hand swabs from farmers. From 84 of the samples, *E. coli* was isolated as the ESBL producer. Resistance to ampicillin, cephalothin and cefotaxime was found in all of the *E. coli*. The presence of the $bla_{CTX\text{-}M}$ and bla_{TEM} gene was confirmed, while the presence of the gene bla_{SHV} was not confirmed. The most prevalent were the $bla_{CTX\text{-}M14}$ and $bla_{CTX\text{-}M32}$ from samples of feces, milk and farmer's hands. The transfer of plasmid from the $bla_{CTX\text{-}M}$ gene in 60 isolates of *E. coli* to the recipient was confirmed by conjugation. According to the Meunier et all 2006 results, *E. coli* resistant to ceftiofur was isolated in France from pigs and cattle with the production of CTX and CTX-M1-M15 enzymes. Genes, responsible for the production of these enzymes $bla_{CTX\text{-}M1}$ and $bla_{CTX\text{-}M15,}$ were detected on plasmids. In the Netherlands, in addition to pigs and cattle, pets and horses may also be the reservoirs of ESBL producers. The most common genes encoding resistance are $bla_{CTX\text{-}M1}$, $bla_{CTX\text{-}M14}$, $bla_{CTX\text{-}M2}$ and $bla_{TEM\text{-}52}$. The same uropathogenic *E. coli* clone was found in humans in hospitals. The transfer of ESBL producers from pets to their owners is becoming more common (Dierikx et al. 2012). On two pig farms in Denmark, in 2005, *E. coli* resistant to ceftiofur and positive for $bla_{CTX\text{-}M1}$ gene was isolated, which represents the first occurrence of an ESBL producer in the primary production of food for humans. The origin of these genes is not known. Aarestrup et al. 2006, confirmed that pigs present a major reservoir of ESBL producers in Denmark. The problem arises with the expansion of these genes in animals used for food production, which may result in unsuccessful treatment of various infections in humans. Hammerum et al. 2014 confirmed that pigs are reservoirs of ESBL producers for people in Denmark, as well as that genes are distributed substantially. Their study has confirmed the presence of *E. coli* with $bla_{CTX\text{-}M1,}$ $bla_{CTX\text{-}M14}$ and $bla_{SHV\text{-}12}$ genes in 79% of the surveyed farms with the use of cephalosporins III and IV generations. These genes were detected in

farmers as well. The transfer of ESBL genes between pigs and farmers was enabled by plasmids (Jorgensen et al. 2007).

CONCLUSION

Bacterial resistance to cephalosporins represents a growing clinical and economical problem both in human and in veterinary medicine. Therefore it is becoming an increasing challenge for clinical microbiologists, medical doctors and veterinarians. To this day pigs and cattle are the main reservoirs of ESBL producers in the primary production of food, although lately there have been more reported cases of pets as reservoirs of ESBL producers. Food, originating from animals which are raised for human consumption, ends up on people's plates, thus threatening their health. The biggest problem is that genes responsible for the resistance to cephalosporins are on plasmids of multiple resistant bacteria. They are transferred from one bacteria to another through plasmids. This way, the horizontal spread of these genes to the environment is enabled. The use of antibiotics should be rational. Choosing antibiotics for animals should be smart and reasonable so we do not end up having a problem with treating human infections. If we comply with these principals, we can have a significant impact on the reduction of antimicrobial resistance, not only to cephalosporins, but also to many other classes of antimicrobial drugs.

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TETRACYCLINE RESISTANCE IN *ESCHERICHIA COLI* ISOLATES FROM POULTRY

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Abstract: The objectives of the present paper arethe analysis and comparison of the results from available literature regarding the prevalence of tetracycline resistance in *Escherichia coli* strains isolated from poultry in different countries. Tetracycline is still one of the most commonly used antibiotic in many developing countries both in human and in veterinary medicine. The main reasons are its relatively low price and availability. Besides that, this class of antibiotics is still used in developed countries. The widespread use of tetracycline in poultry farming could result in horizontal transfer of resistance determinates from poultry to humans as well as to the environment. *Escherichia coli*, ascommensal bacteria both in human and poultry digestive systems, is one of the most important reservoirs of antibiotic resistance and has a significant role in the transfer of various resistance determinants. Some strains of *Escherichia coli* are highly pathogenic and can cause several diseases in poultry which require antibiotic therapy. Positive correlation between the usage of antibiotics both in human and in veterinary medicine and the corresponding antibiotic resistance were reported by many authours. Furthermore, there is also some evidence that the positive correlations were also found between the usage of antibiotics in veterinary medicine and the appereance of antibiotic resistance in bacteia isolated from humans. The need for proper regulation of the usage of tetracycline in poultry production and control measurements are doubtless, as well es permanet monitoring of the presence of the tetracycline resistance.

Key words: *Escherichia coli, poultry, resistance, tetracycline*

HATCHERY STATION LIKE POSIBILITY SOURCE OF ENVIRONMENTAL CONTAMINATION WITH BACTERIA FROM GENUS SALMONELLA

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Abstract: Sallmonelae were two species of those bacteria: *Salmonella enterica* and *Salmonella bongori.* From base of matter of „O" soamtic, H flagelar and Vi capsular antigene Salmonella species were clasificated in serovars. With Kaufmann-Wajt scheme today were recognized moret than 2400 Salmonella serovars. Salmonella are Gram negative bacterial, bacilar type or cocodid shape. They are aerob and faculty anaerob and belonging to familly *Enterobacteriacea.*

Salmonellla in poultry presented one of main causality of human infections via food (meat, eggs, its products) ar by environmental contamination with garbage rom hatchery station or from poultry farms. Hetchery stations like close systempresented ideal sources to reproduction numerous microorganisms including Salmonella. Those induced permanent risk from environmental contamination and potencial health hazard from human and animals.

Material to bacteriological examination were swabs which used from critical point at hetchery stations and embryoned eggs. At total we examined 50 swabs and 450 eggs. Methods of examination were standard microbiological methods to isolation and identification of microorganism.

Results which we obtained show that was necesseilly intensify health control in all hetchery stations and innovate procedure to pretection of biohzard caused by waste product contained during hetchery process.

In order to establish adequate waste management practices, it is necessary to issue appropriate and targeted management and feasibility studies, first at the micro (farm, incubator) and then at the local, regional and national level. In this regard, it should be considered/developed and principally considered adequate safe and economically viable strategy with options of treatment, recycling, transportation and final disposal. The study should include the definition of technical guidelines for the improvement of safe and environmentally sound waste management with step-by-step approach to defining and implementing appropriate management practices. It must also be emphasized the importance of education and training

systems of waste management, not only to operators who manage waste, but also the wider community.

Key words: *Salmonella spp., hatchery station, environment*

INTRODUCTION

Incubator is of particular importance in the technology supply chain of livestock. We have to emphasize its place in the health care of livestock. It can be the center from where they spread many infectious diseases and so must be separated and far enough away from other objects used in poultry. The location to which is to be well insulated or near good roads, water and electricity networks.

The basic rule of incubator station is that eggs enter from one side and out the other chickens. Within the hatchery must not be crossed paths technological eggs, chicks and equipment, nor be mixed workers. The layout of the hatchery is such that logically follows the direction of the technological process. It must ensure even temperature and ventilation. Height of the premises must be at least one meter higher than the height of the equipment (8). Ventilation should be adjusted so that the air pressure is higher at the beginning of the technological process and the lowest at the end of which prevents the movement of air from „dirty" to „clean" part (9)

Incubator must be so constructed that it can be thoroughly cleaned and disinfected. All employees must go through disinfection barriers, take a shower and change and movement within the hatchery shall be limited to the work process, to prevent mixing of people and hence the possibility of transmission of infection. Incubator waste (shells, non-hatch eggs, non-vital chickens, cocks, fluff, etc.) Poses a constant threat, as a source of infection, but must be regularly remove harmless (4). Ecological importance of hatchery as potential contaminants of the environment became current in parallel with their development and increasing production capacity (7, 12).

Microclimate in the hatchery is an ideal environment for the growth and reproduction of various microorganisms including Salmonella, E. coli and other pathogenic bacteria during the incubation of eggs (5, 6). For these reasons, it is necessary to develop methods of safe disposal and proper rolling residues which are directly proportional to hatchery capacity. Our study was aimed to determine the presence of pathogenic microorganisms in the hatchery and embryonated eggs that hatch and the potential risk of environmental contamination bacteriological agents originating from the hatchery (8, 14).

MATERIAL AND METHODS

Material for bacteriological testing swabs was taken from the hatchery to the critical areas and breeding eggs which are provided for placing the incubation process in stages. In total, 50 swabs from the hatchery and 450 breeding eggs. The paper used the standard and accredited methods have been subjected to isolation and identification of salmonella and other microorganisms (1, 3). It should be noted that during the diagnostic procedure Salmonella, including mandatory and reference material, such as in our study, the reference culture ATCC 13076 *Salmonella enteritidis* and *typhimurium* ATCC 14028 – Salmonella manufacturer microbiologists, USA. Pored salmonella bacteria species we identify and present other bacteria such as staphylococci, streptococci, micrococci, Bacillus, Escherichia, pseudomonas and yeast.

RESULTS

Following bacterial flora of hatching eggs, at all stages of production, we found significant contamination by bacteria.

Depending on the length of standing egg comes an increase in contamination of deep inner layers, especially membranes, and then the egg whites. Shell eggs taken from nests in most cases contaminated with *Bacillus sp*. a total of 28 pieces, representing 80.0% of total inspected eggs. Shell eggs were contaminated with Micrococcus sp. In 12 cases, which amounts to 34.28%. *Salmonellae* were isolated in 4 swabs from the shell, and it is 11.4%. *E.coli* were isolated in 6 cases the shell and the membrane 6, which amounts to 34.4%. *Staphylococcus sp.* was detected in 2 cases, and it is 5.7%. *Streptococcus sp* was detected in 3 cases, and it is 8.5%. *Aspergillus sp*. is defined in the shell 2 of the case, and it amounts to 8.5%. Samples of egg white and yolk, chicken eggs out of the nest, were bacteriologically negative. Of the total number of examined hatching chicken eggs (35 pieces) transactions carried out fumigation, the shell were isolated in 34.2% of cases, *Bacillus sp., Micrococcus* in 25.7%, 11.4% in *Staphylococcus, egg* membranes (inner) and egg white the yolk after fumigation bacteriological sterile.

Two days after standing in the warehouse for eggs is the most frequent contamination of shell eggs with *Bacillus sp*. 48.5%. Then he detected *Staphylococcus* also on the shell of a chicken egg which amounts to 11.4%. *Micrococcus* is isolated in 5.7% also on the egg shell. Here are the eye of bacteriological tests membrane, egg white and yolk bacteriological negative.

Bacteriological examination of eggs due after 10 days in storage for eggs from 35 surveyed fertilized eggs in one shell, we established salmonella amounted to 2.8%, and we are here for the first time, isolate *Salmonella* in two cases with a membrane that is 5.7%. With shell we have

isolated *Micrococcus sp.* in 5 cases (14.3%), with a membrane in 4 cases (11.4%), *Bacillus sp.* was isolated in 15 shells (42.8%). Egg white and yolk were bacteriologically negative.

The seventh day of incubation shell contamination expressed in percentages was: 2.86% *Salmonella, E.coli* 5.71%, 25.7% *Staphylococcus, Bacillus sp*. 51.4%. The membrane was in the case 1 and contaminated with *Staphylococcus, Micrococcus,* and *Bacillus sp*., in 9 cases. Egg white and yolk were bacteriologically negative. Bacteriological examination of unfertilized chicken eggs in the seventh day of incubation, the highest number of contaminated eggs was with *Bacillu sp.* in 16 cases, and it is 45.7%, followed by *Staphylococcus sp*. in 4 cases, and it is 11.4%, and at the end of one shell is contaminated with *Micorococcus sp*. and it is 2.8%. Egg white and yolk were bacteriologically negative.

Bacteriological examination of fertilized eggs in the 18-day incubation period established that shell of eggs was contaminated with *Staphylococcus sp.* in 9 cases, which amounts to 25.71%. In 2 cases also with the same microorganisms contaminated the membrane, and it is 5.7%. *Bacillus sp.* we found the shell eggs in 6 cases, which amounts to 17.14%, and 2 cases on the membrane, it was 5.7%. *Pseudomonas sp.* was detected in 3 cases the shell eggs (8.5%) and 2 of the membrane, ie. 5.7%. *E.coli* is contaminated shell eggs in 3 cases, and 2 on the membrane, ie in the deeper layers. in the yolk was detected in 3 cases. Salmonella are here detected in 2 cases in the deeper layers of the egg that is u yolk, and it was 5.7%. *Aspergillus sp.* was not detected in this case on the shell, the membrane, and the albumen of the egg yolk.

In 21-day incubation period, as seen in the shell egg is commonly contaminated with *Staphylococcus sp.* in 10 cases, which amounts to 28.5%, in 4 cases the membrane of fertilized eggs were contaminated with the same microorganism which amounts to 11.43%. This is followed by contamination with *Bacillus sp.* in the 9 cases of the shell which is 25.71% and 2 cases of the membrane (5.71%). *Escherichia coli* were detected in 2 of the shell, the membrane 2 and in the 2 yolk which is 5.71%. Microorganisms *Pseudomonas* species we found 4 shell, on a single membrane, and the yolk in 4 cases. *Salmonella* here we find the yolk in two cases, egg whites here any bacteriological negative. *Micrococcus* is detected on one shell.

DISCUSSION

The corpses of cattle, embryos, non-vital chickens are a significant source of environmental pollution, some of which are first observed air pollution (odor), but also an important source of the disease, either directly or through vectors that attracts. Application for composting solution to the problem of safe disposal of corpses, there are more advantages. The process is inexpensive, applicable throughout the year, environmentally friendly

and suitable for solving problems in the mass deaths of poultry. This procedure prevents the spread of the disease (destruction of pathogenic microorganisms, the influence of temperature that develops in the compost mass (55–65^{o}C during the first one or two days, and then to 60–70^{o}C in the next 7 to 10 days) and the pollution of air, water and land.

Organic waste from the so-called incubator incubator waste consists of unfertilized eggs, shell and membrane eggs and dead (or suppressed) chickens. Traditional methods of destroying this waste include disposal in a landfill, the use of agricultural land as fertilizer, incineration and processing into animal feed (8) found that the incubation composting waste suitable and environmentally friendly way for their destruction. This procedure gets a compost of good quality, especially if the mass of composting by mixing waste from the incubator and manure from the facility for growing broiler hybrids. This way of composting destroys the 99.99% of Escherichia coli as well as a significant percentage of Salmonella.

During our tests for the presence of bacteria on the shell eggs taken directly out of the nest, we found a significant percentage of contamination of eggs and to different types of bacteria, which ranged 85.7%. Dominating the microorganism is detected after microbiological testing is a genus *Bacillus, Micrococcus and then Enterobacteriaceae*. Similar results have come by Ilić (6), Mavwer et al. (10) and Snoeyenbos and Williams (13). Since pathogenic bacteria were detected Salmonella in 11.4% percent of the egg shell. The internal parts of fresh hatching eggs are not formed by bacteria. The results that have come and Harry and Hemsly (4) and Ilić (6).

Comparing our results with the results of foreign and domestic authors (2, 6, 8, 9, 10, 11, 13), we can conclude that they find similar bacterial flora. During production, gassing the parent farms and hatcheries before investing formalin vapors of hatching eggs, is a very effective way of disinfecting and compulsory and unavoidable procedure. These results are important given that biological waste from the so-called incubator waste consists of unfertilized eggs, shell and membrane eggs and dead (or suppressed) chickens. Traditional methods of destroying this waste include disposal in a landfill, the use of agricultural land as fertilizer, incineration and processing into animal feed (8, 14).

CONCLUSION

Based on these results, we concluded that it is necessary to strengthen health surveillance in all hatchery. It is also necessary to introduce a procedure that would enable to reduce pollution biohazardous waste generated in the process of incubation eggs as the rehabilitation of the existing situation in order to preserve and protect the environment.

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BRACHYSPIRA SPP IDENTIFIED IN GROWING PIGS IN SERBIA

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Abstract: Different *Brachyspira (B)* species may be present in growing pigs, where *B. hyodysenteriae* and *B. pilosicoli* are considered the most pathogenic species, producing swine dysentery (SD) and porcine intestinal spirochetosis (PIS) respectively. Others types of *Brachyspira* species that were detected in pigs such as *B. intermedia, B. innocens, and B. murdochii*, are considered as no pathogenic. Nevertheless, some investigations have indicated that they may produce several degrees of colitis and cause productive losses. Therefore, the aim of this study was to identify the species of *Brachyspira* in growing pigs of representative farms in Serbia. In total 42 samples were tested, originated from 14 farms, *spirocheates* isolates were cultivated from the feces and/or caecal contents and tested by the *nox* gene PCR-RFLP analysis. *Spirocheates* were found in the feces and/or caecal contents of pigs from 12 farms. The *B. hyodysenteriae* was identified on ten farms and the *B. pilosicoli* on one farm. Co-infection of *B. hyodysenteriae* and *B. intermedia* was identified on two farms, and a co-infection of *B. hyodysenteriae* and *B. pilosicoli* on one farm. A mixed infection by *B. hyodysenteriae* and *B. innocens* was identified on four farms, and a combination of *B. hyodysenteriae, B. innocens* and *B. murdochii* was found in two farms. On one farm is detected only the presence of *B. innocens* and *B. murdochii*. Different species of *Brachyspira* were identified in growing pigs of Serbia whereby the most frequently isolated highly pathogenic *B. hyodysenteriae*. Concurrently, other two *Brachyspira* species *B. innocens* and *B. murdochii* which are considered nonpathogenic in pigs are widely spread in the local pig population. Beside highly pathogenic species *B. hyodisenterie* and *B. pilosicoli* causing swine dysentery and porcine intestinal spirochetosis, the importance of infection in pigs with „nonpathogenic" *Brachyspira* species should be evaluated in further study since it has been shown that the *B. intermedia* may cause colitis and diarrhea in pigs and *B. murdochii* and *B. innocens* either alone or with other pathogens may have a certain degree of pathogenecity and produce mild clinical or subclinical presentation.

Keywords: *pigs, farms, spirocheates identification, Serbia*

THE HARDERIAN GLAND – IMPORTANT IMMUNE ORGAN IN POULTRY

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Abstract: The Harderian gland is located ventromedial in the orbita of the domestic fowl. It belongs to the tubulo-alveolar glands whose excretory duct opens into the conjunctival sack in the third eyelid. Considering the fowl this gland has two basic functions: production of secretion that lubricates the third eyelid and protects the oculonasally region against external pathogens. Being an organ that is immunocompetent in local immune response, it holds a major role in vaccination against certain diseases where vaccines are administered eye-drop or aerosol method. One of these diseases is infectious bronchitis of poltry. Infectious bronchitis is an acute contagious livestock respiratory disease of viral etiology of all poultry ages which is clinically manifested by catarrhal bronchitis, high mortality in chickens and disorders of the reproductive organs in laying hens. The cause of the disease is a virus from the family Adenoviridae, genus Coronavirus. The application of live vaccines against infectious bronchitis by aerosol method leads to an increase in the number of plasma cells in Harderian gland and histopathological changes in it. These changes indicate that the vaccine virus directly stimulates immunocompetent cells in Harderian gland. For this examination we vaccinated 50 day-old broiler chickens with a vaccine containing live attenuated strain H120 spray method. The amount of antibody titers were followed by ELISA assa and the changes in the structure Harderian gland were followed histologically.

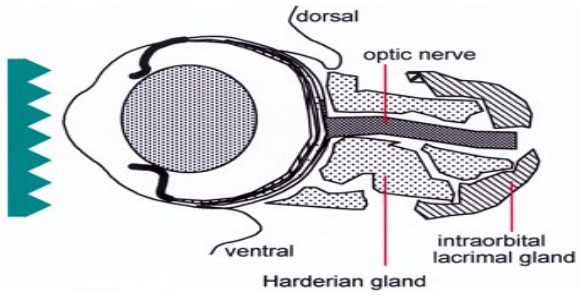
Key words: *Harderian gland, infectious bronchitis*

INTRODUCTION

The first information about Harderian gland (HG) date from 1694, when it was described by Johann Jacob Harder while opserving it on a deer and he described it as a large gland that serves to moisten the surface of the eye and named it „new glandula lacrimalis". This gland is called the enigmatic orbital gland which structure and function is still investigated.

It is located in most terrestrial vertebrates, amphibians, reptiles, birds and mammals and it is not present fish, bats, cows, horses, carnivores and

primates. Functions of the gland are diverse depending on the species and it represents among other things a place of immune response, photo-protective organ, part of the retino-pineal part, source of pheromones, a source of lipids thermoregulatory (Payne 1994). It is located ventromedial in the orbita and very loosely attached to the periorbital fascia below the muscle (Scheme 1).



Scheme 1. Harderian gland (www.getdomainvids.com)

It is tounge shapped in chickens (Walcott et al. 1989) and Shirama (1996) her medial surface is convex and concave lateral. Outside of the gland is surrounded by a thin connective-tissue capsule that has outcrops and that divides it into unequal lobules. It histologicaly belongs to complicated tubular or tubulo-alveolar glands (Wight et al. 1971, Burns 1976) whose excretory duct is morphologically visible after leaving the gland and it opens in a conjunctival bag on the surface of the third eyelid. Interstitial tissue of the Harderian gland is abundant in plasma cells that secretes immunoglobulins, and based on this, the authors consider it as a component of the local immune system in which it plays an important role in protecting the eyes-nasal region of different pathogens (Ohshima and Hiramatsu 2002). The occurrence of a large number of plasma cells in the interstitial HG was first noticed by Bang and Bang (1968) as well as the accumulation of cells organized into follicles with germinal centers. These cellular elements in the opinion of many authors (Miller et al. 1971, Glick 1978, Kowalski et al. 1978) are bursa-depend. Based on research by Mueller et al. (1971) and Burns (1976) bird HG represents bursa – dependent population of plasma cells that produce antibodies against foreign particles and soluble antigens. The application of the antigen in the conjunctive in addition to stimulating immune response within the gland, as written by Mueller et al. (1971), causes proliferation of germinative center, particularly in applications of vaccine infectious bronchitis virus (Devalaar and Kouwenhoven 1976).

These findings have been widely used in the control of infectious bronchitis virus where Harderian gland as highlighted in a local production of antibodies for protection of oculonasally mucosa. Aerosol or/and eye

drop vaccination to protect against infectious bronchitis virus (IB) is now practiced all over the world (Survashe 1979).

Infectious bronchitis is an acute contagious respiratory disease of viral etiology of all ages that is clinically manifested by catarrhal bronchitis, high mortality in chickens and disorders of the reproductive organs in laying hens. The incubation period is short and can be as high as 24–72 hours.

The disease usually lasts less than two weeks with a mortality rate below 30% (Engström et al. 2003). In some cases the disease appears latently (Ignjatovic and Sapats 2000). The disease is widespreaded in all countries with developed poultry and is of great economic importance (Cavanagh and Naqi 2003).

The aim of examination in this paperis focused on establishinf a detailed insight into the role of Harderian gland and the level of immune response in broiler chickens following vaccination against infectious bronchitis.

MATERIALS AND METHODS

For the research we used day old broiler chickens originating from broiler breeders hybrid Hybro PG. The experiment was performed on 100 broiler chickens that were divided into two groups: O1 and K, with 50 individuals in the group. The chickens were placed in separate boxes and fed *ad libitum.*

One day old chicks in experimental group O1 were vaccinated against infectious bronchitis with the vaccine Bronhivet I containing live attenuated strain H120. The vaccine was applied using spray method. The experimental group K was not vaccinated and served as control. The experiment lasted 21 days and during the experiment, daily clinical observation of chickens were carried out. In order to control the immune response of the antigen that was applied we have taken the blood of chickens on first, seventh, fourteenth and twenty-first day of age. Separated sera were inactivated at 56ºC (30 min.) and then tested by ELISA test. Bursa of Fabricius samples for histopathology were fixed in 10% buffered formalin, processed by standard protocol and cast into paraffin blocks. Paraffin 5 µm thin slices were dyed with hematoxylin-eosin (HE).

The results were grouped in the appropriate statistical series and analyzed with the use of several mathematical and statistical methods using Anova program, Stat View, MS Excel 97 and Sigma Plot 4.0.

RESULTS AND DISCUSSION

The results of titre levels of antibodies to infectious bronchitis disease in broiler chickens in the experimental groups are given in Tables 1 and 2.

Table 1. The level of antibody titers in the control group

K control group			
day of age			
1	*7*	*14*	*21*
2567	*1995*	*1054*	*180*
2713	*1256*	*894*	*342*
3257	*1011*	*968*	*1*
1939	*3103*	*970*	*1*
2480	*705*	*1200*	*200*
4566	*664*	*870*	*152*
3404	*1218*	*709*	*1*
5152	*2674*	*850*	*1*
2658	*980*	*704*	*135*
5231	*696*	*902*	*220*

Table 2. The level of antibody titers in the O1 group

O1 experimental group			
day of age			
1	7	14	21
2250	2579	9572	8625
3475	2845	6529	6675
2115	948	5470	4892
1863	1701	5266	6742
4567	1358	5518	4124
5340	1447	4675	5150
2650	2429	5518	6980
3841	1015	8707	6159
4102	2141	5518	5785
3150	1349	12517	7590

Based on the titre levels in experimental group O1 and control group K in one day old chicks a middle value and standard deviation were calculated and on these basis we can notice that the values are uniformed in both groups (Table 3).

Table 3. Middle value and standard deviation of the first day of age in the experimental groups K and O1

	K	O1
Xsr	3396,7	3335,3
SD	1178,5	1142,0

Xsr – middle value, SD – standard deviation

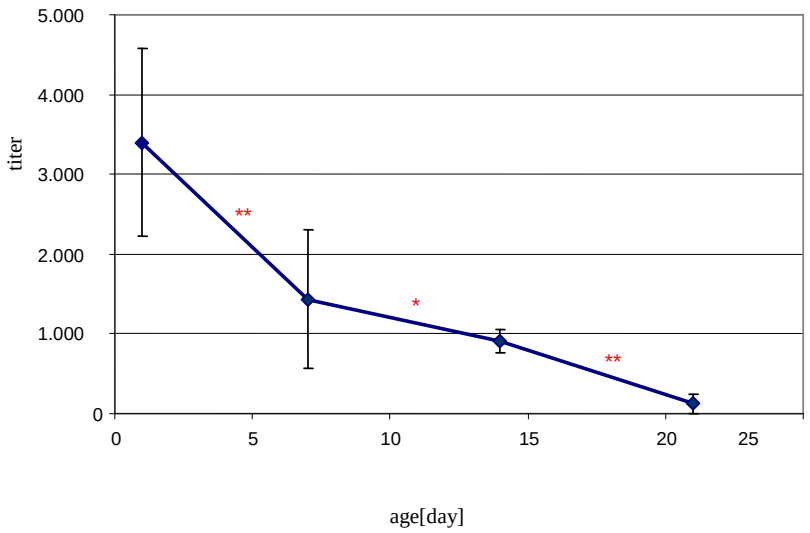
Based on the level of antibody titers we calculated the middle value and standard deviation of the control group (K) according to the days of age (Table 4).

Table 4. Middle value and standard deviation of the control group K according days of age

	age chickens (day)			
	1	7	14	21
Xsr	3396,7	1430,2	912,1	123,3
SD	1178,5	867,3	149,1	118,8

Xsr – middle value, SD – standard deviation

Obtained results are presented graphicall (Graph 1).



Graph 1. The titer of IBV antibodies in the control group over time

The results show that the highest levels of antibody titers were measured on the first day 0and and that they are maternal antibodies. From 1–7 days a decrease in titer was observed which progressively decreases to 21 days of age. Considering that the control group was not vaccinated due to maternal antibody seroconversion time, a decline in antibody title was manifested over time. Using T-test a comparison was performed in mutually series of age and the following results were obtained (Table 5).

Table 5. Values T-test in each series being compared to the age of control group

	between 1–7 days of ages	between 7–14 days of ages	between 14–21 days of ages
p value	0,000286	0,046857315	$1{,}19 \cdot 10^{-10}$
statistical difference	very significant	significant	very significant
mark on the chart	**	*	**

Context: for p > 0,05, no difference
p < 0,05, stattistically significant difference*
p < 0,01, stat.istically very significan difference**

The level of maternal antibodies over time decreases, in research by Darbyshire and Peters (1985). They found that the level of maternal antibodies decline linearly, at the half 5–6 of the day. Davelaar and

Kouwenhoven (1977) in their studies in unvaccinated chickens found high antibody titer after hatching falling to zero in 30-day-old chickens. In our study antibody titers fell by half in 7 days and by the 21 amounted to zero.

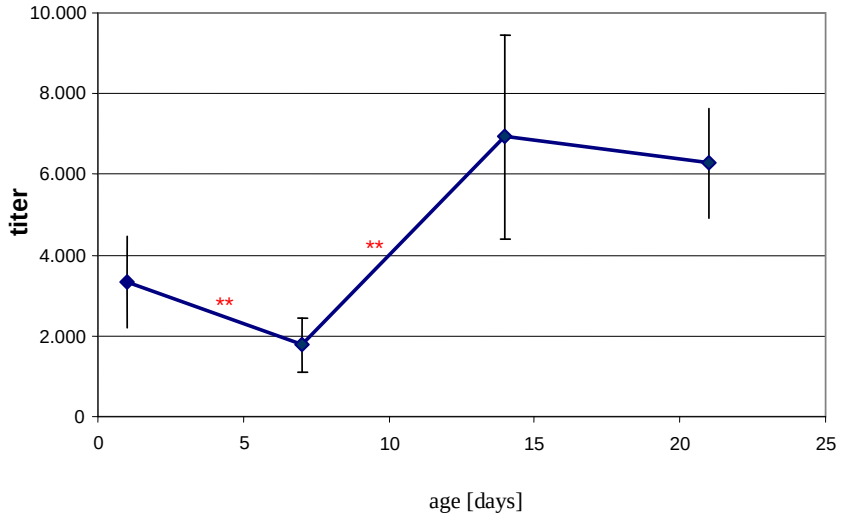
Based on the amount of antibody titer in experimental group O1 we calculated middle values and standard deviations by days of age, which are shown in Table 6.

Table 6. Middle value and standard deviation in experimental group O1

	age chickens (day)			
	1	7	14	21
Xsr	3335,3	1781,2	6929,0	6272,2
SD	1142,0	673,5	2527,2	1341,0

Xsr – middle value, SD – standard deviation

The obtained results were determined graphically (Graph 2).



Graph 2. The titer of IBV antibodies in experimental group O1

The results show that the presence of maternal antibodies decline in 1–7 days of age, then the titer significantly increases and reaches a maximum of 14 days. From 14–21 day no statistical differences in values titers were found. Falling titer of 1–7 days is due to maternal antibody seroconversion, a fact that was carried out in vaccination against IBV on the first day is a logical increase in the age of 7–14. days of age. The difference is not significant in titers between 14–21. of the da A significant increase in antibody titer is the result of immunization against IBV. T-test following results were obtained (Table 7).

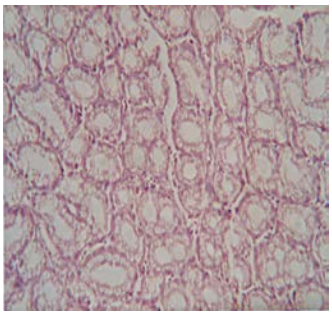
Table 7. Values of T-test in comparison to age experimental group O1

	between 1–7. day of age	between 7–14. day of age	between 14–21. day of age
p value	0,001101	$4{,}37 \cdot 10^{-5}$	0,24
statistical difference	very significant	very significant	insignificant
mark on the chart	**	**	

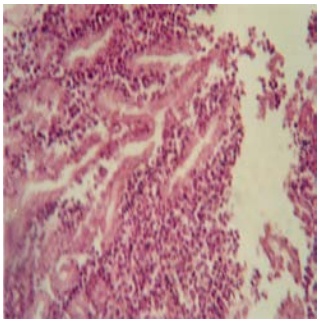
The values of T-test showed statistically significant difference in the amount of IBV antibodies from 1–7 days and 7–14. Between 14 and 21 days statistical difference was insignificant. We notice a significant decrease in the titre of 1–7 day old chicks. The decline of maternal antibodies is explained by seroconversion and the necessity of vaccination on the first day old chicks was explained in works of Davelaar and Kouwenhoven (1981). Conjunctival tissue reacts like a filter to remove particles of virulent (Davelaar Kouwenhoven 1980b). A significant increase in antibody titer was observed in the age of 7–14 days where it reached 14 days peak. Increase in antibody titers in serum two weeks after vaccination against IBV day-old chicks was noticed by Davelaar (1982) and Ignjatovic and Galli (1995) which is consistent with the results we obtained in our study.

Histological examination of the Harderian gland in one-day old chickens showed that the gland wrapped in a thin connective-tissue capsule that provides fibers and parts of the unequal lobules.

Acini occupy cortical part of gland and medullary part consists of tubules that drain into the central channel. Lobules are lined with single-layer epithelium that seamlessly connects with the secondary tubules (Photography 1).

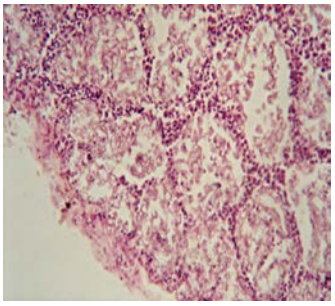


Photography 1. Harderian gland in chickens aged one day with the experimental groups. K and O1. Lobules and ducts H & E, X100.



Photography 2. Harderian gland in fourteenth day of age in the experimental group O1. A large number of lymphocytes and plasma cells fulfills the gland, poor plasma cell destruction H & E, X400

In the experimental group O1 in fourteenth day of age HG is filled with a large number of lymphocytes and plasma cells. We notice a mild destruction of plasma cells, and in the secretory canals excretion of cell detritus. (Photography 2).



Photography 3. Harderian gland twenty-first day of age in the sample group O1.A large number of plasma cells, tubular lumen filled with secretion and detritus epithelium. Present destruction of plasma cells. H & E, X400

On the twenty-first day of age a large number of plasma cells was transferred from the glandular epithelium to the lumen of the tubules, which is filled with discharge. There is destruction of plasma cells and epithelial detritus in the secretory channels (Photography 3).

CONCLUSION

Application of the vaccine against Infectious Bronchitis in one-day broiler chickens stimulates the Harder gland to secrete specific antibodies whose level in two weeks is detected systemic.

Harder gland is involved in the local immune response to the applied vaccine by increasing the number of plasma cells and

immunoglobulinsecretion, but also in partial destruction due to multiplying of vaccine virus.

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THE SIGNIFICANCE OF COCCIDIOSIS IN MODERN FATTENING OF BROILER CHICKENS

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Abstract: Coccidiosis is one of the most common and costly diseases in poultry production despite the progress in management, technology, biosecurity measures, genetics, nutrition, diagnostics and therapy. In the intensive fattening of broiler chickens it is still one of the main economical problems. The annual losses worldwide are estimated to be 2,3 billion euros, and according to data from 2014, up to 3 billion US dollars. The most common species are *E. tenella*, *E. acervulina*, *E. praecox* and *E. maxima*. Considering the significance of coccidiosis and effects it causes in poultry, it is necessary to prevent its appearance in clusters during production. The best prevention method recommended is vaccination, but it is only applicable on parent flocks since it is very expensive. In broilers, coccidiostats are most commonly used in food, during the first five weeks of fattening, in the form of two programs: the „shutlle" program and the rotation program. One of the key things is the timely diagnosis of subclinical coccidiosis, in order to be able to consider whether it is necessary to change the coccidiostatic or stay in the same program. The use of molecular techniques in the diagnosis of coccidiosis has become increasingly important in the last decade. In addition to the application of molecular techniques, traditional methods for diagnosis are being used and improved.

Key words: *coccidiosis, broilers, prevention, coccidiostatic, diagnostics*

INTRODUCTION

Coccidiosis is a parasitic disease caused by protozoa of the genus *Eimeria*, which parasites in the digestive tract. These are intracellular parasites that cause damage to the lining of the digestive tract, diarrhea, difficult absorption of nutrients, the reduction of body weight, they open doors for infections from other diseases and cause high mortality in the flock. Mostly younger animals are affected by this disease, and after the illness they acquire immunity. There is no cross-immunity between the

various *Eimeria* species. The disease may have a mild character if it is caused by a smaller number of oocysts, or it can have a very serious nature if it is caused by a large number of oocysts. Most of the infections have a subclinical character, but there is always the fear of an outbreak of severe forms of the infection. Therefore, in the first five weeks of broiler flocks fattening, coccidiostats are used in feed, in order to prevent the occurrence of infections (Mcdougald et al. 2008, Fadly et al. 2008).

Coccidia from the *Eimeria* species have oocysts on all four round sporocysts. Each sporocyst contains two sporozoites. Coccidia oocysts in chickens are relatively small in size, from 23–27 x 19–22 micrometres, are an oval shape, and have no residual little body and micropile. Sporulation of cysts takes place in the external environment and lasts 48–72 hours, a minimum of 18 hours. The most favorable conditions for sporulation are wet environments with temperatures of 25–30°C. In flocks with poor biosecurity measures, the possibility for mortality can be up to 100%, if appropriate preventative measures are not taken. The pathogen is transmitted mechanically from worker's shoes, equipment, bedding, contaminated food and water (Conway and McKenzie 2007, Pellerdy 1974).

The economical losses caused by coccidiosis in the broiler fattening industry are very high. This particularly applies to broiler flocks, where annual losses worldwide are estimated at 2.3 billion Euros (Sorensen et al. 2006) and according to data from 2014 up to 3 billion US $ (Blake and Tomley 2014). The losses can be divided into those being direct and indirect. Direct losses occur in clinically manifested infections. They are considered to be losses caused by mortality of chickens which are directly caused by coccidiosis. Indirect losses occur in the event of a secondary infection after curing coccidiosis or with subclinical coccidiosis when there is a permanent presence of reduced weight gain in chickens and increased feed conversion. The resulting damage to the gut mucosa are potential causative agents of infections from other diseases, such as *Clostridium perfringens*, S*almonella*, Histomonas meleagridis, Marek's disease, Infectious bursal disease (Shirley et al. 2004, Conway and McKenzie 2007). It is estimated that of all the total losses incurred during the production of broilers in India, 95.61% are from coccidiosis. Research conducted during 2003–2004 show that the maximum loss occurs due to the reduction of growth and due to an increase in feed conversion in chickens (Bera et al. 2010).

MEASURES TO PREVENT COCCIDIOSIS

In modern poultry production, fattening of broiler chickens is impossible to imagine without the use of coccidiostat drugs in food. They stop or inhibit the growth of coccidia, enabling the formation of a latent infection after stopping the drug administration. Coccidiostats are added to

the starter and grover feed mixtures. Caution must be taken when choosing coccidiostats, i.e. and of its rotation. If we used only one type of coccidiostat for a longer period of time, coccidia would be able to acquire resistance. Therefore, it is necessary to combine two or more coccidiostats. There are usually two programs for the application of coccidiostatics: (1) replacing two or more drugs one after the other during the upbringing of chickens („the shutlle program"), (2) rotating different types of drugs with each successive generation of livestock (Gussem 2007, Quiroz-Castañeda et al. 2015).

The main purpose of the coccidiostats program is to prevent the development of coccidia resistance to coccidiostatic drugs, allowing chicks to progress faster and have a higher growth. In „the shuttle program" different coccidiostats are meant to be applied during one production cycle. This means that there will be various other coccidiostats in the starter and grover feed mixes. This type of program is applied in broiler chickens and laying hens. The rotation program is characterized by the use of the same coccidiostat during the production cycle, and then replacing it with another coccidiostat from another group for the next production cycle (Hafez 2008, Chapman 2011).

The best way to prevent coccidiosis is vaccination. It is usually carried out only with parental flocks, because it is expensive. In broiler flocks and flocks of laying hens, coccidiostats are mostly used. After a mild infection, the immunity to this parasite is created. It has been shown that there is no cross-immunity between the various *Eimeria* species, so the vaccination should be targeted. Chickens are susceptible to infections ages 3 to 6 weeks. The infection generally almost never occurs before the third week of chick's life. It often happens that if you vaccinate a flock with a pentavalent vaccine, an infection occurs, because vaccination failed to create immunity to all types of coccidia which are present in the farm. Infections with *E. acervulina, E. tenella, E. mitis, E. mívaiti, E. praecox* and *E. maxima* can be seen from 3–6 weeks of age, and infections with E. necatrix from 8–18 weeks of age. Infections with *E. Brunetti* can be seen earlier and later (Pellerdy 1974).

METHODS FOR A TIMELY DIAGNOSIS

Modern broiler fattening has the aim to achieve the required weight in chickens in the shortest amount of time possible and with the least necessary food consumption. In order to achieve these results we need to keep the chick healthy in order for it to demonstrate its potential. Often during production certain health problems such as coccidiosis must be recognized as soon as possible and in the right way. Usually in flocks of broiler chickens subclinical coccidiosis may be present but later develop into a clinical form. Coccidiosis can be diagnosed the following ways: (1) On the basis of oocyst morphology (size, shape and oocysts color); (2)

Specifics towards the host; (3) The looks and location of lesions in the digestive tract; (4) The prepatent period. In the last couple of years biochemical (metabolic enzyme electrophoresis) and molecular methods (PCR) have been used for diagnosing coccidia. Nine various species of *Eimeria*, which cause diseases in cattle, have been described. These are *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. mívat*, *E. hagani*, *E. necatrix*, *E. praecox* and *E. tenella* (Shirley et al. 2004, Conway and McKenzie 2007).

The morphology of oocysts is investigated by using microscopic techniques. Feces of chickens at ages 3 to 6 weeks are used as samples. To extract the oocysts from faeces the flotation method is used, and to determine the total number per gram, the McMaster technique is used. The size of the oocysts is determined by using slides with numbered scales on them (Long et al. 1976). When visiting a farm and sampling the feces, it is necessary to do a survey and get the following information: name of the owner, the location of the farm (district), address, age of the flock, the number of chickens in the flock, the types of hybrids, the level of biosecurity measures, the coccidiostat being used, the types of the litter, whether coccidiosis has ever appeared on the farm, whether there were any secondary infections after curing coccidiosis. This type of survey will assist us in reviewing the overall management of the farm. Coccidia are classified according to their specificities to the host and by the duration of the prepatent period. Evaluating the level of the lesion on the intestine, caused by the presence of oocysts helps to diagnose subclinical coccidiosis. The recommendation is to sacrifice 5 healthy chickens in a flock, and then observe the small intestine and the cecum. The evaluation of the degree of lesions on the intestine is performed on a scale from 0 to 4, with 0 being normal tissue, and 4 being the maximum degree of the lesion. If changes typical for coccidia are found in large numbers, it is necessary to alter the coccidiostatic in food and find another more efficient one (Zander D. V. 1978).

The use of molecular techniques in the diagnosis of coccidiosis has become increasingly important in the last decade. The aim of their application is to examine what kinds of *Eimeria* are present in production phase, so to be able to pre-plan future vaccination programs for parents. In many countries, molecular techniques are already being used routinely, and studies conducted in India, Brazil, Australia, China, Egypt and Great Britain are proof of this. In these studies, it was found that the most common types of coccidia *E. tenella, E. acervulina, E. praecox and E. maxima* (Gerhold et al. 2015, Carvalho et al. 2011, Rosamond and Morgan 2015, Kumar et al. 2014, Ahad et al. 2015, Sun et al. 2009).

CONCLUSION

Coccidiosis is still one of the biggest challenges for modern poultry production. Despite the application of preventative measures, cases of clinically manifested infections in broilers still remain. This carries the consequences of direct and indirect losses in production. Therefore, it is necessary to improve traditional and new (molecular) diagnostic techniques for subclinical coccidiosis, in order to be able to anticipate a possible infection in a flock faster and more efficient, and apply preventative measures as soon as possible as well as correct the program of the coccidiostats application.

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TICK (Acari: Ixodidae) INFESTATION OF WATER AND BANK VOLES (Rodentia: Arvicolinae)

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Abstract: Small mammal species, especially rodents, are notorious as the important reservoirs and vectors of numerous species of pathogens and parasites. The suitable hosts and different microclimatic conditions vary in quality, intensity and frequency which all affect the presence of the parasites in certain habitats. European water vole (*Arvicola terrestris* Linnaeus 1758) and Bank vole (*Myodes glareolus* Schreber 1780) are herbivorous and habitat specialist species. The aim of the study was to determine the qualitative and the quantitative diversity of ixodid ticks of these vole species as they occupy different ecological niches. The study was conducted from 2011 to 2014, three times per year, at four localities in Vojvodina: Apatin, Bogojevo, Labudnjača and Čelarevo. Using Longworth traps the vole specimens were caught and transported alive to the laboratory, where all ticks were collected using palpatory method and tweezers. After the examination all individuals were returned to their natural habitats. The total number of 1227 specimens of European water vole and 142 specimens of Bank vole were examined. Five tick species from four genera were identified: *Ixodes ricinus*, *I. trianguliceps*, *Dermacentor marginatus*, *Haemaphysalis concinna* and *Rhipicephalus sanguineus*. Only the larval and nymphal stages were present. The average number of tick specimens on the European water voles was 0.828036±0.050265 and on the Bank voles 1.809859±0.216891. The highest prevalence was obtained for larval stages of *I. ricinus* and the lowest for nymphs of *R. sanguineus* found on *M. glareolus*. The highest value of the average infestation intensity index was obtained for larval stages of *D. marginatus* on *M. glareolus*. Furthermore, the larval stages of *I. ricinus* have had the highest values of abundance and infestation index also on *M. glareolus*. *M. glareolus* showed high values of tick infestation as this species is a habitat specialist that prefers forest ecosystems with well developed shrubby vegetation which are also suitable habitats for tick species. Furthermore, both vole species provided spatial and temporal dispersion in different habitats, which is of great importance for tick population maintenance.

Key words: *ticks, European water vole, Bank vole, infestation*

INTRODUCTION

Small mammal species, especially rodents, are notorious as the important reservoirs and vectors of numerous species of pathogens and parasites. According to Kolonin (2007), almost all mammal species could be suitable hosts for 511 tick species, four tick species are described as ornithophilic, and two tick species, besides mammal hosts could feed on reptiles too. Furthermore, 87 tick species are associated with rodents, although the most tick species are usually found on cloven-hoofed mammals.

According to Eisen et al. (2004) the percentage of rodent infestation by ixodid ticks can be extremely high, due to the fact that the microdistribution of the host seeking larvae depends on their dense distribution and movement that is limited to only a few meters, asconsidering the species, tick females lay 2000–3000 eggs at one place. Most of the ixodid ticks species that feed on rodents have a three-host life cycle, where each developmental stage enquire blood meal from a different host species. Usually the immature stages (larvae and nymphs) feed on a large number of small mammals, especially rodents, while the adults parasite the larger mammal hosts (Mihalca et al. 2012).

The suitable hosts and different microclimatic conditions vary in quality, intensity and frequency which all affect the presence of the tick asectoparasites in certain habitats. The tick abundance depends on abiotic (environmental and climatic conditions) and biotic factors (host community, host density, host behavior and host physiological conditions) (Gray 2002, Vaumourin et al. 2015). According to Paziewska et al. (2010) several factors influence the role of rodents as tick hosts in different habitats: (1) suitability of different rodent species as hosts for larval and nymphal stages, (2) the rodent abundance in certain habitats, (3) differences among habitats, primarily in humidity, which have a significant influence on tick survival and (4) differences in large mammal abundance and their movement between habitats, which is directly correlated to tick dispersion within the habitats.

The European water vole (*Arvicola terrestris* Linnaeus 1758) and the Bank vole (*Myodes glareolus* Schreber 1780) are herbivorous and habitat specialist speciesoften found in different types of habitat.

The European water vole inhabits swampy, wet meadows and fields near water, up to 3000 m altitude (Garms & Borm 1981). This species is most frequently found on the banks of rivers, streams and ponds and in all aquatic ecosystems that have a constant water level. *A. terrestris* prefers habitats with well developed vegetation cover, but in the years when

population density is extremely high it could be found in the agricultural fields and gardens too.

Oppositely, the Bank vole prefers deciduous, coniferous and mixed forests with dense and well developed low vegetation cover (Hansson 2002). It is less commonly found in agroecosystems. According to Tsvetkova et al. (2008), this species is codominant with Yellow necked mouse (*Apodemus flavicollis* Melchior 1834) in oak forests at the higher altitudes, but the full dominance shows in all types of deciduous forests and taiga.

The aim of the study was to determine the qualitative and the quantitative diversity of ixodid ticks of these vole species as they occupy different ecological habitats and niches.

MATERIAL AND METHODS

The study was conducted from 2011 to 2014, three times per year, at four localities in Vojvodina: Apatin, Bogojevo, Labudnjača and Čelarevo. Using Longworth traps the vole specimens were caught and transported alive to the laboratory, anaesthetized with ether and all ticks were collected using palpatory method and tweezers. Collected ticks were preserved in plastic tubes (5ml) in 70% ethanol till identification. After the examination all vole specimens were returned to their natural habitats. The rodent species were identified according to: Громов & Поляков (1977), Ognev (1966) and Garms & Borm (1981).The tick species of all developmental stages were identified according to: Nosek & Sixl (1972), Hillyard (1996), Estrada-Peňa et al. (2004), and Georgieva & Gecheva (2013).

The role of rodents as the ixodid tick vectors and their infestation were described using four indices according to Bush et al. (1997): the prevalence (P), the average infestation intensity (MI), the abundance (AB) and the infestation index (K).

RESULTS

The total number of 1227 specimens of the European water vole and 142 specimens of the Bank vole were examined. Five tick species from four genera were identified: *Ixodes ricinus* Linnaeus 1758, *I. trianguliceps* Birula 1895, *Dermacentor marginatus* Sulzer 1776, *Haemaphysalis concinna* Koch 1844 and *Rhipicephalus sanguineus* Latreille 1806. Only the larval and nymphal stages were present.

From the total number of examined European water vole specimens, 40.5% were infested with ticks. The total number of obtained ticks was 1016. The following tick species and developmental stages were detected: *I. ricinus* (larvae and nymphs) 48.43%, *D. marginatus* (larvae) 22.05%, *H. concinna* (larvae and nymphs) 14.76%, *R. sanguineus* (larvae and nymphs) 7.78% and *I. trianguliceps* (larvae) 6.99%. The average number of tick

specimens on the European water voles was 0.828036±0.050265 (for σ=1.760708 and σ^2=3.100094). The highest number of collected ticks per a specimen of *A. terrestris* was 32, found on a male trapped in Bogojevo.

The tick infestation of the Bank vole was higher comparing to the European water vole. From the total number of examined Bank vole specimens, 71.13% were infested with ticks and the total number of obtained ticks was 257. Nevertheless, the same tick species and developmental stages were identified: *I. ricinus* (larvae and nymphs) 51.75%, *D. marginatus* (larvae) 33.46%, *I. trianguliceps* (larvae) 6.61%, *R. sanguineus* (larvae and nymphs) 4.67% and *H. concinna* (larvae) 3.50%. The average number of ticks per Bank vole specimen was 1.809859±0.216891 (for σ=2.584551 and σ^2=6.679902). The highest number of collected ticks per a specimen of *M. glareolus* was 22, found on a female trapped in Apatin.

The values of the prevalence (P), the average infestation intensity (MI), the abundance (AB) and the infestation index (K) for both species are presented in the Table 1. and 2.

Table 1. The prevalence, the average infestation intensity, the abundance and the infestation index calculated for *A. terrestris*

Tick species	Tick stages	n	B	P	MI	AB	K
I. ricinus	Larvae	409	138	**11.247**	2.964	**0.333**	**0.037**
	Nymphs	83	79	6.438	1.051	0.068	0.004
I. trianguliceps	Larvae	71	51	4.156	1.392	0.058	0.002
	Nymphs	0	0	0.000	0.000	0.000	0.000
D. marginatus	Larvae	224	68	5.542	**3.294**	0.183	0.010
	Nymphs	0	0	0.000	0.000	0.000	0.000
H. concinna	Larvae	65	62	5.053	1.048	0.053	0.003
	Nymphs	0	0	0.000	0.000	0.000	0.000
R. sanguineus	Larvae	46	43	3.504	1.070	0.037	0.001
	Nymphs	33	32	2.608	1.031	0.027	0.001
Total		1016	535				

n – the number of tick specimens
B – the number of hosts infested with a certain tick species
P – the prevalence (%)
MI – the average infestation intensity
AB – the abundance
K – the infestation index

Table 2. The prevalence, the average infestation intensity, the abundance and the infestation index calculated for *M. glareolus*

Tick species	Tick stages	n	B	P	MI	AB	K
I. ricinus	Larvae	120	48	**33.803**	2.500	**0.845**	**0.286**
	Nymphs	13	13	9.155	1.000	0.092	0.008
I. trianguliceps	Larvae	17	12	8.451	1.417	0.120	0.010
	Nymphs	0	0	0.000	0.000	0.000	0.000
D. marginatus	Larvae	86	19	13.380	**4.526**	0.606	0.081
	Nymphs	0	0	0.000	0.000	0.000	0.000
H. concinna	Larvae	9	9	6.338	1.000	0.063	0.004
	Nymphs	0	0	0.000	0.000	0.000	0.000
R. sanguineus	Larvae	9	8	5.634	1.125	0.063	0.004
	Nymphs	3	3	2.113	1.000	0.021	0.000
Total		257	112				

n – the number of tick specimens
B – the number of hosts infested with a certain tick species
P – the prevalence (%)
MI – the average infestation intensity
AB – the abundance
K – the infestation index

The both species have had similar distribution of infestation indices, although indices calculated for *M. glareolus*have had higher values. The highest prevalence (P) was obtained for larval stages of *I. ricinus* and the lowest for nymphs of *R. sanguineus* for both species. The prevalence of *I. ricinus* larvae found on *M. glareolus*was three times higher than obtained for *A. terrestris*. The highest value of the average infestation intensity index was obtained for larval stages of *D. marginatus* on *M. glareolus*. Furthermore, the larval stages of *I. ricinus* have had the highest values of abundance and infestation index also on *M. glareolus*.

DISCUSSION

Two dominant tick species found on both, *M. glareolus* and *A. terrestris*, were *I. ricinus* and *D. marginatus*. *I. ricinus* is the predominant tick species in Europe (Barandika et al. 2008, Mihalca et al. 2012). According to Milutinović et al. (2012) this species has a wide ecological amplitude, so it could be found in the different types of habitats: open meadow field and pastures, bogs, deciduous, coniferous and mixed forests, even in the habitats under the direct anthropogenic influence (parks, agro-ecosystems). These habitats are also the habitats with the highest population abundance of European water vole and Bank vole. The high number of larval and nymphal stages on the both vole species could be explained by

the fact that the immature stages of *I. ricinus* are capable of feeding on almost any vertebrate. As stated by Mihalca et al. (2012), *I. ricinus* is the dominant tick species on small mammals.

In Vojvodina, *D. marginatus* follows the distribution pattern of *I. ricinus* as the second most abundant tick species (Petrović 2015). This species prefers deciduous forests, pastures, meadows and all kinds of shrub vegetation along the river banks. According to Estrada-Peňa et al. (2004), *D. marginatus* is a thermophilic species often found in very humid habitats. Milutinović et al. (2012) reported that immature stages of *D. marginatus* exclusively parasite on rodents and that their association could be explained by a unique type of secondary endophilism. Namely, larval and nymphal stages of *D. marginatus* often inhabits the indoors of the rodents burrows and dens and do not exhibit the usual tick behavior of questing the suitable hosts. However, the absence of *D. marginatus* nymphal stages on both vole species could only be explained by the three-host life cycle of the species, where each developmental stage requires blood meal from a different host species.

I. trianguliceps is often found in burrows, dens and nests of small mammals, and so far has not been sampled from the vegetation (Petrović 2015). According to Kovalevskii et al. (2013), this species is thermophilic and therefore found in steppes and deciduous forests, but also could be found in the willow forests and flooded meadows, which are also suitable habitats for *A. terrestris* and *M. glareolus*.

Petrović (2015) stated that *H. concinna* is usually found in forest ecosystems with well developed bushy vegetation and floodplain meadows, but has not been recorded in extremely dense forests. This is a hygrophilic and thermophilic species, often found in open, warm habitats, in the river valleys, floodplains and areas with high groundwater levels (Milutinović et al. 2012).

Oppositely to the results of Paziewska et al. (2010) and Mihalca et al. (2012) the nymphal stages of *D. marginatus*, *I. trianguliceps* and *H. concinna* have not been detected on European water vole and Bank vole, but Petrović (2015) registered their presence on three mouse species (*Apodemus agrarius, A. flavicollis* and *A. sylvaticus*) at the same localities.

M. glareolus showed higher values of tick infestation as this species is a habitat specialist that prefers forest ecosystems with well developed shrubby vegetation which are also suitable habitats for tick species. The obtained results are similar to the results presented by Paziewska et al. (2010), who obtained statistically different values of prevalence and infestation intensity of *I. ricinus* and *D. reticulatus* on the different rodent species, comparing the two types of habitats: forest and meadow ecosystems in northeast Poland. Furthermore, both vole species provided spatial and temporal dispersion in different habitats, which is of great importance for tick population maintenance.

The interface between ticks, rodents, larger mammals and humans can play a significant role in the epidemiology of tick-borne diseases (Paziewska et al. 2010). Within the studied ecosystem, rodents play a significant role in maintaining different tick species populations, enabling and facilitating transstadial continuity in the developmental cycle. Furthermore, based on biological and ecological characteristics, especially the size of the home range during the period of intensive feeding and mating, all the studied species of voles are very good vectors of determined ixodid tick species between different types of habitat, providing them spatial and temporal dispersion, which is of great medical and veterinary interest.

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HONEYBEE DIVERSITY IN VOJVODINA PROVINCE BASED ON MORPHOMETRIC CHARACTERISTICS OF WING INNERVATION

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Abstract: Temperate continental climate in Vojvodina province (Serbia) provides good conditions for beekeeping, because the bees are active over eight months in the year. Geographic features of Vojvodina contribute to the development of modern beekeeping, while the intensification of agriculture imposes its development, as the number of natural pollinators decline. It is assumed that two types of bees are present in the Vojvodina, Apis millifera carnica (domestic corneal bee) and Apis meliifera carnica banatica (a variety of cornual bee i.e. Banat yellow bee). Up-to date scientific research results regarding bee diversity are insufficient and refer to the differences in the color of bees. It was found that Apis meliifera carnica banatica, which is mainly bred in the Banat region, in contrast to Apis millifera carnica, has 2–3 yellow rings on abdomen and slightly shorter tongue. The aim of this work was to determine the presence of bee races in Vojvodina and their prevalence. Morphometric analysis was performed by measuring the wing innervation of 180 samples of bee wings collected from three locations in Vojvodina (Kikinda, Mužlja and Janošik) and 16 angles formed by innervation (A1, A4, B3, B4, D7, E9, G7, G18, H12, J10, J16, K19, L13, M17, O26, Q21). Four indexes (Ci, Pci, Dbi, Ri) were calculated for a total of 20 measures. One way ANOVA was used to determine the differences between angles of wing innervation of different races (Statistica 12) and a comparison of the analyzed bees with DAWINO standards is done for 5 races of bees (Apis mellifera carnica, Apis mellifera macedonica, Apis mellifera mellifera, Apis mellifera ligustica i Apis mellifera caucasica). The analyzed bee samples do not significantly differ in 20% of traits compared to A.m. carnica and A. m. macedonica and for 15% of A. m. ligustica race. The correlation analysis of wing innervations Ci and DBi, using CBeeWing determined that 77% of analyzed bees belong to A.m. carnica race, while 23% of bees do not fulfill standards for this race.

Key words: *honeybee. A. m. carnica, morphometric analysis, wing innervation*

INTRODUCTION

Temperate continental climate in Vojvodina province (Serbia) provides good conditions for beekeeping, because the bees are active over eight months in the year. Geographic features of Vojvodina contribute to the development of modern beekeeping, while the intensification of agriculture imposes its development, as the number of natural pollinators decline. Three rivers, the Danube, Tisa and Tamiš flow through the region, with willow forests on banks, which represent essential spring pasture. Plain, which stretches from Croatia to the west, all the way to Romania in the east and from the Danube River in the south, to the north of Hungary, is a production area for a variety of crops, such as sunflower, phacelia, tobacco, chamomile and the increasingly popular rapeseed. In the south of this region, the Fruška Gora mountain is positioned, which is rich in fruit species that bees can pollinate (Pihler 2012).

It is assumed that two types of bees are present in the Vojvodina, *Apis millifera carnica* (domestic cornual bee) and *Apis meliifera carnica banatica* (a variety of cornual bee named Banat yellow bee) (Ruttner 1988, Mladenović and Simeonova 2010, Mladenović et al. 2011). Most of the scientific studies, up to now, though insufficient, were related to the production results and the differences in the color of bees. It was found that *Apis meliifera banatica* which is mainly bred in the Banat region, in contrast to *Apis millifera carnica banatica*, has 2–3 yellow rings on abdomen and slightly shorter tongue (Radoš 2008). Only Nedić (2011) gives a more accurate morphometric analysis, in a study that involved bees from seven different localities in Serbia, including 12 characteristics of front wing, which covers the bees from the South Banat territory.

MATERIALS AND METHODS

Samples of bees were collected from three locations in Vojvodina (Kikinda, Mužlja and Janošik). For morphometric analyzes, 60 bees were taken from each location. Bees were collected from frames which have sealed brood, to be as safe as possible that a young bee ecloded in the hive. Bees were euthanized in 96% alcohol and kept in a refrigerator until analysis. GPS coordinates for all three locations were recorded during the sampling of the bees.

Table 1. Locations from which the bee samples were taken, with the GPS coordinates of bee hives.

Locality	GPS coordinates
Kikinda (Banat)	45^0 47.650'N; 20^0 26.350'E
Janošik (Banat)	45^0 10.309'N; 21^0 00.364'E
Mužljanski rit (Banat)	45^0 15.484'N; 20^0 19.661'E

Morphometric analysis was performed by measuring the wing innervations, since the ratios of individual sides of the cells that were formed by leaf innervation and angles they form are the best morphometric indicators of a race, because they are the least subjected to changes due to paragenetic factors.

For the analysis of wing innervation of bees, 180 young bees from 3 localities and 6 bee colonies were analyzed. From each colony 30 young bees were analyzed (method by Ruttner 1988).

Bee wing innervation was measured at Zemjodelski Faculty in Skopje (Macedonia), by entering the scanned wing patterns into a Beewings 1.20 program, specially designed for measuring the bee wing innervation.

In the Beewings 1.20 program, 19 reference points are manually marked on each wing (Figure 1.) through which the analysis of 20 characteristics is done on the front right wing of the worker bees.

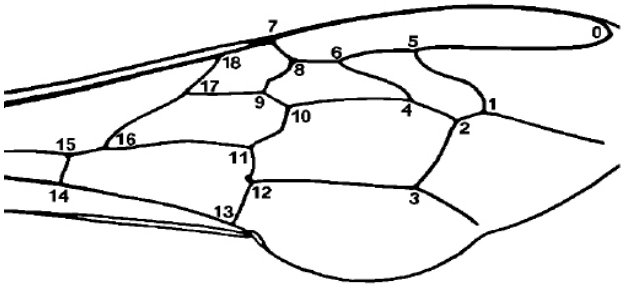


Figure 1. 19 reference points for the calculation of 20 characteristics of wing morphometry.

16 angles (A1, A4, B3, B4, D7, E9, G7, G18, H12, J10, J16, K19, L13, M17, O26, Q21), which a wing innervation forms, were calculated for all 180 samples of bee wings and four indexes (Ci, Pci, Dbi, Ri) were determined for a total of 20 measures.

For each of the 20 analyzed characteristics, a standard descriptive statistics was performed and the following was determined: mean ($\bar{x}$) standard deviation (Sd) and coefficient of variation (Cv).

For determination of the differences between analyzed bees and other bee races, we compared the calculated 20 characteristics of front bee wings with standards for five races (*Apis meliifera carnica*, *Apis mellifera macedonica*, *Apis mellifera mellifera*, *Apis mellifera ligustica* and *Apis mellifera caucasica*) for standards which were obtained by DAWINO method developed by Dol Bee Institute (Czech Republic). Determination of statistical differences was performed using the following formula:

$$Z_0 = \frac{\bar{x}_1 - \bar{x}_2}{Sd \,/\, \sqrt{n}}$$

Where ($\bar{x}_1$) is the mean value of the analyzed trait, ($\bar{x}_2$) the standard value of the specific characteristic, (Sd) standard deviation of specific characteristic and (n) is the number of analyzed bees.

RESULTS

The criteria for race determination by morphometric analysis are many, but the most reliable is the wing morphometry (Rinderer 1986). The minimum, maximum, mean ($\bar{x}$), standard deviation (Sd) and coefficient of variation (Cv) for all observed morphological characteristics of Vojvodina bees wing innervation are presented in Table 2.

Table 2. Minimum, maximum, mean ($\bar{x}$) standard deviation (Sd) and coefficient of variation (Cv) of morphometric traits of bee wing innervation

Trait	Min	Max	Mean ($\bar{x}$)	Std. Deviation (Sd)	Coefficient of variation (Cv)
A1	17.80	39.00	27.11	3.98	14.68
A4	23.00	86.90	29.74	4.85	16.31
B3	48.90	83.00	72.82	3.85	5.29
B4	48.80	123.90	107.03	7.10	6.63
D7	59.10	107.40	96.65	4.31	4.46
E9	18.40	31.10	23.52	2.06	8.76
G7	20.60	28.00	24.97	1.15	4.61
G18	73.60	106.40	92.07	5.35	5.81
H12	11.00	24.40	18.31	2.24	12.23
J10	44.80	66.30	54.72	4.21	7.69
J16	77.10	106.10	93.71	5.20	5.55
K19	67.20	88.10	77.05	3.76	4.88
L13	9.80	23.60	14.53	2.27	15.62
M17	35.00	76.40	55.18	6.73	12.20
O26	21.80	57.20	39.88	5.52	13.84
Q21	33.40	42.40	37.39	1.86	4.97
Ci	1.39	4.64	2.46	0.45	18.29
Ri	1.29	1.92	1.52	0.10	6.58
PCi	2.30	3.34	2.70	0.17	6.30
DBi	0.81	1.34	1.05	0.09	8.57

The analysis of 20 traits of bee wing innervation shows that some traits, such as A1, A4, H12, L13, O26 and Ci, have a very high coefficient of variation, indicating a high variability of these traits in the analyzed bees.

Rutner (1988) presented results of morphometric traits of bees from several European regions and determined the average value of cubital index (Ci) of 2.77 for the Pannonian Plain bees, which is significantly higher than the value of this trait obtained in this study (2.46). On the other hand, the same author obtained very similar results for the wing innervation angle E9 and G18.

Radoš (2008) determined the average value of cubital index (Ci) of 2.4, by analyzing the morphometric characteristics of the Banat bee. These results are consistent with the values (2.46) obtained in this work, for the same trait.

Table 3. Mean ($\bar{x}$) of morphometric characteristics of bee wing innervation from three localities in Vojvodina.

Trait	Kikinda	Janošik	Mužljanski rit
A1	28.10	26.51	26.73
A4	29.58	29.92	29.71
B3	74.43	73.13	70.92
B4	107.14	108.13	105.82
D7	98.70	96.74	94.50
E9	23.07	23.01	24.48
G7	24.90	24.69	25.31
G18	92.64	91.11	92.48
H12	18.76	16.91	19.26
J10	53.62	54.34	56.19
J16	91.05	94.48	95.62
K19	78.46	75.52	77.16
L13	14.39	14.24	14.96
M17	52.36	57.31	55.88
O26	37.39	41.30	40.96
Q21	37.43	37.24	37.51
CI	2.21	2.37	2.78
RI	1.48	1.52	1.57
PCI	2.75	2.74	2.61
DBI	1.01	1.03	1.10

Table 4. Comparison of mean values (z-test) for 20 observed characteristics of wing innervation of Vojvodina bees with DAWINO standards for five European bee races

Trait	*A. m. carnica*	*A. m. macedonica*	*A. m. mellifera*	*A. m. ligustica*	*A. m. caucasica*
A1	11.16**	17.58**	39.18**	15.83**	30.43**
A4	-1.75	-6.53**	-19.95**	-8.93**	-25.21**
B3	-10,72**	-20,99**	-46,36**	-17,37**	-43,94**
B4	-9,72**	-1,54	20,08**	1,08	20,08**
D7	-0,28	-9,44**	-37,46**	-10,52**	-30,46**
E9	1,36	12,65**	54,45**	2,49*	31,86**
G7	13,50**	21,61**	50,00**	9,45**	47,97**
G18	9,87**	3,79**	-7,49**	9,87**	-6,19**
H12	-4,08**	0,07	-11,34**	-11,34**	22,89**
J10	0,64	-0,47	23,81**	9,47**	3,95**
J16	5,87**	25,09**	9,45**	-0,38	28,67**
K19	-11,47**	-9,62**	-23,22**	-7,76**	13,26**
L13	12,59**	0,31	-7,87**	10,54**	-3,78**
M17	42,39**	52,05**	43,42**	37,21**	55,50**
O26	9,60**	13,81**	10,02**	13,81**	19,70**
Q21	8,62**	-2,60**	22,33**	7,37**	19,84**
Ci	-17,86**	-7,50**	33,91**	-7,50**	18,38**
Ri	5,54**	29,99**	54,43**	29,99**	54,43**
Pci	0,27	0,27	-26,71**	0,27	-13,22**
Dbi	-13,28**	11,58**	36,44**	11,58**	61,30**

$P < 0.05$ * i $P < 0.01$**

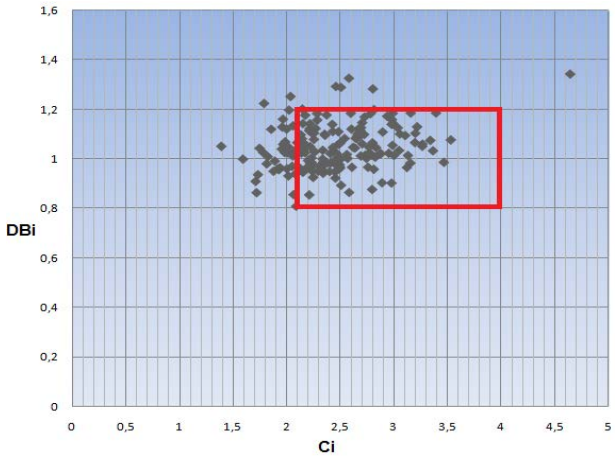
Comparing the values of 20 characteristics of wing innervations, using the z-test, with standard values of the traits for five European races of bees *(Apis mellifera carnica, Apis mellifera macedonica, Apis mellifera mellifera, Apis mellifera ligustica i Apis mellifera caucasica)*, it was found that on the basis of the entire sample there is no statistically significant difference in the A4, D7, E9, J10 and PCi traits of *A. m carnica*, B4, H12, J10, L13 and PCi traits of *A. m. macedonica* and with B4, J16 and PCi traits compared with the *A. m. ligustica* race.

Since Ruttner (1988) stated that in the regions of Austria, Slovenia, Croatia and South Serbia, *A. m. carnica* can be expected in pure breed and that in the southwest it borders with *A. m. ligustica* race and the south with *A. m. macedonica* racem it was expected that we would find traits that are not statistically significant with the same traits in species *A. m. ligustica* and

A. m. macedonica, and also that more traits would not exibit a statistically significant difference with *A. m. carnica* race.

These results we obtained in the examination of wing innervation are consistent with the results obtained by Gajger (2007), who examined the cubital index of continental Croatia bees, and presented its value, which ranged from 1.4 to 3.9 and that only 25.6% of bees belong to cornual bee race, while 61.8% of the bees are in the overlaping zone with other bee races.

According to Ruttner (1988), Vojvodina does not present a natural hybridization zone, or zone of separation of *A. m. carnica* race and races *A. m. ligustica* and *A. m. macedonica*. We can assume that the bees of these races at this location are anthropogenic. Accidentally or on purpose they were brought by beekeepers with some of migrated hives. Vojvodina has a large area with industrial melliferous plants which makes this location very popular for professional beekeepers. On the other hand, the obtained phenotypic values are consistent with the recent studies (Gajger et al. 2007, Radoš et al. 2008) which show values of morphometric traits that do not fit entirely in the standards of the *Apis meliifera carnica* race.



Graph 1. The correlation between the calculated value of Ci (cubital index) and Db (dump bell index) of sampled bees from all three localities

Correlation analysis of wing innervations Ci and DBi, using CBeeWing, determined that 77% of analyzed bees belong to *A.m. carnica* race, while 23% of bees do not fulfill standards for this race.

CONCLUSION

Based on the results obtained by morphometric analysis of 20 traits of bee wing innervation from tree different localities in Vojvodina province (Serbia) and their comparison with DAWIN standards for five European

races of bees, the following can be concluded: the analyzed bee samples do not significantly differ in 20% of traits compared to *A.m. carnica* and *A. m. macedonica* and for 15% of *A. m. ligustica* race. Correlation analysis of wing innervations Ci and DBi, using CBeeWing, determined that 77% of analyzed bees belong to *A.m. carnica* race, while 23% of bees do not fulfill standards for this race.

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FOOT-PAD DERMATITIS INFLUENCE ON POULTRY FERTILITY

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Abstract: Floor-pad dermatitis (Foot pad dermatitis, FPD) is a contact dermatitis of poultry, which occurs from inadequate living conditions, above all quality and humidity of the rugs. The lesions begin with skin discoloration, proceeds with inflammatory processes, hyperkeratosis, the erosion of the skin, and often necrosis. Changes in the soft parts of the feet are sore, and poultry is therefore less mobile, taking less food and water a sexually mature ones avoid copulation. The prevalence of FPD in the flock is highly influenced, except wet rugs, by gender, age, presence of various nutrients in a meal. In our study we examined the influence of FPD in roosters on the fertility of eggs in a single parent flock, at the age of 35 to 45 weeks. The results we obtained suggest that the flock in which the 45% roosters had FPD in diverse intensity has 32.5% fewer fertilized eggs in the same age from a flock which has no problems with FPD.

Key words: *Heavy breeder, roosters, hatching eggs, fertility, floor-pad dermatitis*

INTRODUCTION

Production of hatching eggs is influenced by a number of internal and external factors. Genetic factors, age poultry, nutrition, health, nutritional quality of meals and accommodation all mutually influence the quality and fertility of hatching eggs (Renema et al. 2008). Due to bad accommodation conditions changes in the soft parts of the foot can occur and affect the fertility of eggs which consequently reduces the production of chickens. Changes in the sole foot parts of poultry, known as foot-pad dermatitis, are conditions that are characterized by inflammation and deep or surface necrotic lesions of the soles and fingers. Profound changes can lead to abscesses and thickening basic tissues. Foot-pad dermatitis (FPD) was first described in 1980 in broilers (McFerran et al 1983, Greene et al. 1985).

Genetic predisposed hybrids, immunosuppressive disease of chickens, the composition of the mixture for feeding, unsatisfactory housing

conditions affect the occurrence of wet litter and contribute to the intensive floor-pad dermatitis occurs in one flock. The lesions on the soles of the feet are more often recorded in flocks during the winter months, with the intense weather changes and increased relative humidity in buildings (McIlroy 1987). Most authors are interested in problems of foot-pad dermatitis in broiler chickens and turkeys for economic losses caused as a result of it's presence. The intensity of pain arising due to changes (Sinclair 2015) is different and causes difficulties in moving and reaching drinkers and feeders which than cause lose of weight and cachexia. In heavy breeding flocks, foot-pad dermatitis causes weaker libido in roosters, so in the majority of hybrids a replacement with younger roosters is planned. Flocks with foot-pad dermatitis issues have a smaller percentage of fertilized eggs and consequently a decreased number of chickens. The floor mats and the condition thez are in have a direct impact on the problems of the soft parts of the foot, health and the ability of breeding (Aviagen 2013). In this study the authors followed the production and fertility of eggs in a flock of heavy parents in which the changes occurred on the soles of the feet in the first weeks of production (exploitation) due to increased humidity and poor quality rugs in the house.

THE AIM OF THE WORK

The aim of the study was to determine the impact of floor-pad dermatitis on eggs fertility in heavy line breeders.

MATERIALS AND METHODS

A flock of heavy parents, 7860 individuals (6985 hens and 875 roosters), age 31 weeks, despite the satisfactory production of hatching eggs, had 5% less hatched chickens. After conducting health checks and laboratory tests it was found that the flock contains a number of males with one-sided or double-sided foot-pad dermatitis, varying in intensity. The building had a wire barrier, which allowed us to make a selection of roosters and form two clusters. One cluster was formed with roosters with healthy skin (control group K), while the other one had roosters with changes in the soles of the feet (experimental group O). The other cluster counted 3360 birds (2985 chickens and 375 roosters). Population density in both parts of the building was 5 m^2, and number of feeders and drinkers was appropriate for the requirements of hybrids. After clinical examination of males, it was found that 45% of them had unilateral or bilateral changes of varying intensity on the soft parts of the sole of the foot. A large number of males had necrotic lesions on the soles. Chickens were uniformed, phenotypically and according to body weight, and adequate to the requirements of hybrid technology. Hens and roosters ratio was 1:9 which

is in accordance with the technology of the age of 30–50 weeks. Uniformity of chickens was 95%. An adjustment period lasted 3 weeks. During the adjustment period, the experiment eggs were marked and labeled, placed in drawers organized in order to monitor the percentage of hatching and fertilization.

RESULTS AND DISCUSSION

The egg production for a period of 32–34 weeks of age in the control group (K) and the experimental group (O) are given in Table 1. It shows that egg production, fertility and hatchability decreased with each week of life in the control group, while in 34. week the difference between flocks was over 10%.

Table 1. Results of production during the adjustment period, from 32–34 weeks

Flock age in weeks	Egg production		Control group (K)		Experimental group (O)	
	Control group (K)	Experimental group (O)	fert-ility	hatch-ability	fert-ility	hatch-ability
32	85,2	84,9	91,3	84,5	81,6	72,9
33	84,8	84,2	92,1	83,1	83,7	73,2
34	83,9	83,7	91,9	83,5	78,9	71,9

Results of egg production in an examining groups of 35–45 weeks of age are shown in Table 2.

Table 2. Results of production of 35–45 weeks

Flock age in weeks	Egg production		Control group (K)		Experimental group (O)	
	Control group (K)	Experimental group (O)	fert-ility	hatch-ability	fert-ility	hatch-ability
35	82,1	81,9	88,5	80,5	71,6	63,8
36	81,8	82,2	87,9	80,2	68,5	60,8
37	81,2	81,3	86,8	79,9	66,5	58,8
38	80,1	79,9	86,9	79,3	66,3	58,0
39	79,9	79,9	85,8	77,8	64,1	56,5
40	79,2	79,5	86,5	77,1	60,3	53,9
41	78,9	78,9	85,9	76,8	58,7	51,8
42	77,9	77,5	84,6	75,2	59,9	52,6
43	76,8	77,0	84,2	74,3	53,8	45,2
44	76,5	76,7	83,5	73,3	52,9	43,5
45	76,0	76,5	82,3	72,8	49,8	43,1

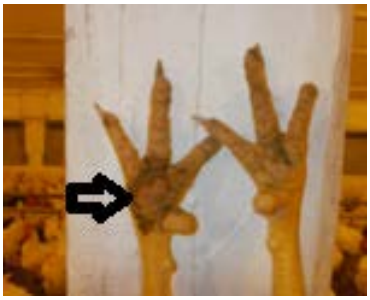
Production of hatching eggs and control of fertility in the experimental groups was carried out in groups consisting of flocks aged 35–45 weeks. Embryonated eggs are candled at day 18 of incubation and all eggs that are not hatched were cracked to determine the percentage of fertility. During the controlled period, percentage of fertilized eggs decreased from week to week. In the 45th week of life, fertility in experimental group O was reduced for 32.5% compared to the control group, and hatchability was 29.7% lower.

Changes in feet

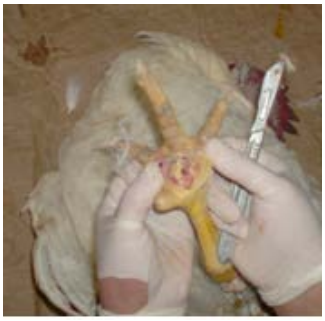
Picture 1. Feet without changes in group K

Throughout the experiment in the control group no clinical changes that would indicate the occurrence of foot-pad dermatitis were observed.

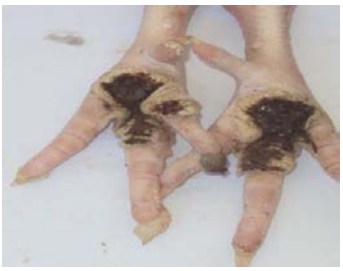
Picture 2. Changes in both sides of feet, group O



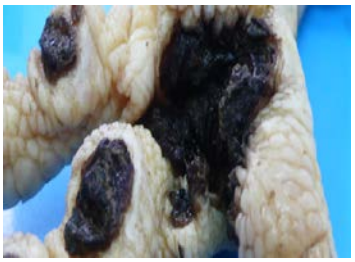
Picture 3. Changes in one side of feet, group O



Picture 4. Changes in 37. Week



Picture 5. Changes in 43. Week



Picture 6. Changes in 45. week

During the experiment with the roosters in the experimental group O, there has been a deterioration and enlargement of changes in the skin (FP dermatitis). At the end of the trial, 25% of males due to the inability of movement and regular food intake became cachectic. In the 46th week of life, a the replacement with younger males was conducted.

Mayne et al. (2007) in their studies found that floor-pad dermatitis in poultry occurs as a result of humid pads, in 41% of flocks. Changes in the intensity varies, from mild lesions to necrosis. In our experiment we extracted roosters who had already developed inflammation, erosion and showed signs of difficulty in movement. During the experiment, changes were getting worse, parts of the sole necrosed, roosters were reluctant to move, which is in line with the research of Sinclair et al. (2015). In this study it was found that livestock that has changes on the soles of the feet

has pain of varying intensity, which causes reduced movement, difficulty in taking food and water and decreased libido.

CONCLUSION

Maintaining a dry floor is an essential prerequisite for reducing the occurrence of floor-pad dermatitis. Issues arising as a result of floor-pad dermatitis significantly affect the quality of life due to pain of varying intensity and the production is economically disbalanced.

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DIVERSITY AND SEX INDICES OF HARD TICKS (Acari: Ixododae) IN URBAN AND SUBURBAN AREAS

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Abstract: All ixodid ticks are habitat generalist and their presence at certain habitats depends on the environmental microclimatic features and the presence of suitable hosts. Ticks are intensively studied because of their permanent presence in the urban and suburban areas as they are proved vectors of numerous and severe pathogens important for human and veterinary medicine. The species diversity and abundance of ixodid ticks were studied during 2015 at 20 localities in Serbia, from April to September, at four types of the urban and suburban areas: parks, urban forests, green areas around schools and kindergartens and river and lake promenade banks. The obtained results were analyzed using the Shannon-Wiener index and sex index. Seven tick species were identified: *Ixodes ricinus*, *Dermacentor marginatus*, *D. reticulatus*, *Haemaphysalis concinna*, *H. punctata*, *Rhipicephalus sanguineus* and *R. bursa*. *I. ricinus* was the dominant species at all prospected localities. *D. marginatus* was the most dominant species at urban forests. *D. reticulatus* and *H. punctata* were sampled only at parks and urban forests, *H. concinna* and *R. bursa* at urban forests and R. sanguineus only at parks. The highest values of the Shannon-Wiener diversity index were obtained in urban forests, such as Poloj (1.004) and Titel (1.000). Females of all identified tick species were more numerous comparing to males with the highest value of sex index (0.90) obtained for females of *D. marginatus* at green areas around schools and kindergartens. The lowest value of sex index was calculated for *R. bursa* (0.33) at urban forests. The obtained results indicate that continuous monitoring and mapping the habitats with high tick abundance is economically justified and necessary, not only for the health safety of human and animals, but also their daily activities as well as the overall environmental protection.

Key words: *ixodid ticks, diversity index, sex index, urban areas*

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THE OCCURENCE OF *SETARIA DIGITATA* IN DAIRY COWS IN SERBIA

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Abstract: *Setaria* spp., onchocercid filaroid nematodes inhabit the peritoneal cavity of many domestic and wild ungulates such as horses, cattle, buffalo, camels, sheep, goats, swine, deer and antelope. *S. digitata, S. labiatopapillosa* and *S. marshalli* are common nematodes found in cattle in the Far East and Asia. Adult worms are generally to be non-pathogenic, although the larvae of *S. digitata* may cause mild fibrinous peritonitis and granulomas in the retroperitoneum and bladder of cattle. Transmission of infective larvae through mosquito and other arthropods vector to non permissive hosts such as goats, sheep, cattle, horse and man, can result in serious and often fatal neuropathological disorders commonly referred to a cerebrospinal nematodiasis or lumbar paralysis (LP). It is also described a case of the uterine tube occlusion by *S. labiatopapillosa* in cattle. Sometimes *S. digitata* may be found in abnormal locations, such as the heart, lungs, and mesenteric lymph nodes where they induced eosinophilic granulomas.

In this study a total of five high-yielding cows, between 4 and 6 years old, which died in early puerperium were necropsied. Cows were died from different pathological conditions as fatty liver, right abomasal displacment and puerperal endometritis. During necropsy as an accidental finding we revealed the presence of parasites, 5–10 cm length in the peritoneal cavity, on the ventral surface of omentum majus. In one cow there were two parasites on the omentum, while in other four there was only one in each. Parasites didn't cause any pathological changes on the omentum. Parasitological examination, using light microscopy, confirmed that found parasites belongs to the genus *Setaria digitata*. To our knowledge, this is the first finding of *Setaria digitata* in cows in Serbia.

Key words: *Setaria spp., dairy cows, omentum*

STREPTOCOCCUS EQUI SUBSPECIES *ZOOEPIDEMICUS* AS CAUSE OF POLYARTHRITIS IN GOAT KIDS: A CASE REPORT

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Abstract: *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) is a Lancefield group C beta-hemolytic streptococcus. *S. zooepidemicus* is commonly found colonizing the mucous membranes of healthy equids. The specific name „zooepidemicus" derives from its wide host range. Mastitis in cattle and goats; pneumonia, septicemia, and wound infections in lambs and dogs; septicemia in chickens and dolphins; and lymphadenitis in guinea pigs are examples of its widely ranging pathogenicity. Infections of humans with *S. zooepidemicus* are associated with consumption of unpasteurized cow and goat milk products, as well as with close contacts with horses.

In this paper we describe a case of polyarthritis in Alpine kids caused by *S. zooepidemicus*. Four Alpine kids (one month old) were submitted for necropsy in order to reveal cause of death. After necropsy, altered tissues and milk samples originated from two goats from the same farm were processed with standard bacteriological methods and with polymerase chain reaction in order to detect *Mycoplasma* spp. DNA. From owner, we got informations that kids several days before death developed joint swelling, apathy, inappetence and inability to move. Kids were held together with other goats, sheep and lambs, horses and llamas. There were no clinical signs of illnes in other animals on the farm.

At external examination of carcasses edema of periarticular tissue was seen. Discrete hyperemia and crust were seen on ears around ear tag. Necropsy revealed severe fibrinous polyarthritis in all examined kids. There were no pathomorphological alterations on other organs. Affected were shoulder, knee and hip joint. Sheets of white to yellow fibrin partially or completely cover the articular surfaces. From all joints *S. zooepidemicus* were isolated while milk samples were negative. No *Mycoplasma* spp. DNA was detected neither from milk or joints.

Although *S. zooepidemicus* induces wide range of pathological conditions in different animal species, as far as we know, our report represents the first described case of arthritis in kids caused by this bacteria. In lack of any signs of illness in other animal species on farm, as well as negative pathomorphological findings in other organs and negative bacteriological results from milk samples, source and route of infection for

kids remain unclear. We assume that source of bacteria were horses present on farm and that lesions on ears represents the portal of entry of *S. zooepidemicus* in kids. It would be tempting to speculate that isolated strain is highly adaptable for goat kids and further molecular studies are needed to investigate that hypothesis.

Key words: *Streptococcus zooepidemicus, goat kids, arthritis*

CARP EDEMA VIRUS DISEASE (CEVD) – NEW VIRAL DISEASE IN CARP AQUACULTURE

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Abstract: Carp edema virus disease or koi sleepy disease is an emerging disease that occures in several countries in Europe and world. The disease was first reported from Japanese koi carp in 1974. In Europe was first detected in England in 2009 in imported koi. Recently koi sleepy disease was detected in Germany, France, Netherlands, Czech Republic and Austria. Carp edema virus is DNA virus belonging to the family Poxviridae. Virus has not been grown in the laboratory by cell culture, but was morphologically characterized by electron micropscopy. Disease occurs in n wild and cultured varieties of common carp (*Cyprinus carpio*) and koi carp. Water temperature is important factor in occurrence of the disease which appears at water temperature between 15–25°C. Mortality can reach up to 80%. Diseased fish laying on the bottom of the ponds and show lethargy. Infected fish will often refuse to eat, which further weakens the fish. Extensive erosions or hemorrhages of the skin with generalized edematous condition are present. Other external signs of infection may include sunken eyes and pale swollen gills. Gill hyperplasia and necrosis can lead to hypoxia. Carp edema virus diagnosis can be reached using modified nested PCR assay. Also, histopathology can support a carp edema virus diagnosis. The best way to prevent koi sleepy disease is to obtain fish free of virus. Also, surveillance of carp edema virus disease in common carp is important and should receive attention.

Key worlds: *carp edema virus, koi sleepy disease, carp aquaculture*

INTRODUCTION

Carp edema virus disease (CEVD), also known as koi sleepy disease was first reported from juvenile colour carp (*Cyprinus carpio*) in Japan in the 1974, and for many years was described only in Japan (Murakami et al. 1976, Ono et al. 1986, Amita et al. 2002). The disease was originally named

„viral edema of carp" (Oyamatsu et al. 1997a). Carp edema virus disease is caused by DNA virus belonging to the family Poxviridae.

CEV has been detected widely across Europe in koi from at least Austria, Czech Republic, France, Germany, the Netherlands, and the United Kingdom (Haenen et al. 2013, Way and Stone 2013, Jung-Shoroers et al. 2015, Lewisch et al. 2014, Vesely et al. 2015). The virus has recently been detected for the first time in the southern emisphere on koi farms in Brazil (Viadanna et al. 2015). In the United States, CEVD/KSD has been ssociated with outbreaks in imported and domestic koi (Hedrick et al. 1997, Waltzek et al. 2014).

Clinical signs of disease are conected with stressful farm conditions and water temperatures between 15 and 25°C (Lewisch et al. 2014). Mortalities can reach up to 80–100% and prevalence to 87.5% (Miyazaki et al. 2005). Clinical signs include extreme lethargy, enophthalmia, generalized oedematous condition, gill hyperplasia and necrosis as well as lamellae clubbing, leading to dyspnoea and hypoxia (Ono et al. 1986, Oyamatsu et al. 1997a, Miyazaki et al. 2005, Lewisch et al. 2014). The severity of disease is greatest in juvenile fish. Typical pathological changes are present in gill tissue and include transformation of epithelial cells of the gill lamellae, with hyperplasia of the filaments. Lesions include infiltrates of eosinophilic granulocytes and macrophages (Ono et al. 1986, Oyamatsu et al. 1997a, Miyazaki et al. 2005). Fish viruses do not infect humans and thus CEV is not harmful to humans.

The diagnosis relied on clinical findings, confirmed by molecular detection of an undefined segment of the viral genome. A nested PCR protocol was initially designed in Tokyo University of Fisheries (Oyamatsu et al. 1997b) and was recently modified in CEFAS-Weymouth Laboratory, England. In order to do an adequate diagnosis of the CEVD/KSD disease koi herpesvirus disease and spring viraemia of carp should be excluded by differential diagnosis. The aim of this paper is introduce with CEVD/KSD and to pay attention to its importance and the need for monitoring in common carp, especially in those countries where it has not been detected, such as Serbia.

CARP EDEMA VIRUS

Carp edema virus is a large, double-stranded DNA virus thought to belong to the poxvirus family of viruses. CEVD/KSD disease should not be confused with another disease referred to as „carp pox", which is caused by a herpesvirus (Cyprinid herpesvirus 1) that causes wart-like growths on the skin in common carp varieties (Hartman et al. 2013). It is a mulberry-like enveloped virus which has been identified in gill cells from diseased fish, and further morphological characterization was provided by electron microscopy (Ono et al. 1986, Oyamatsu et al. 1997a, Miyazaki et al. 2005). Although the disease has been successfully transmitted, using filtrated

suspensions of gill homogenates obtained from sick fish (Oyamatsu et al. 1997a), the virus has yet to be isolated and grown in cell culture; thus, it is currently only detected using PCR-based methods. Currently, little is known about the genetic relationships of CEV and other fish poxviruses. However, comparison of genetic sequences of CEV strains from around the world in common carp and koi have revealed they likely represent a single novel poxvirus species (Way and Stone 2013, Waltzek et al. 2014).

The virus may be transmitted via exposure to contaminated water. Thus, diseased fish shedding virus into the water from gill and skin lesions are likely an important mode of transmission. Whether CEV is vertically transmissible (i.e., from breeders in the eggs or sperm) and whether CEV-exposed fish clear the virus after infection or can harbor the virus as carriers is currently unknown. The length of time the virus remains infectious in water is also unknown.

Common carp and koi carp are the only known susceptible species.

PATHOLOGY

Clinical sings

As the disease name KSD implies, the typical sign of the disease is fish lying on the bottom of the ponds. Fish may swim for a short period of time, but soon settle back into an inactive state on the bottom of the tank (Miyazaki et al. 2005). In CEVD/KSD, weakened juveniles may swim slowly and aimlessly along the margins of the pond or hang motionless just under the surface of the water (Oyamatsu et al. 1997a). Infected fish will often refuse to eat, which further weakens the fish. Diseased carps may have extensive erosions or hemorrhages of the skin with edema (swelling) of the underlying tissues (Miyazaki et al. 2005). Other external signs of infection may include sunken eyes (Haenen et al. 2013), generalized oedematous condition, gill hyperplasia and necrosis (Figure 1, Lewisch et al. 2014). The skin and gill coloration are usually normal, with only a moderate hyperproduction of mucus on the skin (Lewisch et al. 2014). Only when the fish was stressed due to transportation and examination procedures, and when water temperature was raised from 10 to 15°C, clinical signs developed dramatically. Sticky mucus on skin, especially of the fins and gills can be present (Lewisch et al. 2014). Similar external signs (sunken eyes and gill lesions) may also be observed with another important viral disease of common carp-koi herpesvirus disease caused by Cyprinid herpesvirus 3 (Hartman et al. 2013). The disease is typically observed at water temperatures between 15–25°C in koi, and at 6–10°C (Hedrick et al. 1997, Miyazaki et al. 2005, Way and Stone 2013). Recently, CEVD/KSD in common carp and koi has also been reported at lower water temperatures between 7–15°C in Austria (Lewisch et al. 2014).

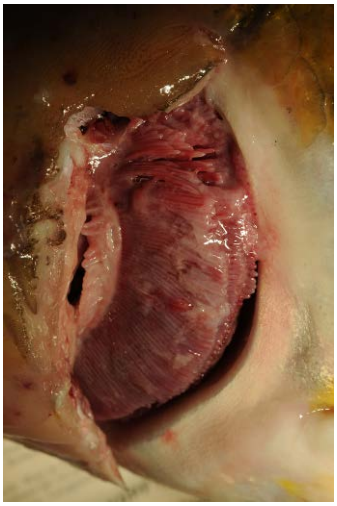

Figure 1. Gill of ommon carp showing pale appearance and abundant mucus (Lewisch et al. 2014)

Histopathology

Typical histopathology lesions include massive chronic gill inflammation, hyperplastic filaments with associated inflammation, showing clubbing and loss of the typical anatomical organization and vacuolation of epithelial cells, extensive destruction of the skin and edema of the underlying tissues (Oyamatsu et al. 1997a, Miyazaki et al. 2005, Lewisch et al. 2014). At necropsy, internal organs of the carp can be adipose, the hepatopancreas can be brittle, pale and patchy. The heart can be enlarged and pale with a spongy consistency of the myocardium. The oedematous gastrointestinal tract also was found without any trace of intestinal contents, but packed with sticky yellow orange mucus (Lewisch et al. 2014). Petechial haemorrhages affecting the swim bladder and oedematous appearance of the internal organs, can be connected with the detection of KSD/CEV as a co-infecting agent.

DIAGNOSIS

Anamnestic information together with the clinical and post-mortem observations can help to better diagnosis of the disease. The high number of secondary infections with ectoparasites and bacteria due to strong immunesuppressive action played by KSVD/CEV can be problem for diagnostic. Carp edema virus diagnosis in common carp can be reached using modified nested PCR assay according to CEFAS-Weymouth Laboratory, England. Also, histopathology can support a carp edema virus diagnosis.

CONCLUSION

CEV/KSD should be considered when spring carp mortality syndrome is observed.

Although an infectious agent is suspected to cause seasonal mortalities in carp populations, no comprehensive investigations are clarifying the role of KSD/CEV.

The best way to prevent CEVD/KSD is to obtain fish free of virus.

It can be helpful to avoid harvest during periods when water temperatures are permissive for the disease.

Always isolate and test sick fish showing suspect clinical signs.

Remove dead fish from ponds immediately to minimize disease transmission.

Freezing, drying out and the disinfection of the fish pond bottom with lime are still very important prophylactic measures.

CEV/KSD should definitively be included in the differential diagnosis during examination for KHVD and SVC.

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PREVALENCE OF CAUSATIVE AGENTS OF SUBCLINICAL MASTITIS IN DAIRY COW

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Abstract: Mastitis is one of the most imprtant economical diseases of high-yielding cattle. The mastitis is mainly caused by a variety of Gram positive and negative bacterial species. There are several different classifications of mastitis. One of the most important is clinical classification on clinical and subclinical mastitis. Diagnosis of acute, clinical mastitis is not difficult. Subacute forms of mastitis represent larger challenge for diagnostic because there are no visible changes in apperance of secreted milk. In spite of several addition tests for diagnosis of subclinical mastitis (California mastitis test – CMT, Somatic cell count, etc) are available, bacterial culture of milk still represent the most reliable method.

Aim of our study was to investigate presence and prevalence of different bacterial species isolated from milk with positive CMT reaction.

In total, we examined 9844 samples of milk in period from 2011 to 2015. From each sample, 0,1ml was taken and cultivated on Columbia blood agar in aerobic conditons on 37^{o}C for 24 to 48 hours. Bacteria were identified by using colony morphology, haemolytic pattern on blood agar and further microsopic examination (Gram staining), standard biochemical methods, polymerase chain reaction (PCR), and commercial testS (BBL Crystal).

From total 9844 samples, bacterial species were isolated in 4381 (44,5%). Most prevalent species were: *Staphylococcus aureus* in 2382 samples, *Streptococcus agalactiae* in 892 samples, *Streptococcus spp.* in 504 samples, *Kllebsiela* spp. in 163 samples, *Escherichia coli* in 133 samples, *Pseudomonas aeruginosa* in 131 samples and *Pasteurella multocida* in 91 samples, *Corynebacterium bovis* in 25 cases and *Trueperella pyogenes* in 14 samples. Beside those, in 25 samples we isolated several different species.

Based upon our results, we concluded that *Staphylococcus aureus* and *Streptococcus agalactiae* represents the most important bacterial pathogens from cases of subclinical mastitis.

Key words: *subclinical mastitis, dairy cow, prevalence, bacterial pathogens*

Plenary lecture

THE EUROPEAN UNION REFERENCE LABORATORY FOR MYCOTOXINS – ACHIEVEMENTS AND WORK AHEAD

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Abstract: Since 2006 the European Union Reference Laboratory (EURL) for mycotoxins acts as key player together the National Reference Laboratories (NRLs) in the EU Member States to assure the harmonization of analytical matters for the determination of mycotoxins in Europe and beyond. Tasks of the EURL are the conduction of comparative tests (proficiency tests) with NRLs and associated reference laboratories. The aims of these tests are next to the benchmarking of laboratory performance the identification of methods used and the identification of robust procedures for future standard methods. Compiling such data on methodologies used in a proficiency test provides not only information to the participants, but also to other laboratories that did not participate in the exercise, thus fostering the improvement of analytical performance on a greater scale.

In addition, the development of new state-of-the-art methods are a key element to meet the demand on the monitoring of increasing amounts of mycotoxins to be monitored. Such state of the art method are usually based on LC-MS multianalyte approaches and the question raises if the performance of methods with a broader scope do differ from those that target a limited number of mycotoxins. To make a prediction, currently generated data is analyzed as we need target performance data to evaluate such methods.

Further, already available data from monitoring, respectively the Rapid Alert System for Food and Feed (RASFF), can be used to identify if there are still options to streamline analytical methods, resulting a reduced workload, while mainlining consumer protection and the demand for monitoring. An example is aflatoxins that might have such potential.

Invited lecture

BENEFITS AND PROSPECTS AFTER 10 YEARS OF THE EUROPEAN UNION REFERENCE LABORATORY FOR ANIMAL PROTEINS IN FEEDINGSTUFFS

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The 23rd of May 2006, the Walloon Agricultural Research Centre (CRA-W) was nominated Community Reference Laboratory (former name for European Union Reference Laboratory – EURL) for Animal Proteins in Feedingstuffs for a mandate of five years renewed permanently in 2011[1,2]. As the other EURLs, its main missions are:

i) to provide a scientific support to the European Commission;
ii) to develop analytical methods;
iii) to coordinate the activities of a lab network composed of the National Reference Laboratories (NRLs);
iv) to assure a quality assistance to the NRLs by organizing proficiency tests and
v) by giving trainings to allow them to keep or improve their skills.

As indicated in the TSE Road map 2[3], the goal of the European Commission is to continue the review of the measures following a stepwise approach supported by a solid scientific basis while holding a high level of food safety. Concerning the feed ban, the framework to any lifting must be in accordance with the following principles: no use of ruminant processed animal protein (PAP), no use of any PAP in feed intended to ruminants and herbivores, no intra-species recycling also commonly named cannibalism. The introduction of the PCR as official method besides the microscopy[4] and the validation of a PCR assay for the detection of ruminant DNA in feed[5] allowed the re-authorization of non-ruminant PAP in feed for aquaculture animals since the 1st of June 2013[6]. The next steps could be the use of poultry PAP for pigs and pig PAP for poultry but only after the validation of analytical tools (e.g. PCR assays) allowing an efficient control. Due to interferences of authorized animal ingredients (e.g. fats, blood products, dairy products…) with the PCR results, additional analytical tools will probably be needed but this point will be described below.

One of the main outputs of the EURL-AP was to create a framework for the analytical methods to have a harmonized application throughout all the Member States. Besides the Commission Regulation No 51/2013 naming the methods of analysis for the determination of constituents of

animal origin for the official control of feed, Standard Operating Protocols (SOPs) provide complements which are most of the time binding to the legal text with well-detailed procedures at the laboratory level. Despite the strict format and the conditions of application, these documents give a better flexibility in case of urgent change in the protocols and the versions in force are easily downloadable on the website of the EURL-AP (http://eurl.craw.eu). European Reference Materials (ERM) are also provided by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC – European Commission) to calibrate the PCR platforms with a high quality reference.

The analytical problems coming from the authorized ingredients of ruminant origin can be managed relatively easily in the case of the aquafeeds. The main source of interference is linked to the use of dairy products but they are not widely utilized for economical (they are costly) and physiological (they are not well-digested by fishes) reasons. These materials can be sometimes included in feeds for fish but only for technological purposes and in very low concentrations. The situation is not the same for the feeds intended for pigs and poultry where much more animal ingredients can be present. For the moment, the control can essentially be conducted with light microscopy but further lifting of the feed ban will need complementary methods to assure the absence of prohibited ingredients. In addition to its own research, the EURL-AP initiated already in 2014 an international network of labs making investigations and developments with alternative techniques such as aptamers (BfR in Germany), mass spectrometry (Fera and University of York in UK, NIFES in Norway and University of Namur in Belgium) and ELISA (CER Group in Belgium). The aim was to stimulate the collaborations between all these institutes to speed up the analytical improvements. Internally, the CRA-W works on Fluorescence In situ Hydribization (FISH) combining light microscopy and molecular biology[7]. Specific ELISAs were also considered for the detection of milk proteins (caseins and β-lactoglobulins). The most promising method is probably the mass spectrometry. These last three years the identification of proteins and peptide biomarkers allowing the detection of PAPs by mass spectrometry gave very interesting results[8] but the efforts must be continued and the way towards a validation and an implementation in the control labs is still long.

Within the ten last years, the knowledge about the detection of PAP by light microscopy, immunological methods or PCR was greatly improved by number of studies conducted by the EURL-AP (http://eurl.craw.eu/en/176/scientific-reports). Challenges are still in front of us: besides the tricky problem of authorized ingredients of animal origin, the use of insects as a new source of proteins in feed is probably the hottest emerging topic.

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PHYTOBIOTICS – MODERN NUTRITIVE SOLUTION IN INTENSIVE FARMING OF NON-RUMINANTS

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Abstract: After the ban of using antibiotics as growth promoters in animal feed (Regulation EC No 1831/2003 of the European Parliament and of the Council) a large interest has developed for finding the appropriate alternative solutions that would primarily support the function of the indigenous microflora in the gastrointestinal tract in the control of pathogenic bacteria. Phytogenic feed additives (phytobiotics or herbal drugs) represent a new generation of growth stimulators and are defined as compounds of plant origin used in animal nutrition in order to enhance their productivity by improving production results of animals, feed properties, as well as the quality of food of animal origin. According to the biological origin, formulation, chemical composition and purity they can be classified into four subgroups: herbs, spices, essential oils and oleoresins. In addition to the proven antibacterial effects, phytobiotics exhibit coccidiostatic, antihelmintic and antiviral effects, while the results of recent studies indicate their anticancer and antioxidant effects. In a series of experiments conducted by Department of Nutrition and Botany of Faculty of Veterinary Medicine, University of Belgrade, the nutritional, medical and economic justification of phytobiotics use in pig and poultry nutrition has been confirmed. Phytobiotics stimulate the secretion of digestive juices, establish eubiosis in the digestive tract of the host, make a positive impact on the morphological characteristics of the lining of the digestive tract (increasing the length of the villi while reducing the depth of the crypt), which directly affects the utilization of nutrients consequently resulting in increased productivity of animals.

Key words: *phytobiotics, non-ruminants, growth stimulants, eubiosis*

INTRODUCTION

Special attention in scientific circles, and certainly among the manufacturers, has always been directed toward finding the adequate nutritional strategies that could improve animal performances and with the same time make production process cheaper. For this reason, during the formulation of feed mixtures, except of basic ingredients, a large number of pronutritive substances are added in the mixtures, ie micronutrients which enter orally in relatively small quantities and improve the nutritional value of meals. On the basis of the existing regulation on the quality of animal feed in the Republic of Serbia (Rulebook of RS 41/09, Article 74) these pronutritives (additives) are classified as follows: 1. Vitamins and provitamins; 2. Minerals and trace elements; 3. Non-protein nitrogen compounds; 4. Amino acids; 5. Growth stimulators; 6. Coccidiostats; 7. Other allowed additives. Within the European Union, European Commission (European Commission) defines feed additives as products used in animal nutrition in order to improve feed quality and quality of food of animal origin, or in order to improve performance and animal health, primarily by increasing the digestibility of nutrients. Based on the categorization of the European Commission listed additives are classified into the following groups: 1. Technological additives (feed preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, silage additives); 2. Sensory additives (flavors, colors); 3. Nutritive supplements (vitamins, minerals, amino acids, trace elements); 4. Zootechnical additives (digestibility enhancers, stabilizers of intestinal flora); 5. Coccidiostats and histomonostats.

Among the many compounds, antibiotics represent the oldest and in the past the most commonly used feed additives in stimulation of animal's growth. The use of antibiotics in rearing pigs nutrition has resulted in increase of performances approximately 4–5%, while in fattening pigs 1.5–2% (Sigvard and Elwinger 1998). In addition to the positive, harmful effects of the use of antibiotics have been described, like the development of resistant strains of *Enterobacteriaceae*, which, still represent a serious problem in the treatment of sick animals, as well as people. The problem of resistant strains is complicated by the cross-resistance, which is the consequence of adaptive capacity of microorganisms and mutagenic effects of antibiotics. Next, common and certainly more significant problem is the presence of antibiotics residues in food of animal origin, as well as possible genotoxic effects of antibiotics and their residues (Sinovec 2000).

Sweden is considered to be the leading country in prohibition of using antibiotics as growth promoters in animal nutrition in Europe and has banned their use in year 1986. Ever since that time, the use of antibiotics is permitted only in treatment or prevention of the disease and exclusively with the veterinary prescription for its use. Successful ban on the use of antibiotics in Sweden and a new approach in animal breeding did not have

important response and the impact on other European countries until the early 90ˋs when has began to strengthen the consumer association and „consumerism‟ as a mode of thinking and marketing. The Scientific Committee of the European Union (Scientific Steering Committee) has performed in 1999 the screening on use of antimicrobial substances in the EU and confined the use of antibiotics in animal nutrition. Following these measures, on 22nd September of 2003, regulation has been given (Regulation EC No 1831/2003 of the European Parliament and of the Council) on additives use in animal nutrition where was outlined that all antibiotics, other than coccidiostats and histomonostats, may be marketed and used as additives only until 31st December of 2005, and that from 1st January 2006, those substances shall be deleted from the Register. In accordance with these restrictive measures, Rulebook on the quality of animal feed in the Republic of Serbia, which became applicable on 1st May 2010, Article 98, states that mixture of animal feed must not contain antibiotics and sulfonamides.

Along with these changes in feeding strategies, a large interest has appeared for developing appropriate alternative solutions, which would primarily support the function of indigenous microflora in the gastrointestinal tract in control of pathogenic bacteria. The last decade is characterized by the introduction of different options and nutritive solutions for control of enteropathogenic bacteria and stimulation the animal growth. A new generation of growth stimulators are probiotics, prebiotics and phyitobiotics, by which use similar effects can be achieved as with antibiotics, but they do not leave residues, nor do they have waiting period.

PHYTOBIOTICS, THE CONCEPT, DEFINITION AND COMPOSITION

Phytogenic feed additives (phytobiotics or herbal drugs) are defined as compounds of plant origin, used in animal nutrition in order to improve their productivity by improving production results of animals, feed properties, as well as the quality of food of animal origin (Windisch et al. 2008). According to the biological origin, formulation, chemical composition and purity they can be classified into four groups: herbs (flowering, nonwoody, and nonpersistent plants), spices (herbs with an intensive smell or taste commonly added to human food), essential oils (volatile lipophilic compounds derived by cold expression or by steam or alcohol distillation), or oleoresins (extracts derived by nonaqueous solvents) (Windisch and Kroismayr 2006).

Plant extracts are one of the oldest food additives known to humanity. For thousands of years herbs and spices were used as flavorings, as well as substances, which due to its antimicrobial properties, participate in the preservation of food. Extracts of plants contain a large number of active substances in a variable quantity and with a different chemical

structure and composition, depending on the extraction method and the plants characteristics. Different authors have point to significant differences in composition between geographic regions or time of collection of plants (Juliano et al. 2000, Faleiro et al. 2002). Essential oils (EO) obtained after harvesting the plants during the summer, immediately after flowering, exhibit the most prominent antimicrobial activity (McGimpsey et al. 1994, Marino et al. 1999), while Delaquis et al. (2002) reported a significant effect of plants part from which the EO is extracted. It can be concluded that the same plant species can produce similar essential oils, however, with a different chemical composition, leading to differences in therapeutic activities. For these reasons it is generally accepted, for the purpose of practical application, the use of the pure derived substance, naturally derived or chemically synthesized, i.e. active principles.

Essential oils or aromatic plant essences (EO) are volatile and aromatic substances with oily consistency, produced by plants. They generally have liquid consistency and different colors ranging from pale yellow to emerald green, or blue to dark brown or red (Balz 1999). Essential oils are synthesized in most plant organs, and then deposited in the secretory cells, cavities, channels, epidermal cells or glandular piles (Bakkali et al. 2008). Different parts of the plants, where essential oils are placed, usually have pleasant smell (Lis-Balchin 1997).

Several techniques are used for extraction of the EO from different parts of aromatic plants, and most commonly used are water or steam distillation, extraction with solvents under pressure as well as super and subcritical water extraction (Kovačević 2004). Distillation as a method for EO extraction is known for more than 2000 years and was used in ancient times in Egypt, India and Persia, while the first written document of their obtaining has presented the Catalan physicist Valanova in the XIII century (Bakkali et al. 2008).

Paracelsus von Hohenheim is the first who used the term „essential oil‟ in the XVI century, which was related to the effective component of the drug as „*Quinta essentia*‟ (Guenther 1948). The first reference of the use of EO in therapeutic purposes was found in the Ebers papyrus (1550 BC), where was in detailed more than 800 medicines and treatments based on EO described. Because of its ability to inhibit the growth of bacteria, myrrh, often mixed with honey and other plants, represented the most important medicinal ingredient. The first experiment on the bactericidal properties of EO was carried out by De la Croix in 1881 (Boyle 1955). However, over time the use of EO in veterinary and human medicine gradually decreased, while their use as flavoring substances increased (Burt 2004).

The essential oils consist on a large number (20–60) of the individual components which are present in various concentrations, whereby the dominant components (2–3) represent up to 85% EO (Table 1). The main components (active principles) determine biological/antibacterial properties of essential oils, but also the presence of other, less common, components

play a important part in the manifestation of those activities (Bakkali et al. 2008, Burt 2004). Identification of active principles which are the representatives of named effects, but also the development of new technological solutions (encapsulation) for their production, extracts obtain an increasingly important role as additives in animal nutrition so that they now represent the latest generation of growth promoters.

Table 1. The main components of EO (adopted from Burt 2004).

The plant	Main component of EO in plant (active principles)	Percentage of main component in EO
Coriandrum sativum (seed)	Linalool	-70
Cinnamomum zeylandicum	Trans cinnamaldehyde	-65
Origanum vulgare	Carvacrol	-80
	Thymol	-64
	g-Terpinene	2-52
	Cymene	-52
Rosmarinus officinalis	a-pinene	2-25
	Bornyl acetate	0-17
	Camphor	2-14
	1,8-cineole	3-89
Salvia officinalis L	Camphor	6-15
	a-Pinene	4-5
	h-pinene	2-10
	1,8-cineole	6-14
	a-tujone	20-42
Syzygium aromaticum	Eugenol	75-85
	Eugenyl acetate	8-15
Thymus vulgaris	Thymol	10-64
	Carvacrol	2-11
	p-Cymene	10-56

ANTIMICROBIAL EFFECTS OF ESSENTIAL OILS

Many authors in the past have investigated antimicrobial properties of essential oils but only in recent decades active principles are identified, as well as relations between their antimicrobial activity, the percentage of participation, the chemical structure, functional groups, and configurations (Dorman and Deans 2000). Essential oils that contain, as the main component, phenols or aldehydes (cinnamaldehyde, citral, carvacrol, thymol, eugenol) have showen the strongest antibacterial activity, while EO which contain terpene alcohols have a somewhat lower activity. Other EO, which contain ketones or esters, such as β-myrcene, α-thujone or geranyl

acetate, exhibit a significantly weaker activity, whereas oils with a terpene hydrocarbons usually are inactive (Dormans and Deans 2000, Ait-Ouazzou et al. 2011). Friedman et al. (2002) examined the antimicrobial effects of 96 different essential oils as well as 23 their constituents (active principles) and demonstrated that cinnamaldehyde, thymol, carvacrol, and eugenol exert the strongest antibacterial activity against strains of *E. coli, Salmonella enterica*, and *L. monocytogenes*.

Direct manifestation of the antibacterial activity of the essential oils is significantly conditioned by size and shape of their constituent molecules, as well as the solubility in water (Shapiro and Guggenheim 1998). Juliani et al. (2009) pointed out the importance of the chemical structure of essential oils finding that the antimicrobial activity of eugenol is higher compared with the activity of methyl-eugenol. Phenols containing an isopropyl group in the para-position, such as carvacrol and thymol, generally exhibit a strong antimicrobial activity. Position of specific groups within the same family of compounds is also important and contributes to the differences in antimicrobial activity. Among the tested isomers of 2-tert-butyl-4-methyl-phenol, 2-tert-butyl-5-methyl-phenol, and 2-tert-butyl-6-methyl-phenol, only the first of the two isomers having a methyl group on meta- or para-position of the benzene ring can exhibit antibacterial activity. Structural features such as the presence of the aromatic ring (thymol and carvacrol), hydroxyl group (thymol, carvacrol and eugenol) or a „major" group such as tert-butyl or isopropyl can change polarity and the topography of the molecule and consequently alter the binding affinity of the EO active principles for bacteria. Table 2 shows the main structure of the plant extracts that are responsible for their antimicrobial activity and their *modus operandi*.

Table 2. Chemical structures involved in antibacterial effects of EO and mechanism of their action (adapted from Cowan 1999).

Class	Subclass	Mechanism
Phenols	Phenols and phenolic acids	Enzyme inhibition
		Membrane disruption
	Quinones	Bind to adhesins, complex with cell wall, inactivate enzymes
	Flavones	Complex with cell wall
	Tannins	Bind to proteins, bind to adhesions, enzyme inhibition, substrate deprivation, metal ion complexation, membrane disruption
	Coumarins	Interaction with eucaryotic DNA (antiviral activity)

Terpenoids, essential oils	/	Membrane disruption
Alkaloids		Intercalate into cell wall and/or DNA
Lectins		Block viral fusion or adsorption
Polypeptides		Form disulfide bridges

Considering the fact that essential oils are composed of a large number of ingredients, it can be assumed that their antimicrobial activity is not tied to a specific mechanism of action but is directed towards slightly different objectives in microbial cell (Skandamis et al. 2001, Carson et al. 2002). Operating modes of EO are degradation of the cell wall, cytoplasmic membrane damage, damage of membrane proteins, loss of cell contents, coagulation of the cytoplasm and the depletion of the proton gradient (Burt 2004). Although the antimicrobial properties of essential oils and their active principles are the subject of investigations of numerous authors, it must be noted that the precise mechanisms of action is still not fully understood (Lambert et al. 2001).

Most authors consider that EO achieve antibacterial effect via two different mechanisms: the first is related to their hydrophobicity, which allows them to be locked into the phospholipid bilayer of the cell membrane, while the second relates to the inhibition of bacterial enzymes and receptors by binding to specific sites inside of cell. Thanks to the hydrophobic structure, EO are able to destabilize and change the permeability of the bacterial membranes (Knobloch et al. 1989, Sikkema et al. 1994, Oosterhaven et al. 1995, Ultee et al. 2000, 2002). Those changes lead to the release of ions from inside the cell into the environment (Oosterhaven et al. 1995, Cox et al. 2000, Lambert et al. 2001), as well as a change in the proton gradient and depletion of intracellular reserves of ATP (Ultee et al. 1999, Sikkema et al. 1995, Davidson 1997). Depletion of ions is usually associated with exit of other cytoplasmic constituents, and that is something what bacterial cells to a certain moment can tolerate without loss of vitality, but if the specified exit increases result is death of the cell.

The possibility of the passage of particulate substance through the cell membrane is dependent on its hydrophobicity, as well as the composition of the membrane itself (Sikkema et al. 1995). Refusal or the possibility of adhesion and the passage of essential oils through the cell membrane is of key importance in determining their effectiveness against a variety of bacteria, especially when tested bacteria with different Gram staining (Lambert et al. 2001). Most authors agree that the antibacterial activity of the EO is more pronounced against Gram-positive than Gram-negative bacteria due to the presence of the outer membrane that surrounds the cell wall of Gram-negative bacteria and limits the diffusion of hydrophobic compounds through the present lipopolysaccharides. Si et al.

(2006) have demonstrated that *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium spp*. show less sensitivity to the effects of carvacrol, thymol and eugenol compared to pathogenic bacteria (*E. coli* and *S. typhimurium*). Similar results reported Manzanila (2005), who conducted the trial on piglets and confirmed positive effect of plant extracts (carvacrol and cinnamaldehyde) on the number and ratio of lactobacilli and enterobacteria in the jejunum. The results indicated the possibility of using essential oils for maintaining a desirable intestinal flora, or eubiosis.

THE USE OF PHYTOBIOTICS IN NUTRITION OF NON-RUMINANTS

The interaction of nutrition, genetics, endogenous regulatory mechanisms and environmental manage the functional development of organ systems, including the development of the gastrointestinal tract and significantly affect the later productivity of animals (Adeola and King 2006). Growth of animals depends in large part on the ability of its gastrointestinal tract to digest and assimilate macromolecules taken into the body, and any reduction in these activities, results in slowing the growth. Postnatal development of the gastrointestinal tract, the introduction and development of intestinal digestive enzymes and intestinal capacity to absorb nutrients are among the most important factors that enable animals to utilize ingested macromolecules (King et al. 2000). At the same time, the mucosa presents physical and immunological barrier against harmful substances, including bacteria, viruses, parasites and allergens (Kato and Oven 1994). Intestinal capacity to fully realize the described functions is not permanent but it changes and adapts to many factors that influence its development, including nutrition, microbial colonization, stress, rearing and environment conditions (Henning 1987, Lebenthal 1989, Mosenthin 1998).

The importance of establishing a stable microbial community in the gut reflects throughout the numerous beneficial effects and symbiotic relationship that is realized with the host. Microbiota forms the most important barrier against pathogens and has an important impact on the morphology of the intestines (Coates et al. 1963), on develop of immunity (Pabst et al. 1988) and digestion of nutrients (Wostmann 1996). Present microbiota prevents the colonization of upcoming bacteria by process called colonization resistance, which represents the first line of defense against the invasion of exogenous, potentially pathogenic microorganisms or the indigenous opportunists (Rolfe et al. 1996, Hooper et al. 1998).

The use of phyitobiotics stimulates the secretion of digestive juices, establishes eubiosis in the digestive tract of the host, achieves a positive impact on the morphological characteristics of the lining of the digestive tract (by increasing the length of the villi while reducing the depth of the crypt), which directly affects the utilization of nutrients and in the final outcome results in increased productivity.

Considering the intense aromatic odor of phytobiotics that may adversely affect the overall feed consumption, of practical interest is to investigate the possibilities of combining different active principles or essential oils in order to reduce the amount of used material and thereby produce the desired antibacterial effect at concentrations that do not cause undesirable changes. In accordance with the aforementioned, Zhou et al. (2007) have organized the experiment where have first determined the lowest concentration (200, 400 and 400 mg/kg, respectively) of cinnamaldehyde, thymol and carvacrol, whose single use exhibited a significant inhibitory effect on the growth of *Salmonella typhimurium*. During concomitant use of the named combination of active principles, (cinnamaldehyde/thymol, cinnamaldehyde/carvacrol and thymol/carvacrol) the authors demonstrated a synergistic effect in relation to a single use. The use of cinnamaldehyde has shown the possibility for decreasing concentration of the previously mentioned thymol and carvacrol down to levels of 100 mg/kg. Michiels et al. (2009) have also used different amounts of thymol and carvacrol and confirmed synergistic effect of this combination toward the total number of anaerobic bacteria in the jejunum of piglets.

Along with foreign researchers, Department of Nutrition and Botany from Faculty of Veterinary Medicine, University of Belgrade, have organized a series of experiments in order to examine the effects of using phytobiotics in pigs and poultry nutrition on health status and growth production results under commercial production conditions characteristic for our country. The results of two experiments are shown in Tables 3 and 4.

The first trial lasted 40 days and was divided into two phases, each lasted 20 days. The experiment included 24 pigs, crossbreds of Swedish Landrace and Pietrain, weaned from the sow at the age of 35 days. Tests were conducted on piglets of both sexes, with an average body weight 8.61 ± 1.59 kg, which were immediately after the weaning assigned in one of two feeding treatments. The control group was fed diet without growth stimulators, while the experimental group received a diet containing preparation of phytobiotic (Enviva EO 101, Danisco Animal Nutrition) in the amount recommended by the manufacturer (0.1 kg/t). Feed mixtures for piglets were formulated in accordance with the recommendations of NRC (1998) and AEC (1993) and fully satisfied their nutritional requirements. During the experiment, there was no disturbance of health condition and/or appearance of clinical signs of disease. The use of preparation of phytobiotic as growth stimulator has resulted in better production results compared to the control group, based on the achievement of a higher body mass (27.19 ± 4,77kg), higher average daily gain (0.46 ± 0.09 kg) and better feed conversion (2.043).

Table 3. Production results achieved during the trial (average body weight, average daily gain, average daily feed intake and feed conversion-feed to gain ratio)

The tested parameter [kg]		Experimental group	
		Control group K	Experimental group O-I
Initial average body weight***** [1st day]		8,53±2,20	8,82±1,40
Period of trial 1-20.days			
	Average daily gain *****	0,23±0,15	0,23±0,09
	Average daily feed intake	0,49	0,49
	Feed conversion	2,130	2,130
Period of trial 20-40.dan			
	Average daily gain *****	0,61±0,13	0,68±0,16
	Average daily feed intake	1,29	1,40
	Feed conversion	2,115	2,059
Period of trial 1-40.dan			
	Average daily gain *****	0,42±0,12	0,46±0,09
	Average daily feed intake	0,89	0,94
	Feed conversion	2,119	2,043
Final average body weight***** [40th day]		25,32±6,31	27,19±4,77

***** Values are expressed as $\overline{X}$ ±Sd

Simultaneously with recording the performances during the trial microbiological tests were also performed in the proximal and caudal segments of the intestine (total number, as well as the identification of bacterial species) and morphometric examination of the lining of the intestine. Adding the phytobiotic in diet resulted in reducing of total number of bacteria in the jejunum and in the cecum (10^6 vs. 10^7 and 10^8 vs. 10^9, respectively) compared to the values determined in the control group of piglets, with significantly narrowed range of species of present bacteria and at the same time an increase in the presence of bacteria, characterized as "useful" microflora (*Lactobacillus spp.*). The results of morphometric analysis indicated the positive effect of phytobiotic. The height and width of the villi in the jejunum increased and the crypt depth lowered compared to the control while the depth of cecum crypts was higher hence increasing the absorptive capacity of the lining of the intestine.

In the second experiment, a total of 240 broilers, Cobb 500 provenience, were used, divided into four groups (control – K and three experimental groups O-I, O-II and O-III) with 60 animals in each. The animals were fed standard diets (NRC 1994) for broilers (starter 0–7 days,

grower 8–20 and finisher 22–42 days), with a difference that the experimental group receaved a meal with preparation of phytobiotics, added in quantities recommended by the manufacturer: O-I group, phytogenic additive (Enviva) containing thymol and cinnamaldehyde, 100 g/t of feed; O-II group, phytogenic additive (Digestarom) containing caraway seeds and a mixture of essential oils of mint, clove and anise, 150 g/t of feed and O-III group, phytogenic additive (Kokciguard) containing thymol, 750 g/t of feed. Observing the entire period of the experiment, broilers from all experimental groups with added phytobiotics achieved a higher average daily gain, lower daily food intake with lower (better) feed conversion. Results of the trial are shown in Table 4.

Table 4. Production results achieved during the trial (average body weight, average daily gain, average daily feed intake and feed conversion).

The tested parameter **[g]**	**Experimental group**			
	K	O-I	O-II	O-III
Average body weight of broilers 1st day	46,80±3,27	46,93±3,83	46,33±3,57	46,40±2,91
Experimental period 1–20 days				
Average daily gain	697,27±81,37	706,43±70,77	710,83±86,35	724,27±85,11
Average daily feed intake	66,53	61,21	59,23	65,15
Experimental period 21–42 days				
Average daily gain	$1686,90^{A,B}$±162,52	$1814,33^{A}$±237,29	1766,07±192,03	$1825,93^{B}$±209,84
Average daily feed intake	167,38	159,96	156,20	156,49
Experimental period 1–42 days				
Average daily gain	$2384,67^{A,B,a}$±139,22	$2520,77^{A}$±221,66	$2466,60^{a}$±182,82	$2550,20^{B}$±220,87
Average daily feed intake	115,37	109,13	110,81	109,27
Feed conversion *	2,03	1,82	1,89	1,80
Average body weight of broilers 42nd day	$2430,97^{A,B,C}$ ±139,14	$2567,70^{A}$±222,46	$2526,23^{B}$±183,94	$2596,60^{C}$ ±220,82

The same letters within the row A, B, C, D, F, G, H ($p<0,01$), a,b ($p<0,05$)* [kg]

LEGAL ASPECTS OF USING PHYTOBIOTICS IN ANIMAL NUTRITION

According to the current regulations of the European Union (EC No 1831/2003) on feed additives, phytobiotics are in the Register (European Union Register of Feed Additives), mainly classified as second category of supplements, defined as sensory additives. Only a small number of commercial phytobiotic products falls under the fourth category of supplements called zootechnical additives. The abovementioned regulation defines sensory additives as substances that after adding to feed can enhance or modify its organoleptic properties or the visual characteristics of the food of animal origin that are used in human nutrition. Sensory additives are divided into two groups (color and aromas). Aromas are defined as substances that after addition in different feed can enhance their smell and taste (palatability). Under the term aroma are included natural flavors or their corresponding synthetic, chemically defined compounds and natural products of plant origin (essential oils, extracts, tinctures and oleoresins). EFSA (2009), also states that phytobiotics, like all other feed supplements of plant origin, belong to and are subject to the regulations of the sensory additives-aromas. Food Safety and Drug Administration (FDA, 2013) have marked a large number of essential oils, oil resin, and natural extracts as substances that are generally accepted as safe for use (Substances geneally recognized as safe-GRAS). In the Republic of Serbia, on the basis of the Regulation on the quality of animal feed (Rule book of RS, No. 41/09, Article 88) phytobiotics are classified under the category of growth stimulators.

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Invited presentation

MONITORING OF FEED FOR THE PRESENCE OF ANIMAL PROTEINS IN SERBIA – DECADE OVERVIEW AND CLOSE PERSPECTIVE

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Abstract: Transmissible spongiform encephalopathies (TSE) were among more than 30 new diseases detected during the last decades of the 20th century. They have caused great attention as they are fatal and caused by prion, non-typical infectious agent. Bovine spongiform encephalopathy (BSE) has a special place on the epidemiology world map because it is linked to the variant-Creutzfeldt-Jakob illness in humans. It is food-born disease, so from the moment when the theory of contamination through the infected ruminant protein in meat and bone meal (MBM) has been set up, legislation has been established to avoid MBM entering the food chain. Total MBM ban for all farm animals, except fish meal for non-ruminants, has been established in EU legislation. In Serbia, until 2011 presence of these nutrients was officially banned just in the diets for ruminants. Such partial limitation caused more frequent cross contamination of feed for ruminants with prohibited ingredients. Therefore, feed producers were obligated to separate lines for ruminant feed, or to dispose MBM and fish meal. Official monitoring of implementation of regulations has been carried out from 2006 by laboratory testing of feed for the presence of constituents of animal origin by classical microscopy in the Scientific Institute of Veterinary Medicine of Serbia in Belgrade and the Scientific Institute of Veterinary Medicine in Novi Sad. One decade summarized analysis (2006–2015) show declining trend of usage of forbidden feedingstuffs. Comparison of the latest results and those from early years, demonstrate the improvement in Serbian feed industry. Success of harmonization of legislation and control system is also obvious. But, new challenges and adjustments are ahead. Loosening of the feed ban in EU started in 2013 with permission to use MBM in feed for aquaculture. Such situation required new feed control system which can achieve sufficient sensitivity and also be able to identify animal species, so EURL for animal proteins in feed validated and published appropriate real-time PCR protocol. In order to go side by side with new European requirements, closer cooperation with the EURL is needed. Therefore it is necessary to form a national reference laboratory (NRL).

Key words: *feed, microscopy, TSE*

INTRODUCTION

Processed animal proteins (PAP) for use in animal nutrition are obtained largely as by-products of dairies, slaughterhouses and fish processing industry. These feedingstuffs are characterized by a high percentage of proteins (over 50%, or some types even close to 80%) of high biological value, because of the ideal amino acid composition. For this reason, especially in developed countries, they had been often used in breeding of highly productive animals. However, after the outbreak of bovine spogiformne encephalopathy (BSE), commonly known as „mad cow disease" and diagnosed in the UK in 1986, it was found that it spreaded by feed through the infectious ruminant proteins processed into meat and bone meal (MBM). Eradication started immediately and one of the most important measures was to establish the regulations to avoid entry of these nutrients into the food chain (Nesic and Radosavljevic 2014).

EU Regulations 999/2001 (Off. J. EC, 2001) and 1234/2003 (Off. J. EU, 2003) prohibit the application of PAP, which include the various types of MBM, to all farm animals entering the human food chain, except fish meal to non-ruminants. Also, regulation 1774/2002 (Off. J. EC, 2002) repealed by regulations1069/2009 (Off. J. EC, 2009) and 142/2011 (Off. J. EC, 2011), prescribed general guidelines for the safe use of by-products of animal origin and prohibited the use of proteins originating from the same species in animal nutrition. Such rigorous measures were made due to the fact that incriminated ingredients pose potential to cause an infection with prions not only for the animals, but also indirectly for people, through food of animal origin.

On the other hand, a total ban on meat and bone meal usage in the diet of animals, although very successful in terms of eradication of transmissible spongiform encephalopathies (TSE), is rejection of highly valued proteins, which has brought great losses in the economic and environmental sense. It is believed that annual production of animal by-products from the meat, milk and eggs supply in the European Unionis is about 17 million tons. Therefore, not only because of the nutritional importance, but also in terms of sustainability, re-introduction of processed animal proteins in the diet of farm animals would have had numerous advantages. It is estimated that this step would provide an annual profit of about 350 million euros (Woodgate et al. 2006).

The control system in Serbia, in comparison with the countries of the European Union, was somewhat more complex, with the differences in regulations and preventive measures that have been applied. In 2001 amendments to the Directive on quality and other requirements for feed

(Pravilnik, *Sl. list SRJ* 28/2001) for the first time officialy banned animal by-products in the diet of ruminants, while their presence was still allowed in diets for monogastric animal species. Identical measures were prescribed by Directive on the quality of feed (Pravilnik, *Sl. glasnik RS* 4/2010) which came into force on 1st of May 2010. However, Directive on determining, diagnosis and preventing of transmissible spongiform encephalopathies (Pravilnik, *Sl. glasnik RS* 96/2010) from 1st of April 2011 introduced a total ban of MBM for all farm animals, equal to the prevention in Europe. Partial feed ban, although preferable for economic and nutritional reasons, caused more frequent cross contamination of feed for ruminants with prohibited ingredients. Therefore, according to the Veterinary Law, Article 110 (*Zakon o veterinarstvu, Sl. glasnik RS* 91/2005) feed producers were obligated to separate lines for ruminant feed, or to dispose MBM and fish meal. Control of these conditions has been conducted by laboratory tests of feed for the presence of meat and bone and fish meal using classical microscopy, as prescribed by EU regulations, since 2006. From 2011 this monitoring has become a part of the annual state Programme of measures for animal health care.

However, changes in EU regulations that lead to the relaxation of ban began from mid-2013 with the introduction of animal proteins derived from poultry and pigs in feed for aquaculture. By the Regulation 51/2013 (Off. J. EU 2013), in addition to classical microscopy, PCR method is approved for the detection of species of animals from which the ingredients originate. EU Reference Laboratory for animal proteins in feed has validated real-time PCR protocol for ruminant DNK. On the 1st of April 2016 amendments on Directive on determining, diagnosis and preventing of transmissible spongiform encephalopathies (Pravilnik, *Sl. glasnik RS* 33/2016) were published as a result of harmonization with the EU legislation.

The aim of this paper is to present the results of one decade work in this area in Serbia and to point out the tendencies and tasks that lie ahead.

MATERIAL AND METHODS

In order to monitor the implementation of feed regulations for the prevention of TSEs, official program has started in 2006 and has been carried out annually. Samples of complete feed for cattle, sheep, goats, pigs and poultry have been taken by veterinary inspectors in feed mills all over the country and sent to the Scientific Institute of Veterinary Medicine of Serbia in Belgrade and the Scientific Institute of Veterinary Medicine in Novi Sad for laboratory testing on presence of ingredients of animal origin. Applied method was classical light microscopy, ISO 17025 accredited and performed in accordance to Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, Annex VI (Off. J. Eur. Union 2009) and Regulation (EC) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as

regards the methods of analysis for the determination of constituents of animal origin for the official control of feed (Off. J. Eur. Union 2013). The total number of samples for one decade period (2006–2015) was 6803 with variations among years.

RESULTS AND DISCUSSION

During 2006 total of 274 samples of feed mixtures for different animal species were tested by classical microscopy (Table 1). Most of them were feed samples for pigs and poultry – 160 in total and among them 21.25% were found positive for the presence of particles of animal origin, MBM or fish meal, which were both allowed at that time according to legislation (Pravilnik, *Sl. list SRJ* 38, 2001). This was the cause of the relatively high percentage of cross-contamination of feed for ruminants: from 8.26% positive samples of cattle feed to even 25% of feed for sheep, although only four of these samples was not enough for the relevant statistical analysis. Such feed was excluded from further usage.

Table 1. Results of microscopic examination of 274 different feed samples in 2006

	cattle	sheep	goats	Pigs and poultry
TOTAL	109	4	1	160
Negatives	100	3	1	126
Positives	9	1	-	34
% of positives	8.26	25.00	0.00	21.25

In 2007 more extensive control was undertaken with total of 1802 samples examined all over Serbia (Table 2). Thanks to the educational programs that began in parallel with the monitoring, more feed manufacturers became aware of the necessity of applying preventive measures in order to avoid cross contamination. Since then, more of them were choosing to stop the use of MBM, while only a small number was able to separate the lines for production of feed which contained those ingredients (*Zakon o veterinarstvu, Sl.glasnik RS* 91/2005). It was still 7.2% of feed for cattle and 12.5% of feed for sheep declared unusable.

Table 2. Results of microscopic examination of 1802 different feed samples in 2007

	cattle	sheep	goats	Pigs and poultry
TOTAL	736	32	0	1034
Negatives	683	28	0	748
Positives	53	4	0	286
% of positives	7.20	12.50	0	27.66

During the next years further decline in the number of positive and inadequate results was noted. As it is showen in the Table 3 in 2008 among total of 917 samples, 577 samples of feed for cattle were analysed and 4.68% of them were positive. Also 10.34% of feed for sheep and 17.36% of feed for pigs and poultry contained particles of animal origine.

Table 3. Results of microscopic examination of 917 different feed samples in 2008

	cattle	sheep	goats	Pigs and poultry
TOTAL	577	29	0	311
Negatives	550	26	0	257
Positives	27	3	0	54
% of positives	4.68	10.34	0	17.36

During laboratory tests in 2009 even lower percentages of positives were detected (Table 4), except for one sample of feed for goats, which can not be considered a relevant indicator.

Table 4. Results of microscopic examination of 657 different feed samples in 2009

	cattle	sheep	goats	Pigs and poultry
TOTAL	421	35	1	200
Negatives	412	34	0	176
Positives	9	1	1	24
% of positives	2.14	2.86	100.00	12.00

In the Table 5, among 547 feed samples, 1.82% of feed for cattle made them unusable as intended, because they did not correspond to the Regulation (Pravilnik, *Sl. glasnik* 4/2010, Pravilnik, *Sl. glasnik* 96/2010).

Table 5. Results of microscopic examination of 547 different feed samples in 2010

	cattle	sheep	goats	Pigs and poultry
TOTAL	329	23	6	189
Negatives	323	23	5	176
Positives	6	0	1	13
% of positives	1.82	0	16.67	6.88

From 2011 a total ban of MBM for all farm animals, equal to the prevention in Europe, was introduced (Pravilnik, *Sl. glasnik RS* 96/2010), so all positive results, even of feed for pigs and poultry, refer to the presence of fish meal (Table 6). Since then MBM has been used only in feed for animals that are not part of the food chain to humans.

Table 6. Results of microscopic examination of 540 different feed samples in 2011

	cattle	sheep	goats	Pigs and poultry
TOTAL	267	16	1	256
Negatives	264	16	1	242
Positives	3	0	0	14
% of positives	1.12	0.00	0.00	5.47

In the years that followed (Table 7 and Table 8) less and less of positive results were detected. In general, Serbian feed industry started to use alternative sources of proteins, even in feed for pigs and poultry where presence of fish meal was still allowed.

Table 7. Results of microscopic examination of 968 different feed samples in 2012

	cattle	sheep	goats	Pigs and poultry
TOTAL	451	49	1	467
Negatives	450	49	1	457
Positives	1	0	0	10
% of positives	0.22	0.00	0.00	2.14

Table 8. Results of microscopic examination of 344 different feed samples in 2013

	cattle	sheep	goats	Pigs and poultry
TOTAL	137	21	1	185
Negatives	136	21	1	182
Positives	1 R	0	0	3
% of positives	0.73	0	0	1.62

However, in 2014 cross contamination of feed for cattle by fish material dropt to zero level (Table 9), as well as feed for other ruminants in 2011. Only 0.9% of feed for pigs and poultry contained fish meal.

Table 9. Results of microscopic examination of 453 different feed samples in 2014

	cattle	sheep	goats	Pigs and poultry
TOTAL	219	12	0	222
Negatives	219	12	0	220
Positives	0	0	0	2
% of positives	0	0	0	0.90

Almost identical situation was maintained during 2015 (Table 10) with the exception of mixtures for pigs and poultry which more frequently contained fish particles in the analysed samples.

Table 10. Results of microscopic examination of 301 different feed samples in 2015

	cattle	sheep	goats	Pigs and poultry
TOTAL	122	7	0	172
Negatives	122	7	0	168
Positives	0	0	0	4
% of positives	0	0	0	2.32

Based on the one decade results of microscopic examination of feed for the presence of constituents of animal origin in Serbia, it could be concluded that during this period establishment of new system of prevention and conducted control raised our feed industry to higher level. In this period international standards HACCP and GMP were widely applied, what made our manufacturers competitive on foreign markets. On the other hand, new regulations harmonized to EU legislation and corresponding laboratory methods contributed to further improvement of the safe feed to safe food concept.

But, it is not possible to remain at this level, nor the present system can stay static. The need to use feedstuffs of animal origine in nutrition of non-ruminants, as an irreplaceable source of biologically valuable proteins, will not disappear. On the contrary, predictions are that it will definitivly grow. Following the European legislation, from this year in Serbia the use of non-ruminant MBM in feed for aquaculture is also allowed (Pravilnik, *Sl. glasnik RS* 33/2016). Further exemptions to the MBM prohibition lie ahead. One thing is certain, the prevention of use of proteins derived from the same species (cannibalism) will remain strictly controlled. This means that the classical microscopy will not be able to meet all requirements and new methods will be included in the official monitoring. Such laboratory tests should allow determination of animal species. It seems that real time PCR method is the backbone of the future system of control and prevention of TSE.

The validation and publication of official protocols at the EU level is maintained by EURL for animal proteins in animal feed. The only way to be informed on time, keep pace with the predictions and equally update our monitoring system is to become part of the network of European laboratories. Therefore, it is necessary to finally determine the national reference laboratory in this field in Serbia.

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A TWO-YEARS SURVEY ON THE PRESENCE OF SULPHITES IN MEAT PRODUCTS COMMERCIALISED IN SOTHERN ITALY

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OBJECTIVE OF THE STUDY

Sulphites are antimicrobial and antioxidant substances recognised as additives by EU Regulation 2003/114. These compounds are added to different food types in the form of sulphur dioxide (E220), potassium metabisulphite (E224), sodium bisulphite (E222), potassium bisulphite (E228). These compounds if bound with oxymyoglobin (a pigment rich in iron and present in the muscle which conferring the typical red appearance) prevents the conversion into metamyoglobin (dark red), a valid index of protracted oxidationprocess and physical-chemical integrity of the product. Indeed, the oxidation processes confers a dark coloration to the meat. These chemicals are harmful compounds which have mutagenic, cytotoxic and allergological effects.

The use of sulphites is absolutely forbidden for fresh meat, according to Italian and European regulations. In this work a total of 1324 fresh meat samples were examined between 2013 and 2014 in order to have a comprehensive epidemiological dataset about the use of these substances in Sicilian territory (Southern Italy).

MATERIAL AND METHODS

The sulphites levels of the meat samples (hamburgers, sausages, minced meat and meat balls) were detected by the implementation of an ion chromatography method. A calibration curve was carried out using five concentration levels of μgSO_4^{-2}/ml (1-10-20-30-50).

RESULTS

Results obtained revealed only 35 cases of sulphites positivity during all the monitoring plan, with maximum concentrations on meatball and minced meat samples (over 2900 mg/Kg). Over 97% of the samples examined showed sulphites concentrations under the Limit of Detection of the method (4.2 mg/Kg). Very low positivity cases were detected in sausage samples. The use of sulphites was mostly found in meatball samples, with

prevalence values between 3.3% and 10.1%. No significant differences were detected across years of sampling ($p<0.05$), although the use of these substances seems to have a decreasing trend.

CONCLUSIONS

The present study aims to investigate the use of sulphites in meat products. Further studies are needed in order to evaluate possible chemical interaction between several flavouring products in transformed meat products (such sausages) and sulphites. Finally, constant epidemiological studies are needed in order to ensure the consumer's health.

STREPTOCOCCUS SUIS, FROM PEN TO THE FOOD CHAIN

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Abstract: *Streptococcus suis* infection is one of the major health problem in the swine industry worldwide.During the last decade, the numberof reported human cases due to *S. suis* has dramatically increased, andwhile most sporadic human cases of infection appear to be due to closeoccupational contact with pigs/pork products, particularly in Westerncountries (farmers, veterinarians, butchers, food processing workers, etc.), two epidemics were recorded in China in 1998 and 2005. Slaughterhouse pigs are a major reservoir of *Streptococcus suis* serotype 2 capable of causing human infection. Consuming of uncooked or partially cooked pork meat in Asian countries is major risk factor for infection. Different from Asian countries infected persons in Western countries are usually adult males and this can be readily explained, since many acquire the disease following occupational exposure to pigs or pork products. Affected humans had usually close contact with pigs or meat, but the real route of infection is still unknown. It is very difficult to implement effective preventive measures for persons that come into close occupational contact with pigs and pig products, especially employees of the meat industry. Therefore it is suggested that scientific laboratories do regular monitoring of possible antimicrobial resistance until some protective measures can be implemented and measures that prevent infection can be established.

Key words: *Streptococcus suis, pigs, meat, infection*

INTRODUCTION

*Streptococcus suis*infection is one of the major health problemin the swine industry worldwide. This pathogen is the most prominent cause of meningitis and septicemia in the porcineindustry, however, otherpathological conditions have also been described, such as arthritis, endocarditis, pneumonia, and septicemia with suddendeath.

Although *S. suis* isprimarily considered a major swinepathogen, it has been increasingly isolatedfrom a wide range of mammalianspecies and from birds. These findings suggest the existence of complex epidemiologicalpatterns of the infection, since other animal species might alsobe a source of swine infection (Gotschalk et al. 2010b). Human *S. suis* infections haveusually been considered sporadic (Arends and Zanen 1988). During the last decade, the numberof reported human cases due to *S. suis* has dramatically increased, andwhile most sporadic human cases of infection appear to be due to closeoccupational contact with pigs/pork products, particularly in Westerncountries (farmers, veterinarians, butchers, food processing workers, etc.), two epidemics were recorded in China in 1998 and 2005 (Goyette-Desjardins et al. 2014). However, the important outbreak in China thatoccurred in 2005 and that affected morethan 200 people with a mortality rate ofnearly 20% changed the perspective onthe threat of *S. suis* to human health.

S. SUIS: GENERAL CHARACTERISTICS OF THE MICROORGANISM

Streptococcussuis is a facultative anaerobic, gram-positive coccoid bacterium that has the ability to synthesize a capsule and secretehaemolysin. It has components of cell wall antigens similar to those displayed by group D streptococci (Stanojkovic et al. 2012). However, *S. suis* is not genetically associated with group D streptococci (Kilpper-Balz and Schleifer 1987).

Previously, *Streptococcus suis* had been classified into 35 serotypes (serotype 1/2, and 1–34) (Higgins and Gottschalk 1995) and then reduced to 33 serotypes because serotypes 32 and 34 were determined to be Streptococcus orisratti, streptococci that can be often isolated from rats (Hill et al. 2005). More recently, it was proposed to remove serotypes 20, 22, 26 and 33 from the Streptococcus suis taxon (Tien et al. 2013). Hence, it is currently considered that there are 29 true Streptococcus suis serotypes.

S. SUIS AND THE PIG FARM

The natural habitat of *S. suis* is the upper respiratory tract of pigs, more particularly the tonsils and nasal cavities, but also the genital and digestive tracts (Goyette-Desjardins et al. 2014). Almost 100% of pig farms worldwide have carrier animals, and that puts *S. suis* as one of the most important bacterial pig pathogens with quite established infection patterns.

Transmission of *S. suis* among animals is considered to be mainly through the respiratory route. Indeed, investigation of presence of alpha hemolytic streptococci, enterococci and streptococci-like bacteria in tonsil and nose swabs of clinically healthy pigs in one research (Stanojkovic et al. 2011) showed that most species belonged to *S. suis* (64%). The presence of other alpha hemolytic streptococci was far lesser: *Streptococcus sanguinis*

(13.8%), *Streptococcus salivarius* (5.6%), *Streptococcus mitis* (5.6%), *Streptococcus parasanguinis* (2.7%) and *Streptococcus oralis* (2.7%) and *S. bovis* (5.6%). *S. suis* strains are very good colonizers of the palatine tonsils of both clinically ill and apparently healthy pigs (Mwaniki et al. 1994).

The sow is also a source of infection. Gilts and sows mayharbor *S. suis* in the uterus or vagina, but no male reproductive organs have beenshown to be infected. Piglets born to sows withuterine or vaginal *S. suis* infections become infected at birth, before birth, or soonafter birth (Robertson and Blackmore 1989), but mostly when passing through the birth canal.

Transmission of virulent strains betweenherds usually occurs by the movement of healthycarrier animals. The introduction of carrier pigs harboringvirulent strains (breeding gilts, boars, weaners) into a noninfected recipient herd may result in the subsequentonset of disease in weaners and/or growing pigs (Higginsand Gottschalk 2005). Horizontal transmission is important especially during outbreaks when diseased animals shed higher numbers of bacteria, increasing transmission by direct contact or aerosol (Cloutieret al. 2003).

S. suis typeis an important contaminant of feces, dust and water. In water, theorganism survives for 10 min at 60°C and for 2 h at 50°C. At 48°C, *S. suis* cansurvive in carcasses for 6 weeks (Clifton-Hadley et al. 1986). At 0°C, the organism cansurvive for 1 month in dust and for over 3 months in feces, whereas at 25°C, it can survive for 24 h in dust and for 8 days in. Dee and Corey (1993) have also been shown that to transmition of *S. suis* strains can be with fomites, such as manure-covered work boots and needles. *S. suis* can be inactivated using many disinfectants, such as diluted bleach. Organic matter reduces effectiveness of chemical disinfectants and should be completely removed with thorough washing prior to application. Even though *S.suis* survives in water up to 2 hours at 50°C butonly 10 minutes at 60°C, use of heated pressure washers compared with nonheated is of limited valuesince water cools rapidly on surfaces negating potential benefit (Clifton-Hadley and Enright 1984).

Vectors of *S. suis* can play a role in disease transmission. Houseflies can carry *S. suis* strains for 2 for 5 days, and have been shown to easily transmit the disease migrating between farms (Enright et al. 1987). Mice can be experimentally infected orally or intranasally with *S. suis* type 2, and the transfer of organisms from orally infected mice to uninoculated mice has been established (Williams et al. 1988, Robertson and Blackmore 1990). Transmission of disease between mice and pigs is believed to occur (Williams et al. 1988).

All categories of pigs can be affected by the disease caused by *S. suis*, including suckling piglets, older piglets and fatteners. *S. suis* carriage rates mayvary between herds and can range from 0% to up to 80–100% (Amass et al. 1997). More thanone serotype of *S. suis* often colonizes individual pigs. In one study, 31% of pigs had only one serotype of *S.suis* in

their nasal cavities, 38% had two or three serotypes, and 6% had more than four serotypes (Monter Flores et al. 1993). According to Silvonen et al. (1988) even if all the pigs in the herd are infected with some strains of *S. suis* clinically apparent disease varies and is usually below 5%. The prevalence of and the morbidity and mortality from *S. suis* vary among herds.

Even when the pig carrier rate is near 100%, the incidenceof the disease varies from period to periodand is usually less than 5% (Clifton-Hadley et al. 1986a). Clinical signs can vary between herds, depending on the pathogenesis of the disease. Pigswith per acute *S. suis* infection maybe found dead with no previously noticed signs of disease or die within hours of the onset of clinical signs. In the acute form of the disease, clinical signs may include fever (up to 42°C), depression, anorexia and lassitude, followed by one or more of the following: ataxia, incoordination, tremors, opisthotonus, blindness, loss of hearing, paddling, paralysis, dyspnea, convulsions, nystagmus, arthritis, lameness, erythema, and/or abortion (Staats et al. 1997). So, we can conclude that meningitis is the major feature of *S. suis* infection in pigs but other organs (joints, heart, lungs, reproductive organs etc.) can also be affected.

PREVALENCE OF *S. SUIS* STRAINS

During the last 12 years, more than 4500 serologically confirmed strains recovered from diseased pigs have been reported. Globally, the most dominant serotypes isolates from clinical cases in pigs are serotypes 2, 9, 3, 1/2 and 7, while 15,5% were so called non-typable strains. However, there is clear geographical distribution of serotypes.

Goyette-Desjardins et al. (2014) summarize strain prevalence in Europe and America. In Canada the most prevalent serotype is serotype 2 while in United State serotype 3 is the most prevalent. In these countries there is only slight difference in percentages of prevalent strains, demonstrating similar distribution of serotypes when data from Canada and the USA are combined. Both, serotypes 2 and 3 are the most prevalent from diseased pigs with 24,3% and 21,0% prevalence respectively, followed by serotypes 1/2, 8 and 7 (Goyette-Desjardins et al. 2014). This can be explained by easy and freely movement of animals from United States to Canada and vice versa. In South America, all results came from Brasil, stating that serotype 2 being the most prevalent with 57,6% reported cases followed by serotypes 1/2, 14, 7 and 9. In Asia majority of results regarding serotype affiliation came from China and South Korea. In China the most prevalent serotypes of infected pigs are in decreasing order of prevalnce, serotype 2, 3, 4, 7 and 8. On the contrary in South Korea serotype 2 had a prevalence of only 8,3%, the same ase serotypes 8 and 33 while the most dominant were serotypes 3 and 4 with 29,2% and 20,8% respectively, while serotypes 16 and 22 had distribution of 4,1%. Other Asian countries

reported many human cases of disease but strains isolated from pigs only refer to slaughterhouses and healthy pigs. Similary, in Japan there have been reported 10 human *S. suis* cases reported but studies on the distribution of isolates from ill pigs have not been reported lately and all of the research dates before 1987 year. In Cambodia, Philippines, Laos and Singapore, human cases were diagnosed recently but there are no data avilable on the epidemiology of *S. suis* infections in pigs.

In Europe the largest number of *S. suis* serotypes isolated from clinically ill pigs belongs to serotypes 1 to 8 (Reams et al. 1996, Higins and Gotschalk 2001). Most of the *S. suis* serotype distribution reports date before year 2000. *S. suis* serotype 2 was the most common in clinical cases in Italy, France and Spain, whreas serotype 9 was more frequent in the Netherlands, Germany and Belgium. Recent conducted reaserch on serotype distribution in Spain suggest that serotype 2 is no longer the most prevalent serotype, and that serotype 9 is the one most frequently isolated from diseased pigs. Behind serotype 9 is serotype 2, followed by serotypes 7, 8 and 3 (Luque et al. 2010). In Netherlands, serotype 9 was the most prevalent in data collected between 2002–2007 followed by serotypes 2, 7, 1 and 4. Contrary to the fact that serotype 9 becomes most prevalent in some countries, there were no human cases reported that were associated with this serotype. In Belgium and United Kingdom serotype 1 was the predominant in ill pigs while in Denmark serotype 7 was the most frequent one. In Southern Europe serotype distribution was done in Serbia where serotype 2 was the only serotype found in piglets that had clinical symptoms of meningitis (Stanojkovic et al. 2012). Beside that various *S. suis* serotypes were found in healthy animals (Stanojković 2012).

STREPTOCOCC SUIS IN SLAUGHTERHOUSES AND MARKETS – DANGER TO HUMAM HEALTH

Streptococcus suis infection is often mentioned as an „oldneglected zoonotic infection“ (Gotschalk et al. 2010b), and the scientific community still considers *S. suis* one of the most important emerging infectious diseases in Asian countries, where the majority of people have regular contact with raw pork meat. In Western countries, *S. suis* diseasehas been considered a rare event inhumans. Most cases of human infection are related to close contact with meat or live animals: pig farmers, abattoir workers, persons transporting pork, meat inspectors, butchers, and veterinarian practitioners (Tang et al. 2006). Two countries in Europe consider *S. suis* infections in humans an industrial disease: France and the United Kingdom (Gotschalk et al. 2010b). According to Arends and Zanen (1988) the annual risk of developing *S. suis* meningitis among abattoir workers and pig breeders has been estimated to be 3.0 cases per 100,000 population while the risk is lower for butchers, at 1.2 cases per 100,000 population in developed countries.

Different from pigs infection, the main route of entry of *S. suis* in humans is thought to be through contact of cutaneous lesions, most usually on the hands and arms, with contaminated animals, carcasses or meat, although in some cases, no wound was detected; bacteria may colonize the nasopharynx, as observed in swine; and the gastrointestinal tract, as suggested by diarrhea as a prodromal symptom (Fongcom et al. 2001, Wertheim et al. 2009). The outbreak in China in 2005 caused by *S. suis* affected more than 200 people, with almost 20% mortality rate. This epidemic has completely changed the perception of the danger which this pathogen presents to human health (Stanojkovic et al. 2014). Period of incubation ranges from just a few hours to few days (Fongcom et al. 2001). Just like in pigs *S. suis* produces meningitis as the main feature of disease but cases of endocarditis, pneumonia, peritonitis, arthritis and other less common clinical signs can be seen as the part of generalized septicemia (Arends and Zanen 1988). Also, there have been described per acute infections related to this pathogen which were usually in the form of streptococcal toxic shock − like syndrome (STSLS) that has been associated with most of the death cases in China 2005 epidemics. In China 2005 epidemics there have been 215 cases of infection while 38 of them died mainly as the results of STSLS.

Streptococcus suis studies regarding prevalence are mainly directed to clinical cases in pigs and humans and discharges, tonsils, blood, brain and spinal fluids as a specimens. There are little date of this pathogen prevalence in raw meat.

According to Hoa et al. (2011) slaughterhouse pigs are a major reservoir of *Streptococcus suis* serotype 2 capable of causing human infection. But these results were obtained from tonsil samples at the slaughterhouse. Same authors found that *S. suis* serotype 2 was the most common serotype isolated fromthe sampled pigs, indicating that *S. suis* serotype 2 is highly prevalent in slaughterhouse pigs in southern Vietnam. In contrast to above mentioned, in a study of slaughterhouse pigs in Korea, *S. suis* serotype 2 strains were absent, while serotype 9 was the most common serotype (Han et al. 2001). Cheung et al. (2008) examined 78 samples of raw pork lean meat from retail markets and wet markets and determined that *S. suis* can be found in every sample although in different levels (MPN/g). Authors concluded that sometimes standard culture methods can't efficiently recover *S. suis* from samples. *S. suis* was isolated from 6.1% of raw pork meat from 3 of the 6 wet markets in Hong Kong. Similar meat prevalence in Serbia (Stanojkovic et al. 2016) showed serotype 2 was the most isolated serotype from fresh pork with 46,1 % of isolated *S. suis* serotypes followed by serotype 9, 7, 3, 1 and 4. Slaughtered pigs had similar prevalence of *S. suis* strains just like those data reported for clinically ill pigs.

Stanojkovic et al. (2016) found that there was a significant difference in the presence of *S. suis* strains on the basis of sample collected. In above

mentioned authors research hog head was highly contaminated with *S. suis* serotype 2 strains (prevalence of 25%). That kind of result is maybe expected since *S. suis* is normal inhabitant of respiratory system such as tonsils, and also slaughtered pigs are held in that kind of position that allows water to spread bacteria from hind part of the body to the head. Same authors found that presence of *Streptococcus suis* serotype 2 in liver, kidneys, shoulder, ham, loin and belly was 20%, 12%, 5%, 5%, 5%, and 10% respectively. Noppon et al. (2014) have found overall prevalence of *S. suis* serotype 2 in pork of 12,8%. Same authors mentioned that prevalence of *S. suis* serotype 2 in fresh meat was 10,8% but it was not clear referring to the part of the body that fresh meat was taken from. These authors detected 15,4% prevalence of *S. suis* serotype 2 in liver and other offal. Nakayama et al. (2011) demonstrated that *S. suis* accumulates in the kidney during *S. suis* infection.

It can be concluded that processing and consuming of uncooked or partially cooked pork meat in Asian countries is major risk factor for infection. Local cuisine specialties such as raw or half-cooked intestines, uterus, tonsils or fresh pig blood can be important sources of infection. In Thailand for example there is an increasing trend of the incidence of the disease, mainly because of consumption of half-cooked/baked meat. Wangsomboonsiri et al. (2008) determined that the majority of *S. suis* infected patientshad a history of eating under-cooked pork or internal organs which is a major route of transmission of *S. suis* from pig to human. Because of the local culture, people in Thailand usually eat raw or under-cooked pork, internal organs and fresh pig's blood.

Different from Asian countries infected persons in Western countries are usually adult males and this can be readily explained, since many acquire the disease following occupational exposure to pigs or pork products. Affected humans had usually close contact with pigs or meat and very often small cuts on their hands (Stanojkovic 2012). Stanojkovic et al. (2012) found that *S. suis* can readily isolated from butchers knives. Also, there are reports that confirm carrier state in humans, especially abattoir workers (Sala et al. 1989, Rohas et al. 2001). Strangmann et al. (2002) determined nasopharyngealcarriage rate of *S. suis* serotype 2 in the high-risk group (butchers, abattoir workers, and meat processing employees) was 5.3%, while those without contact with pigs or pork consistently tested negative. This kind of nasopharyngealcarriage ratehas also been shown in pigs (Higginsand Gottschalk, 2005).

CLINICAL FEATURES OF THE DISEASE IN HUMANS

In humans, *S. suis* usually produces a purulent meningitis but endocarditis, cellulitis, peritonitis, rhabdomyolysis, arthritis, spondylodiscitis, pneumonia, uveitis, and endopthalmitis have alsobeen reported (Gotschalk et al. 2010b, Wertheim et al. 2009). Also, there have

been described per acute infections related to this pathogen which were usually in the form of streptococcal toxic shock-like syndrome (STSLS) that has been associated with most of the death cases in China 2005 epidemics (Lun et al. 2007). The most important often mentioned sequela of *S. suis* infection are vestibular dysfunction or unilateral or bilateral hearing loss.

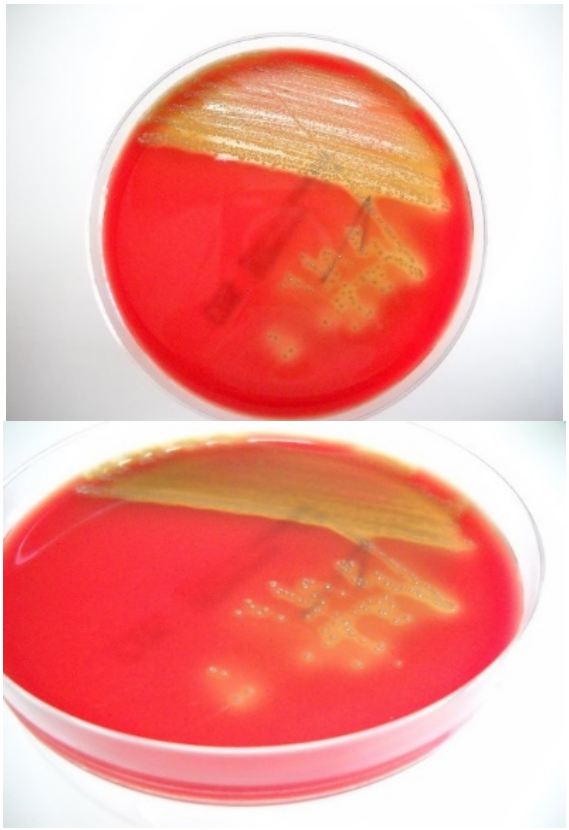
DIAGNOCIC AND ISOLATION OF *S. SUIS*

A preliminary diagnosis of *S. suis* infection in pigsis usually made onthe basisof clinical signs andmacroscopiclesions.However, the diagnosis is confirmed by bacteriaisolationand detectionof microscopiclesionsin tissues. As mentioned above it is demonstrated that *S. suis* accumulates in the kidney during *S. suis* infection and these findings might be useful for diagnosis of streptococcal infection (Nakayama et al., 2011).

Different samples of blood, meninges, brain, spleen, joints, liver or kidneys from diseased or dead pigs are required for microbiological identification of organism. In diseased humans cerebrospinal fluid (CSF) is the sample for culturing. Wangsomboonsiri et al. (2008) found that 86% hospitalized humans had blood samples positive for *S. suis*.

S. suis is encapsulated, gram positive species , that occur single, in pairs or occasionaly in short chains. organism grows well on media usually used for isolation of streptococci, most frequently sheep blood agar, and forms glistening, round, slightly grey alpha haemolitic colonies (Picture 1).

S. suis can grow well in aerobic conditions but the growth is enchanced by microaerophilic atmosphere. Isolation and identification of strains is relatively easy, especially in the cases of diseased animals. *S. suis* has vary variable biochemical properties (Stanojković et al. 2014) and thus must be confirmed by serotyping. Although *S. suis* can be easily identified by veterinary laboratories that are aware of pathogen, many human veterinary laboratories misidentify it as enterococci, Streptococcus pneumoniae, viridans streptococci or even Listeria monocytogenes and mainly by use of rapid multitest biochemical kits (Gottschalk et al. 2010b).



Picture 1. Alpha haemolitic colonies of Streptococcus suis on CNA 5 %sheep blood agar

This confusion may have led to the misdiagnosis of *S. suis* infections in the past. Serotyping is the only accurate method for definitive diagnosis of *S. suis* infection. This can be done by two methods. First, by serological method that is performed by either co-agglutination, capillary precipitation test or Neufeld's capsular reaction using reference antisera. Second method is serotyping using PCR method in which CPS (capsular) genes are amplified by either simplex or multiplex PCR. Polymerase chain reaction (PCR) tests have been used to directly detect *S. suis* DNA from samples with a high sensitivity. Some *S. suis* isolates do not agglutinate with any of the antisera directed against capsular antigens and these strains are identified asnon-typable isolates.

CONCLUSION

Streptococcus suis is a swine pathogen that causes important economic losses in the swine industry worldwide. This bacterium causes wide range of diseases in pigs, including meningitis septicemia and endocarditis, but also

cases of sudden deaths. It is also emerging zoonotic pathogen and the causative agent of serious disease in humans. Veterinarians, physicians and especially microbiologist should be aware of this microorganism and the infections that it causes. There is some dispute regarding the preventive measures that might be due to the high rate of contamination of pigs with *S. suis*. It is very difficult to implement effective preventive measures for persons that come into close occupational contact with pigs and pig products, especially employees of the meat industry. Therefore it is suggested that scientific laboratories do regular monitoring of possible antimicrobial resistance until some protective measures can be implemented and measures that prevent infection can be established.

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FINDING OF *SALMONELLA* SPP. IN MECHANICALLY SEPARATED MEAT

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Abstract: Salmonellosis is among the most frequently reported foodborne diseases worldwide. While numerous potential ways of transmission exist, commercial chicken meat has been identified as one of the most important food vehicles for these organisms. Although specific data on the burden of foodborne disease associated with Salmonella in poultry is limited, the role of poultry is considered to be significant in this respect; however, the risk in different countries varies according to control measures and practices implemented along the chain from primary production to final preparation of the meat for consumption.

In our laboratory *Salmonella* was detected in two cases in mechanically separated meat. It was a total of 52 samples. The procedures for isolation of Salmonella from food is given in protocol follow the ISO-6579. For Pre-enrichment it use non-selective medium (buffered peptone water). Then selective enrichment in Tetrathionate broth (Müller-Kauffmann) and Rappaport Vassiliadis soy peptone (RVS) broth and subcultivation on Xylose Lysine Desoxycholate (XLD) agar and on Brilliant Green agar (BGA) were done (or another selective agar media).

Most important control measures at primary production are: the elimination of Salmonella in grandparent and parent flocks, all-in all-out production at the broiler farm, to avoid any carry over during processing, logistic slaughter planning scheduled to avoid pathogens being transferred from contaminated processing equipment to another flock and finally, satisfactory cleaning of transport crates.

Important moments that take account of levels of contamination when carcasses leave the processing plant, thereafter using inputs for storage time in retail stores, transport time, storage times in homes, and the temperatures carcasses were exposed to during each of these periods. The presence and level of *Salmonella* in this step is very much country specific, since the level of infection when leaving the processing step will vary between the countries in relation to the methods used at the processing plant.

Key words: *Salmonella, poultry, mechanically separated meat*

INTRODUCTION

Salmonellosis is one of the most frequent foodborne diseases, being an important public health problem in almost all industrialised countries (D'Aoust 1997). In the last decades, significant increase in the incidence of salmonellosis has been reported in Europe by „WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe" (Schmidt 1998). In the United States, between 1993 and 1997, Salmonella accounted for the largest number of outbreaks and cases of foodborne disease (CDC 2000).

Although Salmonella is ubiquitous, the primary reservoir is the intestinal tract of animals and the colonisation is favoured by intensive animal production. Poultry products are frequent vehicles in the transmission of Salmonella, dominating other foods of animal origin as potential source of infection (Bryan and Doyle 1995, D'Aoust 1997).

The incidence of Salmonella has been recently studied in poultry meat in many countries such as UK (Plummer et al. 1995), Malaysia (Rusul et al. 1996), Greece (Arvanitidou et al. 1998) and Belgium (Uyttendaele et al. 1998, 1999).

In meat processing plants, muscles cut from animal carcasses are often further processed into raw meat products such as minced meat and other meat preparations. In order to obtain as much meat as possible, mechanically separated meat (MSM) is produced from poultry carcasses from which whole muscles have been cut. According to (EC) Regulations 853/2004, the term „minced meat" refers to „boned meat that has been minced into fragments and contains less than 1% salt", whereas „meat preparations means fresh meat, including meat that has been reduced to fragments, which has had foodstuffs, seasonings or additives added to it or which has undergone processes insufficient to modify the internal muscle fiber structure of the meat and thus to eliminate the characteristics of fresh meat". MSM is a term for „the product obtained by removing meat from flesh-bearing bones after boning or from poultry carcasses, using mechanical means resulting in the loss or modification of the muscle fiber structure". Likewise other types of meat products, minced meat, meat preparations and MSM must fulfill foodsafety criteria which means „a criterion defining the acceptability of a product or a batch of foodstuff applicable to products placed on the market" (EC Regulation no. 2073/2005). The acceptability of the fore mentioned products where they are eaten and cooked is solely assessed based on Salmonella sp.'s absence in 10 or 25g, in the case of meat other than poultry and poultry, respectively (EC Regulation no. 2073/2005).

Mechanically separated meat (MSM) presents a high potential health risk to the consumers, depending on the ways of its extraction. The chicken meat and the red meat extracted in that way could be a source of pathogens such as *Salmonella* spp., *Campylobacter* spp., enterohaemorrhagic *E. coli* (like *E. coli* O157:H7), *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus* and others, rotten microorganisms like *Pseudomonas*. Therefore of a great significance are all the sanitarian and hygiene measures for the raw materials and production of the mechanically separated meat has to be followed and controlled closely. Microbiological criteria for safety and hygiene of the mechanically separated meat's production are presented in Regulation (EO) No 2073/2005 and includes *Salmonella* spp. in 10 g product, aerobic colonies between $m=5\times10^5$ cfu/g, M=500 cfu/g are allowed and *E. coli* between m=50 cfu/g, M=500 cfu/g are allowed.

MATERIALS AND METHODS

Sample collection

From Januar to December 2015, a total of 52 samples of mechanically separated meat were analysed. Samples were transported to the laboratory after being collected in a portable cooler at a temperature of 4 ° C and microbiological analysis were carried out immediately.

Isolation and identification procedure

Isolation and identification of Salmonella was performed according to standard methods (SRPS EN ISO 6579:2008). For the isolation of Salmonella 10 g of minced meat and meat preparations was homogenized with 90 ml of Buffered Peptone Water (Oxoid, Basingstroke, Hampshire, UK) using Stomacher lab blender for 2 min and then pre-enriched at 37 ± 1^0C for 18±2h. As a next step, the culture obtained from the pre-enrichment was subcultivated in two selective enrichment broths, a Rappaporte Vassiliadis medium with soya and a Müllere Kauffmann tetrathionate novobiocin broth (both Oxoid), which were incubated for 24±3h at 41.5 $\pm0.5^0$C and 37 ± 1^0C, respectively. After incubation, a loopful from these enrichment broths was streaked on the selective plating media Xylose Lysine Deoxycholate and Brilliant Green agars (both Oxoid) and incubated at 37 ± 1^0C for 24±3h. Colonies of presumptive Salmonella were further subcultured and identification confirmed by biochemical and serological tests as described in the ISO 6579:2008 standard.

The plates were examined for the presence of typical colonies of Salmonella, i.e. red coloured colonies on BG agar and red colonies with black centres on XLD agar. Suspected colonies (maximum five) were randomly selected from each plate and confirmed by biochemical tests including fermentation of glucose, lactose and sucrose, hydrogen sulfide

production, urease activity, phenylalanine deamination, lysine decarboxylation, citrate, methyl red and indole tests. One isolate per sample with the typical biochemical profile of Salmonella was confirmed using the API 20E system (BioMerieux, France) and submitted to serological classification by agglutination tests with several specific O antisera (Torlak, Serbia).

RESULTS

In total from 52 tested, 2 samples were positive for *Salmonella spp.* Table 1. shows the prevalence of *Salmonella* in in mechanically separated meat.

Table 1. Prevalence of *Salmonella* spp. in mechanically separated meat

Product	Number of Negative samples (%)	Number of Positive samples (%)	Total (%)
Mechanically separated meat	50 (96%)	2 (4%)	52 (100%)

DISCUSSION

Salmonella spp. is the cause of the second most frequent foodborne illness in the European Union (European Food Safety Authority (EFSA) 2011), and accounted for 108,614 confirmed human cases in 2009. In Poland, Salmonella sp. causes about 33% of foodborne outbreaks (Baumann-Popczyk and Sadkowska-Todys 2011). In 2010, a total of 9,732 salmonellosis cases were noted in Poland. Most of the cases were noted during summer (Sadkowska-Todys et al. 2012) and were caused by consumption of meals prepared from eggs (22.5% of outbreaks, 12.9% of total cases), from at least three different types of raw meats (9.3% of outbreaks, 14.3% of total cases), poultry and eggs (6.2% of outbreaks, 5.1% of total cases) and meat other than poultry (2.3% of outbreaks, 3.8% of total cases) (Baumann-Popczyk and Sadkowska-Todys 2012).

As previously stated, the consumption of poultry products is a major source of salmonellosis. It was estimated that Salmonella sp. occurs in 50% of flocks (Arsenault et al. 2007, as cited by Carrasco et al. 2012). The bacteria from the gastrointestinal tract of birds infected at farms spreads during slaughtering and further processing of the meat (Carrasco et al. 2012). Also porcine meat is a significant source of salmonellosis mainly due to its large consumption (Baer et al. 2013). Healthy pigs may be a carrier of the bacteria because Salmonella infections, with the exception of S. Choleraesuis and some strains of S. Typhimurium, usually do not produce severe disease in pigs (Botteldoorn et al. 2003). Such animals are

the source of Salmonella spp. in slaughterhouses, which results in contamination of the plant's environment and therefore creating a risk of products' contamination (Botteldoorn et al. 2003). Cattle are also carriers of the bacteria and consumption of ground beef has been a cause of salmonellosis outbreaks.

An increase in the number of animals infected with Salmonella is primarily caused by its intense industrial production. Animals are kept in a relatively small space, which makes their natural resistance weaker and enables cross-infections with the bacteria (Chajecka-Wierzchowska et al. 2012). The products that are of a special concern are raw, smoked or lightly cooked beef products (Carrasco et al. 2012). Thus, this study was conducted to determine the frequency of Salmonella spp. in mechanically separated meat which is not significant. In Serbia prevalence Salmonella spp is only 4% .

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ESCHERICHIA COLI IN FISH MEAT – CURRENT STATUS AND PERSPECTIVES

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Abstract: The aims of the present paper are the analysis and comparison of the results from available literature regarding the prevalence of *Escherichia coli* strains, including *E. coli* 0157:H7 isolated from fish in different countries. Outbreaks of food-borne diseases arising from the consumption of fish contaminated with pathogenic bacteria are of major public health and economic concern to the aquaculture. *E. coli* is reported as one of the most common causes of food-borne diseases in many countries worldwide. In addition, the presence of *E. coli* as fecal coliform in fish has been used as an indicator of water pollution. Their presence in fish intended for human nutrition may represent a potential hazard both as the cause of disease and because of possible transfer of antibiotic resistance from fish bacteria to those which infecting humans. *E. coli* O157:H7 has been implicated as the causative agent in major outbreaks of diarrhea and haemolytic uremic syndrome associated with the consumption of raw meat, especially beef but the fish can also be a vehicle for this microorganism. Pathogenic bacteria such as *E. coli* O157:H7, when present in fish and in fishery products, are usually found at low levels and if these products are properly cooked, food safety hazards are not significant.

Key words: *Escherichia coli, fish meat, food-borne diseases, thermal treatments*

INTRODUCTION

Fish and fishery products represent valuable sources of all nutrients, so they are very important from a nutritional point of view. Fish meat is highly variable in quality due to differencies in species, nutrition, season, age and other factors (Ćirković et al. 2012) and because of that it is very difficult to control the quality of fish meat. It is very perishable and furthermore fish meat can be a carrier of several microbial and other health

hazards. Fish and fishery products have been recognised as carriers of health hazards such as diseases causing different bacteria, parasites, natural toxins, histamine, heavy metals and other pollutants (Ljubojević et al. 2015).

FOOD-BORNE DISEASES ASSOCIATED WITH CONSUMPTION OF FISH

Outbreaks of food-borne diseases arising from the consumption of fish contaminated with pathogenic bacteria are of major public health and economic concern to the aquaculture. *Escherichia coli* O157:H7 has been implicated as the causative agent in major outbreaks of diarrhoea and haemolytic uraemic syndrome associated with the consumption of raw meat, especially beef but the fish and fishery products can also be a vechicle for this microorganism (Asai et al. 1999). The pathogenic strains of *Escherichia coli*belong to the group of bacteria which are present in fish as a result of contamination with human or animal feces or otherwise introduced into the aquatic environment, refered as non-indigenous bacteria (Feldhusen 2000). Moreover, hazards may occur during post-harvest handling or processing. When *E. coli* are present in fresh fish, they are usually found at low levels, and if fish are adequately cooked, food safety hazards are insignificant. *E. coli* are often used as an indicator of fecal contamination and it have been isolated from unpolluted warm tropical waters where they are part of the natural microbiota (Hazen 1988). Besides, some strains are capable of causing food-borne diseases. Shiga toxin-producing *E. coli* (STEC) O157:H7 which can cause haemolytic uraemic syndrome and death in some cases, is a global public health concern. The use of animal manures, particularly bovine, as pond fertilizers presents a risk of the presence of pathogenic strains of *E. coli* in the pond water (Jenkins et al. 2011). The contamination of fish can occur prior to harvest, during capture, processing, distribution or storage. People can be exposed to *E. coli* through the ingestion of fish that have been harvested from contaminated water or which have come into contact with contaminated water during processing. Fish can be contaminated during processing and pathogenic agents present on the raw material may survive and thus be present on the final product and contamination with new pathogens is also possible. Recontamination of heat treated fish and fishery products before packaging is another serious safety concern especially where only a low infective dose is required to cause disease, such as case with *E.coli* O157:H7.

E. COLI

E. coli is the type species of the genus *Escherichia*, family *Enterobacteriaceae*. It is associated with gastrointestinal disease including

strains of many different serotypes, categorized into five major groups according to their virulence mechanisms: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC or STEC), and enteroaggregative (EAEC) (Cohen et al. 2005). *E. coli* is reported as one of the most common causes of food-borne diseases in many countries worldwide, including Europe, United States, Africa, South America and the Far East. Diseases caused by *E. coli* with diarrhoea symptoms are often self-limiting. However, the systemic infections sometimes can cause death in vulnerable people with diminished immunity. Verotoxigenic *E. coli* (STEC) can cause gastrointestinal diseases in humans and furthermore life-threating hemolytic-uremic syndrome (HUS) (Gerber et al. 2002). STEC produces stx1 and/or stx2 Shiga toxins and production of stx2 type Shiga toxin increased the risk of the development of HUS (Friedrich et al. 2002). EHEC are a subgroup of STEC that together comprise hundreds of O:H serotypes (Ferens and Hovde 2011). Notwithstanding the fact that most EHEC strains produce stxs, EHEC O157:H7 are especially virulent and are responsible for the majority of HUS cases worldwide (Gyles 2007). Non-EHEC STEC strains can also be pathogenic to humans (Gyles 2007). It is important to note that the stx genes are not necessarily associated with morbidity, and STEC may be carried asymptomatically by humans (Jenkins et al. 2003). EHEC of the O157:H7 serotype are zoonotic pathogens responsible for the majority of severe cases of human EHEC disease (Ferens and Hovde 2011) and the prevalence of EHEC O157:H7 is probably underestimated. Besides farm animals, mainly cattle, EHEC O157:H7 occur in amphibian, fish, and invertebrate carriers, and also can colonize plant surfaces and tissues. Transmission of EHEC O157:H7 to humans occurs primarily via ingestion of inadequately processed contaminated food or water and less frequently through contact with manure, animals, or infected people (Ferens and Hovde 2011). Moreover, the greatest number of disease outbreaks caused by *E. coli* O157:H7 are food-borne. The possibility of croscontamination should not be underestimated especially having in mind that the infectious dose for *E. coli* O157:H7 is very low and that infection can occur after consumption of contaminated food in which bacteria can survive and not necessarily grow. Also, *E. coli* O157:H7 can remain viable in soil for greater than 4 months and possess the capability to adapt easily to environmental stresses (Jones 1999). The public health significance of *E. coli* O157:H7 was recognised in 1982 when it was associated with a large outbreak of infection at a fast-food restaurant in Oregon and Michigan (Riley et al. 1983). The number of reported infections with *E. coli* O157:H7 is small in comparison to other enteric pathogenic bacteria such as *Salmonella* or *Campylobacter*, but *E. coli* O157:H7 has become an increasing public health problem because of its rise in incidence and because of the severity of its complications (Jones 1999). Furthermore, *E. coli* O157:H7 outbreaks have now been reported throughout most of the

world and have occurred in most countries in the European Union (Newell et al. 2010). Moreover, a very low infection dose with the ingestion of only 10 to 50 cells for full symptoms to develop is required (Licence et al. 2001). Infection with *E. coli* O157:H7 can lead to a wide spectrum of clinical symptoms, including hemorrhagic colitis (bloody diarrhoea), haemolytic-uraemic syndrome (HUS), non-bloody diarrhoea and thrombotic thrombocytopenic purpura (TTP), particularly in susceptible patients such as children, pregnant women and the elderly (Gerber et al. 2002). *E. coli* O157:H7 is a facultative anaerobe and therefore can survive and growin both aerobic and anaerobic environments including themodified atmospheres which are used for food storage. STEC are increasingly being recognized as the causative agents of serious gastrointestinal diseases in humans, and have been responsible for several foodborne outbreaks. Fish caughtnear a site of slaughter of zebu (*Bos indicus*) in central Africa harbored *E. coli* O157:H7 (Tuyet et al. 2006). Although *E. coli* O157:H7 is currently the most common serotype in many countries, serotypes O26, O103, O111, O145 are considered equally important in causing hemorrhagic colitis (Perelle et al. 2007). Occurrence of STEC has been reported in a number of food products such as beef, pork, lamb, poultry and fish (Samadpour et al. 1994). However, most reported outbreaks of foodborne diseases have been from developed countries possibly due to underreporting of outbreaks in developing countries.

THE FINDINGS OF E. COLI INCLUDING E. COLI O157:H7 IN FISH

E. coli is reported as predominant enteric bacteria from intestines of fresh water fishes (carp, tilapia and catfish) and pond water (Niemi and Taipalinen 1982). High levels of fecal coliform, *E. coli* in pond water lead to breakage of the immunological barrier of fish and invasion of fish muscles by pathogens (Guzman et al. 2004). There are reports of the occurrence of pathogenic strains of *E. coli* from market fish and seafood from different parts of the world (Samadpour et al. 1994; Teophilo et al. 2002). Quines (1988) isolated *E. coli* in fish species *Oreochromis niloticus*, *Cyprinus carpio* and *Ophicephalus striatus*. Samadpour et al. (1994) examined fresh meat, poultry, and seafood purchased from Seattle area grocery stores for the presence of STEC and none of *E. coli* isolates recovered from fish were of the O157:H7 serotype and the frequency of detection of SLTECs in the study may reflect the higher frequency of SLTECs of other serotypes in the fish. Kumar et al. (2001) reported that about 3% of fresh sea fish marketed at Mangalore, India, have been detected positive for non-O157:H7 STEC. Kumar et al. (2001) examined the occurrence of Shiga toxin-producing *E. coli* in fresh fish, shellfish and meat sold in open markets in Mangalore, India and found that two of the 60 fish samples were positive for stx and hlyA genes by PCR and that none of

the samples was positive by PCR for rfbO157. Results obtained by Basti et al. (2004) detected *E. coli* in intestine of silver carp and common carp in Iran. They bacteriologically investigated a total of 120 cultivated fish (common carp and silver carp) for pathogenic organism in their intestine and obtained *E. coli* in 65% of the examined fish samples. Furthermore, biochemically identified *E. coli* were serologically investigated and the sera: O2K1, Ol 19B14, O127B8 and O114K9 were detected. Manna et al. (2008) determined the microbiological quality of fresh and ice-preserved Indian major carps (*Labeo rohita, Catla catla, Cirrhinus mrigala*), tilapia (*Oreochromis mossambica*), catfish (*Heteropneustes fossilis*, *Clarias batrachus*) and shrimp (*Penaeus monodon*) from Kolkata, India with special emphasis on *E. coli* O157 and they reported that few samples were contaminated with non-O157 serotypes of *E. coli* which produce enterohaemolysin and Shiga toxin. Also, fish were contaminated with coliforms, including *E. coli*, indicating poor hygiene and sanitary conditions. Results obtained by Manna et al. (2008) showed that 1 (7.69%) ice-preserved Indian major carps and 1 (5.55%) shripm were contaminated with sorbitol negative *E. coli* belonging to serogroups O101, O105 and that *E. coli* O101 and O105 expressed EHEC haemolysin and the O101 isolate harbored stx2 gene. They suggested that stx2-positive and/or EHEC haemolysin-positive *E. coli* might represent a public health risk. The probability to be infected with *E. coli* due to consumption of fish is very low. Tavakoli et al. (2012) reported that the incidence of *E. coli* in carp was 8% and in shad 2% in Iran. The results obtained by Bonyadian (2015) showed that the rainbow trout meat may have a role as a vehicle to transmit the verotoxigenic *E. coli* to human. They determined the prevalence of verotoxigenic *E.coli* and their virulence factors isolated from coldwater fishes (Rainbow trout) in Iran. Non of the isolate was *E. coli* O157:H7, but the other non-O157 verotoxigenic strains were isolated and stx1, stx2 and eae genes were detected in 14% of skin isolates and 4% of fecal isolates. Elsaidy et al. (2015) examined the microbial water and fish quality due to feeding of chicken manure and fermented chicken manure to fish in ponds, using Nile tilapia (*Oreochromis niloticus*). They isolated *E. coli* from chicken manure, but not from fermented chicken manure. *E. coli* were isolated from water and fish raised in ponds receiving either chicken manure or fermented chicken manure with higher incidence in those with chicken manure. However, all water and fish samples tested were free from *E. coli* O157:H7. The results obtained by Elsaidy et al (2015) proved the influence of chicken manure on water and fish quality and recommend the use of fermented chicken manure as a bacteriologically safe fish pond fertilizer. Pao et al. (2008) found *E. coli* in 1.4, 1.5, 5.9 and 13.2% in trout, salmon, tilapia and catfish respectively but did not find *E. coli* O157:H7. On the other hand, Asai et al. (1999) isolated STEC O157:H7 from processed salmon roe which had been a suspected food item in sporadic infections which occurred in Japan in 1998. According to Ozer and Demirci

(2006) four fish and shrimp associated outbreaks with *E. coli* O157:H7 were reported between 1990 and 2002. Furthermore, they stated that about 73000 cases of *E. coli* O157:H7 occur annualy in the USA and most of vehicle was unknown. Tuyet et al. (2006) reported *E.coli* O157: H7 in water and fish in the Central African Republic due to contamination of the field surface water and described the novel strains of serogroup O157:NM isolated from fish and a variety of assays indicated that these strains belong to the enteropathogenic pathotype. *E. coli* O157:H7 strains were isolated from 6 (2.3%) of the 260 water samples collected at N'Goila, and in 3 fish (4.7%) of the 64 fish captured in the Oubangui River. All strains had the genes encoding both Shiga toxins 1 and 2. They concluded that the carriage of *E. coli* O157:H7 by fish, and contaminations of the of field surface water are important contributing factors to the high prevalence of hemorrhagic colitis and HUS in Bangui and suggested that *E. coli* O157:H7 was able to spread over large distances, perhaps via waterways or fish.

CONCLUSIONS

There are a little risk of fish-borne diseases caused by *E. coli* in Europe due to the production condition in aquaculture and heat treatment of fish before consumption as well as the activities of the European food-related authorities. Pathogenic bacteria such as *E. coli* O157:H7, when present in fish and in fishery products, are usually found at low levels and if these products are properly cooked, food safety hazards are not significant (Feldhusen 2000). Even though there is no official report regarding finding of *E. coli* in fish as cause of disease in humans in Republic of Serbia, the results of the previous investigation indicate that possible foodborne illnesses caused by *E. coli* strains are major concerns surrounding fish and fisheries products. Therefore, further research and a broad control system are needed to improve safety of these products and to retain the fish and fisheries product quality characteristics to satisfy consumer demands while controlling this potential safety hazards. The results suggest that changes such as adding natural antimicrobial and antioxidant ingredients and different process modifications and especially thermal treatment in fish and fisheries products should be applied to improve safety.

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IMPACT OF TRADITIONAL STORAGE SYSTEM ON THE PRODUCTION OF FUMONISINS IN MAIZE IN SERBIA

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Abstract: Fumonisins are secondary metabolites of the moulds *Fusarium moniliforme* Sheldon, *Fusarium proliferatum* (Matsushima) and *Fusarium nygamai*. This group of mycotoxins is considered the most common fungal contaminant of maize and maize products. Occurrence of fumonisins in raw maize is highly dependent on climatic conditions before and during harvesting, as well as by storage conditions. With an aim of assessing the influence of traditional system of maize storage in individual households in Serbia, 35 samples were collected from different households from 8 localities in northern Serbia. The samples were collected immediately upon storage and again in spring next year. Immediately upon sampling, stored samples were analyzed for moisture content to establish its possible correlation with toxin production during storage. Fumonisin content in maize samples collected before and during storage was analyzed using enzyme-linked immunosorbent assay (ELISA). Statistical analysis did not reveal any significant correlation between moisture content before storage and fumonisins concentration before (r=0.189; p=0.276) and after (r=0.171; p=0.326) storage. Regardless of somewhat higher average value of total fumonisins after storage (0.413 µg/kg) as compared with the results obtained immediately upon harvesting (0.227 µg/kg) and the number of positive samples (60 and 51 after and before storage, respectively), the examination of stored maize samples revealed that traditional storage method in barns does not significantly influence the production of fumonisins in maize.

Key words: *fumonisins, maize, storage, barns*

INTRODUCTION

Toxin-producing fungi are broadly divided into two groups, the „field" fungi invading crops and producing toxins before harvest and „storage" fungi that are associated with post-harvest toxin contamination. In the first case, the invasion is enhanced by plant host – fungus and some biological interactions (e.g. insects). In the second case, toxin production is stimulated by crop nutrients, environmental conditions, that is, abiotic (temperature, humidity) and biotic factors (Miller 1995).

Fusarium moniliforme and *Fusarium proliferatum* are „field" fungi, which produce fumonisins in both, field and storage conditions. Association of *F. moniliforme* with fumonisin production has been reported worldwide, including Africa, USA, France, Poland, Italy, Brazil, Argentina, Indonesia, Thailand, Philippines etc.; however, higher fumonisin levels are characteristic for regions with warm climate (Miller 2001). Namely, *F. moniliforme* grows well at higher temperatures, and grain rotting and fumonisin accumulation are associated with draught, grain damage by insects and inadequate climate conditions for planted hybrid (Miller 2001, Parsons and Munkvold 2012). The incidence of fumonisin contamination of maize is also dependent on geographic location, technology of agricultural production as well as the maize hybrid itself (Shelby et al. 1994, Jackson and Jablonski 2004, Soriano and Dragacci 2004). Environmental factors such as temperature, humidity, draught and prolonged rainy periods before and during harvest as well as maize storage under inadequate humidity conditions can result in further accumulation of fumonisins (Bacon and Nelson 1994). Toxin production can continue even throughout the post-harvest (storage) period unless the maize grain is dried below 14% moisture content (Ono et al. 2002). In that respect, rapid grain drying is of critical importance at the initial stage of drying considering that *Fusarium* sp. do not grow below 0.9 water activity, thus preventing fumonisin production during storage.

As it can be seen, *Fusarium* moulds can develop in a field environment, thus the most extensive toxin production occurs before harvest. However, grain handling during harvesting and storage conditions can substantially facilitate mould growth and toxin production. In that respect, we investigated the effects of aforementioned factors on fumonisins contamination of cereal crops under characteristic conditions for agricultural production and storage in our region.

MATERIAL AND METHODS

To investigate the effects of traditional maize storage system in individual households maize samples were collected during fall 2005 from a number of household barns from eight locations in northern part of Serbia. The samples were collected immediately after harvesting and again in

spring 2006 in the region of Bačka (Lipar, Temerin, Rumenka), Banat (Nakovo), Srem (Putinci, Ruma, Sremska Kamenica) and Mačva (Bogatić). Immediately after sampling, 1000 mg of each sample was prepared by grinding in a laboratory mill to pass the 0.8 mm sieve. The sample was than homogenized by mixing and moisture content was determined to establish potential correlation between toxin production and duration of storage period. The samples were either immediately analyzed or placed into plastic bags and stored in deep freeze at -20ºC until analysis. Prior to analysis, the samples were tempered at room temperature. Fumonisin content in collected maize samples was tested using ELISA method (*ELISA Ridascreen® Fumonisin R3401* (R-Biopharm, Darmstadt, Germany). Photometric measurements of ELISA plates were performed using Thermo Scientific Multiskan FC ELISA reader (Shanghai, China; Figure 3.4). Special software Rida®Soft Win (Z9999, R-Biopharm, Germany) was applied for data evaluation and calculation of toxin concentration.

The analysis of the obtained results was performed using statistical tests – comparative *t*-test and linear regression analysis as well as the PAST software (version 2.12, Oslo, Norway).

RESULTS AND DISCUSSION

The results on moisture content before storage and fumonisin level in the examined maize samples before and after storage are displayed in Table 1. As can be seen, total fumonisin content after storage is somewhat higher as compared to those measured immediately after harvesting. However, the results of both comparative *t*-test (at the level 0.05) and linear regression analysis (r=0.030, p=0.880) revealed no statistically significant difference between fumonisin contents before and after storage. Even though the increase in concentration is not substantial, especially in relation to maximum permitted levels, further analysis of the obtained data (Table 2) revealed an increase in fumonisin content in a number of samples (54%). Changes were not observed in 20% of samples, in which fumonisins have not been previously detected either before or after storage. Decrease of fumonisin concentration was observed in 26% of samples. However, this result should be considered with some reservations – it is questionable whether it could be attributed to actual fumonisin decomposition or to the sampling procedure itself, i.e., uneven toxin distribution in maize. Similar results of decreased toxin concentration during storage are reported in the literature (Atukwase et al. 2012). Correlation of moisture content and variation in fumonisin levels (Table 2) indicates that neither average moisture content nor the range suggest major effects of moisture on the aforementioned changes. Statistical linear regression analysis did not reveal any significant correlation between moisture content immediately post-harvest and fumonisin contents before (r=0.189; p=0.276) and after storage (r=0.171; p=0.326). According to the obtained results, we may conclude

that traditional storage system in barns is acceptable, that is, such conditions of drying and storage of maize are not associated with significant increase in toxin production.

Table 1. Fumonisin content in maize before and after storage in barns

Locality	House-hold	Grain moisture content immediately after harvesting (%)	Content of fumonisins (mg/kg)	
			Immediately after harvesting	After storage
Nakovo	1	17.39	ND	0.054
	2	21.48	ND	ND
	3	14.88	ND	0.034
	4	22.55	0.066	ND
	5	22.04	0.054	ND
	6	23.68	ND	0.083
	7	16.94	0.288	ND
	8	20.19	ND	ND
Putinci	1	25.56	ND	3.30
	2	22.56	0.620	0.651
	3	27.78	ND	0.383
	4	23.45	0.472	0.482
Ruma	1	24.65	0.072	0.606
	2	23.87	0.052	0.493
Sr. Kamenica	1	20.09	ND	ND
Rumenka	1	20.30	0.034	0.339
	2	16.10	0.036	0.151
	3	17.42	ND	0.056
	4	21.47	0.034	ND
	5	13.39	ND	0.162
Temerin	1	20.55	ND	0.079
	2	22.23	0.351	0.707
	3	25.55	0.654	ND
Lipar	1	26.94	0.085	ND
	2	27.57	ND	0.202
	3	27.01	0.069	0.303
	4	26.94	ND	ND
	5	26.10	ND	ND
	6	27.13	0.076	0.031
	7	24.55	0.042	ND
Bogatić	1	25.72	ND	ND
	2	21.88	ND	ND
	3	21.78	0.038	0.046
	4	29.65	ND	0.129
	5	27.79	1.04	0.388

No of positive samples (% of positive samples)	/	18 (51)	21 (60)
Average value	22.78	/	/
Average value in positive samples	/	0.227	0.413
Concentration range	13.39−29.65	0.034−1.04	0.031−3.30
Median value	22.56	0.070	0.202

ND – not detected; GD=25 μg/kg

Table 2. Effects of storage conditions on the production of fumonisins in maize

Changes in the concentration of fumonisins	Number of samples (% of positive samples)	Moisture content (%)	
		Average	Range
Increase	19 (54)	22.09	13.39−29.65
Decrease	9 (26)	23.88	16.94−27.79
No changes	7 (20)	23.20	20.09−26.94

The results of this research on the effects of storage conditions on fumonisin levels in maize in Serbia are similar to those obtained in other countries. Investigation of effects of 12-month storage period on growth of *Fusarium* sp. and fumonisin production conducted by Ono et al. (2002) revealed decrease in number of fungal population without any significant changes in toxin production. The maize samples were dried to 11% and 14% moisture content, thus, the authors are of the opinion that drying is of vital importance in preserving the quality of stored maize. Orsi et al. (2000) reported decrease in number of *Fusarium* colonies and decreasing tendency of fumonisin B_1 concentration (in spite of variation in concentration) after 140-day storage in wooden barns. The authors emphasized the importance of sample size for the assessment of variations in fumonisin levels. Traditional storage system in Uganda positively influenced the drying of maize (averagely from 19.2% to less than 14% moisture content during 2-month storage period), while incidence of fusarium-moulds significantly decreased throughout six months (by 31.9%). Moreover, fumonisin concentration dropped from 5.7 to 2.8 mg/kg (Atukwase et al. 2012). According to cited authors, decrease in fumonisin content is most likely related with ambient (temperature and relative humidity) and internal (moisture and metal ion contents) factors, which play an important role in fumonisin degradation. However, better understanding of fumonisins fate still requires further research.

CONCLUSION

The examination of stored maize samples revealed that traditional storage method in barns does not significantly influence the production of fumonisins in maize. Although the grain moisture content before storage exceeded 14%, the drying of maize in barns was adequate, thus, significant toxin production did not occur. Still, it should be emphasized that maize included in this research originated from harvest in a production year characterized by typical climate conditions in Serbia. Consequently, there were no significant deviations with respect to precipitation amount and air temperature as well as increased presence of corn pests. However, important climatic changes have been recorded in Serbia during past several years characterized by abrupt changes of extremely hot and extremely rainy weather. Comprehensive further research on the effects of such extreme climate conditions should be continued, particularly targeting the toxin production during storage of potentially mouldy or insect-affected maize.

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SEASONAL VARIATION OF AFLATOXIN M1 IN DAIRY PRODUCTS DURING 2015 IN SERBIA

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Abstract: During the last two years, official regulations about aflatoxin M1 (AFM1) level in milk are periodically changed. The cause of the variation in the concentration levels of AFM1 in milk during different seasons. The Serbian Government does not have the required regulations for the level of AFM1 in dairy products. The official regulations exist only for raw and heat treated milk.

The aim of this study was to monitor the level of AFM1 concentration in two groups of commercial dairy products (fermented dairy products and milk drinks) during different seasons (autumn, spring, winter and summer) in 2015 year. The level of AFM1 varied during the season and the higher level of AFM1 recorded during the autumn, while during the summer period AFM1 level is much lower. However, level of AFM1 in different seasons was not significantly different and generally is much less than in previous years.

The study indicates that current level of AFM1 in fermented dairy products and milk drinks is satisfactory. As it known, changes in the concentration of AFM1 in dairy products are a direct result of changes in the concentration of AFM1 in raw milk. However, based on literature data and results of this study it could be concluded that raw milk could be contaminated due to improper feed of cows, but is not accepted by dairy processors. These results indicate that situation regarding safety of dairy products has been improved, but still is essential to introduce monitoring programs for primary milk producers in order to avoid their losses.

Key words: *Aflatoxin M1, dairy products, season, Serbia*

INTRODUCTION

Milk is a highly nutritious food, and it is a source of necessary macro and micronutrients for the growth, development and maintenance of human health. However, it may also be a source of natural food contaminants that

may cause disease. Milk and dairy products contamination with aflatoxin M1 (AFM1) is important problem worldwide especially for developing countries in Mediterranean and Middle East region for the last ten to twenty years (Prandini et al. 2009). The presence of this mycotoxin in dairy products is important issue, especially for children and infants, who are more susceptible than adults.

Because aflatoxins are carcinogenic to animals and humans, they are monitored closely in the food supply. Milk that is sold commercially is checked for aflatoxin M1. When aflatoxin M1 is found at concentrations of 0.5 parts per billion (ppb) or greater, the milk is discarded because it cannot be used for products that go into the human food supply. The AFM1 concentration in milk and dairy products within EU is usually at very low level and below MRL (Prandini et al. 2009) indicating a strong control and continuous monitoring of feedstuff.

Opposite to that the Serbian Government because of poor supervision and monitoring of aflatoxin in milk during different seasons, finds a solution in frequent changing regulations and limits for the presence of AFM1 in milk. In order to harmonize regulations with the EU, the Serbian government established MRL of AFM1 in raw milks and heat treated at 0.05μg/kg (Serbian Regulation 2011, 2014). However, in 2013 a big crisis in terms of the AFM1 contamination in milk (Tomašević et al. 2015) led to the fact that the Serbian Government increased the maximum tolerance level from the EU level of 0.05 to 0.5μg/kg (Serbian Regulation 2013). 2014 as a result of the step taken to reduce the level of AFM1 in milk and dairy products, there have been new changes in legislation and the limit is again restored at the 0.05μg/kg (Serbian Regulation 2014). The latest legislative change occurred in the end of 2015 (Serbian Regulation 2015), which is the limit for the level of AFM1 milk was set to 0.025 mg/kg.

The aim of this study was to investigate the occurrence of AFM1 in dairy products and its seasonal variations during 2015 years. In a matter of fact, we wanted to check how moving the level of aflatoxin M1 in dairy products, as a direct result of the quality of raw milk during different seasons, regardless of the prescribed permissible level of AFM1 in raw milk by the Serbian Government. We hope that this research will improve the monitoring and control of AFM1 raw milk and as a consequence to mark consistently and better quality of dairy products in Serbia.

MATERIAL AND METHODS

Sampling and preparation

The chemicals and supplies used in the study were: AFM1 commercial kit (r-biopharm, RIDASCREEN, Aflatoxin M1, Germany); methanol and hexane, GLC grade-pesticide residue grade, (Fisher Chemical Scientific, UK).

A total of 660 samples including fermented dairy products (yoghurt and sour cream) and milk drinks (pasteurized, UHT and chocolate milks) were stored at 2–8°C until further analysis of AFM1. Duplicate analyzes were performed for each test sample.

Milk drinks

The liquid samples were cooled to 10°C and shaken manually to ensure sample homogeneity before being opened. Centrifuge milk drinks samples for 10 min, and after centrifugation, remove upper cream layer completely.

Fermented dairy products – yoghurt and cream samples

Bottles of yoghurt were shaken manually for 1 min before being opened to ensure that the mixtures were homogeneous. Preparation of yoghurt samples is based on the dilution of the 5g sample with 5ml water, after cooling at 10°C and shaking, the mixture was centrifuged for 10 min. The upper layer was aspirated and discarded. A 200 mL supernatant was diluted with 200 mL sampler buffered.

Cream samples (2.5g) were mixed with 10 mL methanol and incubated in water bath for 30 min at temperature 50°C. Then, samples were cooled to 10°C and centrifuged for 10 min. 2 ml of the supernatant placed in a test tube where 2 ml of hexane were added, stirred and cooled to 10°C. After centrifugation of about 10 min upper layer was removed completely. A 50 mL supernatant was diluted with 200 mL sampler buffered.

Aflatoxin analysis by ELISA (procedure and method validation)

Determination of AFM1 was done by Enzyme Linked Immuno-sorbent Assay (ELISA) method using standard validated commercial kit (r-Biopharm, RIDASCREEN, Aflatoxin M1, Germany). The test kit is sufficient for 96 determinations (including calibration curve). Kit is designed for the detection of AFM1 in milk, milk powder and cheese. Because of the advantages of the method, Elisa test is recommended for aflatoxin M1 in milk control, where screening method is necessary.

The analytical quality of the ELISA method was assured by the use of certified reference material (CRM). Milk powder with certified AFM1 content of 36.1±6.8 ng/kg (RealCheck AFLAM1 MilkHigh-Level MI211UK) was used as reference material. The validation parameters (Table 1) were calculated and expressed according to the requirements of British standard BS EN ISO 14675: 2003 and their values were in accordance with this standard. The quality of the results was tested through participation in a proficiency test organized by TEST VERITAS (Italy) of lyophilized milk. The proficiency test results were satisfactory according to the calculated z-score values of -0.40 and 0.30, respectively (acceptable range $-2 \leq z \leq 2$).

Table 1. Validation parameters for ELISA test

Validation parameters	Milk	Milk powder	Cheese
LOD (µg/kg)	0.006	0.006	0.021
LOQ (µg/kg)	0.020	0.020	0.070
RSDr (%)	0.88	0.31	1.45
Recovery (%)	96.00	98.00	101.00

LOD: limit of detection (µg/kg); LOQ: limit of quantification (µg/kg); RSDr: relative standard deviation calculated under repeatability conditions

RESULTS AND DISCUSSION

Seasonal variations and the presence of AFM1 in dairy products

Seasonal variations and the presence of AFM1 in dairy products during the different seasons of 2015 year (autumn, spring, winter and summer) are shown in Table 2.

Table 2. The overview of level AFM1 in dairy products during different seasons of 2015 in Serbia

Year	Seasons	Total no of sample	Range of AFM1 concentration (µg/kg) number of samples (frequency distribution. %)				Total % exceed EU (>0.05 µg/kg)	Mean ±SD	Min	Max
			≤ 0.025	0.026–0.05	0.051–0.5	> 0.5				
Milk drinks	Winter	105	91 (86.67%)	4 (3.81%)	10 (9.52%)	0 (0%)	10 (9.52%)	0.018 ±0.047[a]	0.005	0.310
	Spring	32	27 (84.38%)	2 (6.25%)	3 (9.37%)	0 (0%)	3 (9.37%)	0.015 ±0.037[a]	0.005	0.187
	Summer	24	21 (87.50%)	3 (12.50%)	0 (0%)	0 (0%)	0 (0%)	0.007 ±0.006[a]	0.005	0.048
	Autumn	197	138 (70.05%)	51 (25.89%)	8 (4.01%)	0 (0%)	8 (4.01%)	0.018 ±0.013[a]	0.005	0.149
	Total	**358**	**277 (77.37%)**	**60 (16.76%)**	**21 (5.86%)**	**0 (0%)**	**21 (5.86%)**	**0.018 ±0.021[A]**	**0.005**	**0.310**
Ferm-ented dairy pro-ducts	Winter	73	68 (93.15%)	1 (1.37%)	4 (5.48%)	0 (0%)	4 (5.48%)	0.017 ±0.047[a]	0.005	0.320
	Spring	18	16 (88.89%)	1 (5.55%)	1 (5.55%)	0 (0%)	1 (5.55%)	0.012 ±0.032[a]	0.005	0.185
	Summer	19	17 (89.47%)	2 (10.52%)	0 (0%)	0 (0%)	0 (0%)	0.007 ±0.006[a]	0.005	0.048
	Autumn	192	138 (71.87%)	51 (26.56%)	3 (1.56%)	0 (0%)	3 (1.56%)	0.022 ±0.015[a]	0.005	0.149
	Total	**302**	**239 (79.14%)**	**55 (18.21%)**	**8 (2.64%)**	**0 (0%)**	**8 (2.64%)**	**0.019 ±0.024[A]**	**0.005**	**0.320**

[a,b] values denoted with the same small letter are not significantly different at the level of 5% (P>0.05);
[A, B] values denoted with the same capital letter are not significantly different at the level of 5% (P>0.05).

The average AFM1 concentration in dairy products in 2015 year for milk drinks and fermented dairy products were 0.018 and 0.019 µg/kg and 5.86 and 2.64% of samples exceeded EU MRL. In 2013 and 2014 year, about 38% of dairy products were above EU MRL. The high AFM1 contamination of dairy products was a consequence of high raw milk contamination due to bed climate conditions in 2012 year and improper feeding of dairy cows in 2013 year (Škrbić et al. 2014). Big crisis in 2013 was followed by great public attention and as mentioned above Government solved it by regulation change.

Our unpublished data indicated that incidence of contamination of raw milk samples with AFM1 at the end of 2014 year was high (29.95% samples was above EU MRL) indicating that problem reappeared. However, compared to 2013 year, current situation is quite different. From results present in this study it could be seen that safety of dairy products were significantly improved but contamination of raw milk still exist and strongly depend of season (unpublished data). Hence, it can be concluded that dairy processors responded to the pressure aroused from the previous crisis (in 2013) and took more responsibility by refusing the reception of contaminated raw milk from farmers. Due to that we could say that the consumers are more protected in term of AFM1 intoxication, but the primary milk producers are still dealing with financial losses. A high occurrence of AFM1 in milk and dairy products were presented in numerous studies (Fallah 2010, Golge 2014, Iqbal et al. 2013, Kos et al. 2014, Tavakoli et al. 2012), but usually in countries where both climatic and technological conditions stimulate their formation.

Results of this study also indicate that seasonal variations of AFM1 concentration in dairy products were not found. However, the highest level of AFM1 concentration was found during winter and autumn seasons in both dairy products group. Seasonal variation of AFM1 concentration was presented by many authors. The most of them reported higher concentration of AFM1 in cold seasons as compared to hot seasons (Bilandžić et al. 2015, Hussain and Anwar 2008, Xiong et al. 2013). Elevated concentrations of AFM1 determined in milk in different countries in dry periods or during winter are influenced by the use of greater amounts of mixed supplementary feedstuff, dry hay and corn contaminated with high levels of AFB1 (Asi et al. 2012, Fallah 2010). Tomašević et al. (2015) reported that seasonal variation of AFM1 concentrations in heat treated milk samples followed the trend observed in raw milk analysing a big set of data in 2013 and 2014 year. They found that in these years the significant higher AFM1

concentration was during winter and spring as consequence of bed quality feed produced in previous year.

CONCLUSION

Results of this study showed quite satisfactory situation regarding AFM1 contamination in dairy products. Despite the raw milk contamination that appears in some seasons this data indicate that dairy processors introduced much more control and refuse to accept contaminated raw milk.

However, primary milk producers still need to improve conditions and introduce more control in order to reduce AFM1 in raw milk and avoid financial losses.

Strict regulations and continuous feed control in Serbia would contribute to AFM1 level reduction to the EU level. The current advancements in analytical techniques could help to continuous implementation of strict regulation. Also, increasing the awareness and education level of primary milk producers can contribute to minimize of AFM1 occurrence in raw milk and dairy products.

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THE USE OF ORGANIC ACIDS IN ANIMAL NUTRITION

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Abstract: During the last years there has been reported an increase in the use of organic acids as feed additives, which puts them at the center of scientific attention. The European ban of antibiotic growth promoters in 2006, in order to avoid the risk of developing cross-resistance of pathogens, but also to avoid their residues in meet, led to the need for finding the alternative supplements to replace the antibiotics. Among others (enzymes, probiotics, prebiotics, herbs, essential oils, immunostimulants…), organic acdis and their salts are pointed out as effective. Organic acids have been used for decades in commercial compound feeds, mostly for feed preservation, because of the decrease of pH value and the buffering capacity as well as antibacterial and antifungal effects in the feed. These effects are also manifested in gastro-intestinal tract, where they inhibit gram-negative indigenous microflora (*Salmonella spp.*, *Escherichia coli*, *Campylobacter jejuni*...). Organic acids reduce the pH value by release of hydrogen ions in the stomach, thereby activating pepsinogen to form pepsin consenquently improving protein digestibility (optimum pH for the activity of pepsin is 2,5–3). Further, they improve the digestion of minerals (P, Ca, Mg, Zn) from nutrients, due to the increased enzyme activity at lower pH (microbial phytase) and the formation of soluble complexes of minerals and organic acid anions. Organic acid improve energetic utilization in the intermediate metabolism. While the main action of organic acid in poultry is mainly antimicrobial, in pigs has been shown that short chain fatty acids like acetic, propionic and n-butyric acid in the GIT may increase the proliferation of epithelial cells and have stimulatory effects on both endocrine and exocrine pancreatic secretions. Lack of consistency in demonstrating an organic acid benefit in poultry is related to the fact that organic acids are rapidly metabolised in the craw, which reduces their impact on growth performances of broilers, but also on layer performances (egg production, egg quality).

Key words: *organic acids, antimicrobial activity, growth performance, pigs, poultry*

INTRODUCTION

During the last years there has been reported an increase in the use of organic acids as feed additives, which puts them at the center of scientific attention. The European ban of antibiotic growth promoters in 2006, in order to avoid the risk of developing cross-resistance of pathogens, but also to avoid their residues in meet, led to the need for finding the alternative feed supplements to replace the antibiotics. Among others (enzymes, probiotics, prebiotics, herbs, essential oils, immunostimulants…), organic acdis and their salts are pointed out as effective. Organic acids have been used for decades in commercial mixtures, mostly for feed preservation, but their positive effects on production performances and animal helth have been also demonstrated in a number of trials. As they increasingly capture the attention of the feed and livestock industry, there is a growing need to define the several acids and their salts.

Organic acids are carboxiliyc acids (general structure R-COOH), of which short-chain acids (C1–C7) have antimicrobial activity. Most of the organic acids used in animal nutrition have an aliphatic structure (formic, propionic, lactic, acetic, sorbic or citric acids; benzoic acid is built on aromatic ring). Formic, acetic, propionic and butyric acids are monocarboxilyic acids, lactic, malic, tartaric and citric acids bind an hydroxyl group (usually on the α carbon), werhease fumaric and sorbic acids are unsaturated. These are weak acids, which can be used as additives to drinking water or as feed acidifiers (Dibner and Buttin 2002). Also, organic acids can be microencapsulated in protective lipid matrix, which allows slow release of the active ingredients in the GIT. Consequently, they reach the distal part of the intestine, where they can modulate the microbial ecosystem by decreasing coliform populations (Grilli et al. 2015, Piva et al. 2014). In animal nutrition acidifiers and their salts exert their performance promotion effects via: decrease of pH value and the buffering capacity as well as antibacterial and antifungal effects in the feed; reduction of pH value in the stomach and small intestine (thereby activating pepsinogen to form pepsin and improving protein digestibility); antimicrobial activity in the GIT; improved energetic utilization in the intermediate metabolism.

ORGANIC ACIDS IN FEED

Under favourable conditions, especially at higher moisture level and in a warm environment, microbes can rapidly multiply during storage of the feed. In order to reduce microbial growth and count different conserving agenst have been used. Among others, organic acids have been used, usually in lower concentrations than for promotion of growth performances. For each acid its specific inhibitory effect on bacteria, yeast or mould, along with minimal inhibitory concentration, is determined (Freitag 2009). For example, formic acid is not suitable to suppress moulds in compound feeds

or grains, because it may promote the growth of aflatoxin-forming *Aspergillus* species (Freitag 2009). Thus, the use of additives that contains more than 50% of formic acid, to treat aerobically stored, high-moisture grains, is prohibited in the EU.

Besides that hygienic effect, reduction of the pH value and the buffering capacity of feed, can have beneficial impact on animal health, especially in pigs. At weaning period, disruption of milk intake, lead to the reduction of bacterial fermentation and production of lactic acid, while secretion of HCl is still low. The high acid-binding capacity of the feed, because of the high content of the crude protein and minerals in feed, helps to further raise the stomach pH. In those environmental conditions, pepsin activation and pancreatic enzyme secretion are reduced, impairing nutrient digestion. Thus, lowering of buffering capacity of feed has beneficial impact on feed digestion. (Suiryanrayna and Ramana 2015, Freitag 2009).

REDUCTION OF pH in GIT

While in poultry the main action of organic acids is antimicrobial, in pigs that is the reduction of stomach pH. Protein digestion in pigs begins in stomach, by activating of pepsin from pepsinogen, as the enzyme precursor. Conversion of pepsinogen to pepsin is enhanced at lower pH (optimum pH for the activity of pepsin is 2,0 to 3,5) (Suiryanrayna and Ramana 2015). Inadequate reduction of stomach pH inhibits pepsin activity and protein digestion, particularly at weaning pigs, in which, due to the low hydrochloric acid output, transition from milk to solid food, and high acid-binding capacity of the feed, there is an increase of pH level in stomach. Organic acids reduce the pH value by release of hydrogen ions in the stomach. Also, duodenal secretion of pancreatic enzymes is reduced at high pH, which impairs overall digestion. Supplementation with organic acids leads to the decrease of duodenal pH, thus improving nutrient digestibility and N retention (Freitag 2009, Overland et al. 2000). Pancreatic exocrine response is the highest for formic acid, followed by lactic acid, then pyruvic, acetic, butyric and propionic acid (Suiryanrayna and Ramana 2015).

IMPACT ON NUTRIENT AND MINERAL UTILIZATION AND GUT MORPHOLOGY

It has been proven that short chain fatty acids, such as propionic and n-butyric acid, may increase the proliferation of epithelial cells. Supplementation with sodium butyrate, postweaning, increased the mucosal thickness, and increased the densites and numbers of enteroendocrine and SST cells in the fundic mucosa of pigs (Mazzoni et al. 2008). Lu et al. (2008) proved an increase in villus height and villus height to crypt depth ratio at the small intestinal mucosa of the pigs supplemented with 1000

mg/kg sodium butyrate. Galfi and Bokori (1990) demonstrated an increase in the length of the microvilli in the ileum and the depths of the crypts in the caecum in growing pigs when fed with 0.17 % of sodium butyrate.

Organic acids improve the digestion of minerals (P, Ca, Mg, Zn) from nutrients, due to the increased enzyme activity at lower pH (microbial phytase) and the formation of soluble complexes of minerals and organic acid anions, thus improving the digestion of these minerals and reducing their excretion (Suiryanrayna and Ramana 2015).

ANTIMICROBIAL ACTIVITY

The antimicrobial activity of organic acids *in vivo* may be a resultant of various factors including: the carbon chain length and inclusion level; the proportion of dissociated to undissociated forms; intra-luminal digesta acidity and acid-binding capacity; the time of retention/exposure of digesta in particular gut segments; specific potency of pathogens for colonization and enterotoxin production (Mroz et al. 2006). Mode of action of organic acids and their salts is based on two fields of their impact. Organic acids and their salts manifest their antimicrobial activity by lowering the pH level of the environment, and by direct effects of anions and protons on microbial cell. Growth rate of many microbes (*Cl. perfrigens*, *E. coli*, *Salmonella spp*.) is reduced below pH 5, while acid tolerant microorganisms are unharmed, like *Lactobacillus spp.*, which counts stay unaffected or may even be enhanced (this helps eubiosis in post-weaning pigs) (Freitag 2009).

On the other hand, organic acids exert their direct antimicrobial activity due to the fact that they are lipophilic and can diffuse across the cell membrane, where, in the base environment of the cytoplasm, they dissociate, release protons, and consequently reduce the internal pH in the microbe cell (Freitag 2009).

Organic acids exert their bactericidal effect due to their ability to dissociate according to the pH of the environment. Only in its un-dissociated form, they can pass throught the cell walls of bacteria and fungi. Inside the cell, pH of the cell matrix is higer then pK of the acids, because of which a large proportion of the acid will dissociate and release its hydrogen ion. Lowering of the pH value of the cytoplasm alert metabolism of the cell, and its enzymes activity, which leads to the inhibition of its growth. In the attempt to pump out the hydrogen ions (H^+) via the H^+-ATPase pumps, the microbial cell consumes enormous amounts of energy that lead to cell death (Dibner and Buttin 2002, Freitag 2009, Lückstädt and Mellor 2011, Suiryanrayna and Ramana 2015).

The efficiency of an organic acid to inhibit the growth of the microorganisms depends on its pKa value, which describes the pH value at which the acid is available 50% in its dissociated and undissociated form respectively (Table 1). Only in its un-dissociated form the organic acid has its bacteriostatics and bactericidal effects. That means that the antimicrobial

efficacy of organic acid is higher in acidic conditions, like in the stomach, than at neutral pH, like in the intestine. Also, organic acids with a high pKa value are weaker acids and thus more effective preservatives for feed, as, being present in the feedstuff with a higher proportion of their un-dissociated form. Therefore, the lower the pKa of the organic acid is, the greater is effect on the reduction of stomach pH and the lower is antimicrobial effect (because of the higher proportion of its dissociated form) (Freitag 2009).

Table 1. Chemical properties of selected acids and salts

Acid/Salt	pK value	Solubility in water	Molecular weight (g)	Gross energy (KJ/g)	Physical condition
Formic acid	3,75	very good	48,0	5,8	liquid
Acetic acid	4,75	very good	60,1	14,8	liquid
Propionic acid	4,87	very good	74,1	20,8	liquid
Lactic acid	3,08	good	90,1	15,1	liquid
Fumaric acid	3,03/4,44	low	116,1	11,5	solid
Citric acid	3.14/5.95/6.39	good	210,1	10,3	solid
Ca-formate	-	low	130,1	3,9	solid
Na-formate	-	very good	68,0	3,9	solid
Ca- propionate	-	good	16,6	16,6	solid
Ca- lactate	-	low	10,2	10,2	solid

(Kirchgessner and Roth 1991)

Additionally, there are some specific effects against yeasts, moulds and bacteria of each acid, which cannot be explained by the pKa value. The rate at which organic acids kill bacteria depends on the time of exposure, ambient temperature and specific properties of the acid used. Minimum inhibiting concentrations of each organic acids against bacteria species are determined under *in vitro* conditions (Table 2). For example, G- bacteria are only sensitive to acids with less than eight carbon atoms, whereas gram positive bacteria are sensitive to longer chain acids (Partanen 2001). Strauss and Hayler (2001) state that low concentration of lactic acid can stimulate the growth of *Clostridium perfrigens*, if this acid was used in the dosage below 0,2%. Other authors found enhanced development and increased mycotoxin formation when propionic, benzoic and sorbic acids were used at subinhibitory concentrations (Strauss and Hayler 2001).

Table 2. Minimal inhibitory concentration (MIC) of different organic acids

	Formic acid (%)	Propionic acid (%)	Lactic acid (%)
Salmonella typhimurium	0,10	0,15	0,30
Escherichia coli	0,15	0,20	0,40
Campylobacter jejuni	0,10	0,20	0,25
Staphylococcus aureus	0,15	0,25	0,40
Clostridium botulinum	0,15	0,25	0,30
Clostridium perfringens	0,10	0,25	0,30

(Strauss and Hayler 2001)

All organic acids are corrosive products, even if they are sprayed on a carrier. That's why salts of organic acids, like calcium propionate, sodium formate or sodium benzoate, may be used as their solid and non-corrosive form. Organic acids salts added to feed, reduce the buffering capacity of the compound feed, by increasing the concentration of cations (e.g. Ca^{2+}, Na^{+}). However, activity of the organic acid salts depends of free hydrogen ion, which will activate this molecule. Actually, in the presence of the stronger acid, the weakar one is released from its salt. That means that in the acid stomach pH, begins a reaction in which will be formed organic acid, from HCl and salt of organic acid (e.g. NaCOOH (sodium formate) +HCl (hydrochloric acid) → HCOOH (formic acid) +NaCl (sodium chloride)). In pigs nutrition this will improve the digestion at adults, better than in young pigs, whose stomach produce less HCl. Organic acid salts don't have direct acidifying effect, they can't reduce the pH level of the environment, thus their activity is determined by their anion (Freitag 2009).

The importance of anions in overall benefits for performance can be demonstrated by comparison of acids and salts in feeding trials. Kirchgessner and Roth (1987) compared formic acid and Na-formate in trial in which equal amounts of anions were added to both groups respectively. Growth promoting effects, such as daily gain and feed conversion ratio, were around 50% in the salt group compared with those receiving formic acid. This appears to be due to the effects of anions, whereas the higher effects observed for the formic acid group were due to pH reduction. Conversely, reduction of gut microbe counts seems to be due to the anionic effect. Numbers of *E. coli* and *Enterococcus* ssp. can be reduced to similar levels with either Ca-formate or formic acid (Kirchgessner et al. 1992).

Additionally, change in microflora of GIT, cause changes in the intestinal environment, consequently reduce concentrations of ammonia and lactic acid in stomach and small intestine. Reduction in total count of bacteria in GIT reduces the metabolic needs of microbes, which leads to the better absorption rate of nutrients, especially energy and amino acids, and

enhanced feed efficiency and daily gain (Overland et al. 2000). Further, eubiosis of intestinal flora and the reduction in the activity of microbes, leads to the reduced intestinal ammonia, amine and toxin concentrations and consequently less digestive disorders, e.g. diarrhoea. Hellweg et al. 2006, demonstrated significantly increased dry matter content in the caecum of pigs, after supplementation with potassium diformate, thus in this respect salts can be as effective as acids. Diarrhoea can also be prevented by inhibition of pathogens like *E. coli* to adhere to the gut wall. Most microbes can only proliferate, alter epithelial function or produce toxins after binding to specific receptors on the gut wall. Again, these effects seem to be more closely related to anions rather than to undissociated acids (Freitag 2009). However, sufficient amounts of anions are essential, as acids with low molecular weight, e.g. formic acid, seem to have more pronounced activities than those with higher molecular weights, e.g. fumaric or citric acid (Eidelsburger 1997).

ORGANIC ACIDS IN METABOLISM

Organic acids are absorbed throught the intestinal epithelia by passive diffusion, and they carry the conisderable amount of energy. Thus, they act as energy source, as these are intermediate products of tricarboxylic acid. Short chain acids can be envolved in cytric cycle, as precursors of ATP (for example, 18M ATP is generated from 1M fumaric acids). Sorbic acid is metabolised via ß-oxidation, as long chain fatty acids (Freitag 2009). Kirchegessner and Roth (1988) suggested that pigs can utilize fumaric acid as energy source as efficient as glucose. The same is true for citric acid, while acetic and propionic acid need 18% and 15% more energy for 1M ATP synthesis (Kirchgessner and Roth 1988). Blank et al. (1999) reported that there is a possibility that fumaric acid as a readily available energy source may have a local trophic effect on the mucosa in the small intestines.

ORGANIC ACIDS IN PIG NUTRITION

It has been proven that in adequate doses organic acids can improve growth performances in pigs to level comparable with antibiotic growth promoters.

Øverland et al. (2009) added 0.8% or 1.2% potassium diformate to diets for primiparous and multiparous sows, during gestation and lactation. The performance of the piglets of these sows was also recorded and compared. The authors found that sows fed potassium diformate had increased back fat thickness during gestation, although daily feed intake and body weight gain did not change. Feeding potassium diformate also tended to be associated with a heavier birth weight of piglets, average daily gain, and consequently greater weaning weight, irrespective of dose.

Partanen et al. (2001), proved significant feed-to-gain improvements at weaned-piglets and fattening-pigs, after supplementation with formic, fumaric, and citric acids and potassium diformate. Weight gain and feed intake effects were significant for formic acid and potassium diformate (Dibner and Buttin 2002).

Creating and maintaining a healthy intestinal environment has become essential to productivity and food safety programmes. Improved hygiene and biosecurity measures alone improved *Salmonella* status, but to a much lesser extent than when it's included dietary acidification. Correge et al. (2010) concluded that potassium diformate reduced the percentage of salmonella-positive pigs by 50% and decreased salmonella ELISA scores in pork meat juice by 46% in grower-finishing pigs. The antimicrobial activities of organic acids differ from acid to acid depending upon concentration and pH. As examples, lactic acid is more effective in reducing gastric pH and *Coliforms* whereas other acids like formic and propionic acids have broader range of activity on *Salmonella, Coliforms* and *Clostridia spp*. (Suiryanrayna and Ramana 2015). Additionally, adaptation to acidic environments is recognised as an important survival strategy for many micro-organisms. This should be monitored when acids are added to feed over a longer period of time (Freitag 2009).

ORGANIC ACIDS IN POULTRY NUTRITION

Improvements in broiler performance and hygiene in response to organic acids are often reported. Improved broiler performance by supplementation with single acids was noticed for formic acid and fumaric acid. However, an important limitation is that organic acids are rapidly metabolised in the crew, which will reduce their impact on growth performance (Lückstädt and Mellor 2011). Double salts of organic acids, such as potassium diformate and sodium diformate, which reach the small intestine, have a significant impact. Selle et al. (2004) demonstrated the effects of potassium diformate, et doses 0,3–1,2%, on nutrient utilization. Furthermore, diformates reduced numbers of pathogenic bacteria (*Salmonella, Campylobacter and Enterobacter*) in broiler chickens and increased numbers of *Lactobacilli* and *Bifidobacteria* (Khan et al. 2016). The mode of action of acidifiers in poultry is mainly antimicrobial, whereas in pigs that is reduction of stomach pH (Lückstädt and Mellor 2011). There is data about beneficial impact of organic acids on layer performances and egg quality (particularly egg shell thickness). Drinking water acidification is preferred in the modern broiler and layer industry for improving performance (Khan et al. 2016).

CONCLUSION

Literature available to date reveals that organic acids and their salts can be used to enhance animal health and performance, and as such they can be used to replace sub-therapeutic antibiotic growth promoters. Their effects include several levels of action: in feed, in GIT- effects on enzyme secretion, gut morphology, nutrient and mineral digestion, antimicrobial activity… Economic benefits result from a reduction in feed costs and a shorter time to market, in pigs and poultry production, but above all, these are products generally recognized as safe in livestock and food industry.

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LC-ESI-MS/MS DETECTION OF FUNGICIDE RESIDUES IN BROWN HARE

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Abstract: The liquid chromatography – tandem mass spectrometry technique combined with electrospray ionization, LC-ESI-MS/MS, was applied for the detection of 22 fungicides in the adipose tissue of brown hare. The extracts of fifteen samples were obtained using the acetonitrile-based QuEChERS preparation technique for fat samples. This was equipped with a reversed-phase C18 analytical column of 50×4.6 mm and 1.8 μm particle size (Agilent Zorbax Eclipse). For the mass spectrometric analysis, the Agilent 6410B Triple-Quad LC/MS system was applied. Agilent Mass Hunter software was used for the method development, data acquisition and quantification. The validated method was used for the determination of fungicide residues in fatty tissue, using carbofuran-D3 as an internal standard. The basic validation parameters include the calibration which ranged from 5.0 to 250.0ng/ml. The recoveries were investigated at the 10, 50 and 100 μg/kg levels. The average recoveries for all analites were 53.0 to 101.4% (RSDs 8.21–11.47%). The total of 8 fungicides was detected in the analysed samples (azoxystrobin, carbendazim, cyproconazole, cyprodinil, difenoconazol, pyrimethanil, tebuconazole and trifloxystrobin). The most frequent fungicides were cyprodinil (6 samples) and difenoconazol (4 samples). Pyrimethanil, tebuconazole and trifloxystrobin were detected in three samples. The regulated MRLs values which are within the EU Pesticide database, supervised by the European Commission, do not comprise pesticide residues in fatty brown hare tissue. But for most animals the MRLs were set and they are the same for different animals. Taking into account the EU MRLs, all the detections of cyprodinil were above the MRL of 0.02 mg/kg and they were in the range of 0.023 to 0.027 mg/kg. In two samples the concentrations of difenoconazol and trifloxystrobin were above the MRLs of 0.05 and 0.06 mg/kg, respectively. The high concentration of tebuconazole was detected in one sample and it was 1.500 mg/kg, while the MRL for this pesticide is 0.1 mg/kg.

Key words: *fungicide residues, LC-MS/MS, brown hare, adipose tissue*

INTRODUCTION

Pesticides are modern chemical substances for plant protection. They can be used against microorganisms (bactericides and fungicides), insects (insecticides) and weeds (herbicides). As very toxis agents pesticides can affect the game, directly or indirectly, through contaminated food and water (green crop, weed seeds, and other plants, insects etc.) (Beuković and Popović 2014). By drastic increase in areas under crops where the pesticides are intensively used along with the decrease in areas under fodder crops, the possibility for proper brown hare nutrition is reduced while its typical habitats have been changed (Bursić et al. 2015).

At the registration of the preparations for use with the Ministry in charge special care is taken of the toxicity of crops themselves and their PHI, i.e. the time after which it is possible to use agricultural products in the nutrition of people and animals without any hazards to their health. However the game under all these circumstances presents a „collateral damage" since there are no particular studies on game and the concern about their protection is reduced to declarative statements, „poisonous to game, fish and amphibia, not to be used in the vicinity of watercourses" (Topić 2013). Some of the undesired consequences of the pesticide application on the population of hare and other animals is the presence of pesticide residues in fat tissues and organs, higher mortality, reduced immunity to diseases, shorter life cycle, reduction of fecundity and fertility as well as behavioral changes (Šovljanski and Lazić 2007).

The causes of lower number of hares have not been positively confirmed but it is supposed that they are the result of the changes of the hare environment (Bošnjak 2006). The application of the mechanization and chemical substances has very unfavorable effcct on this kind of game (Beuković et al. 2011).

That is why the aim of our study was to determine the pesticide content from the group of fungicides by validated multi residual method using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

MATERIAL AND METHODS

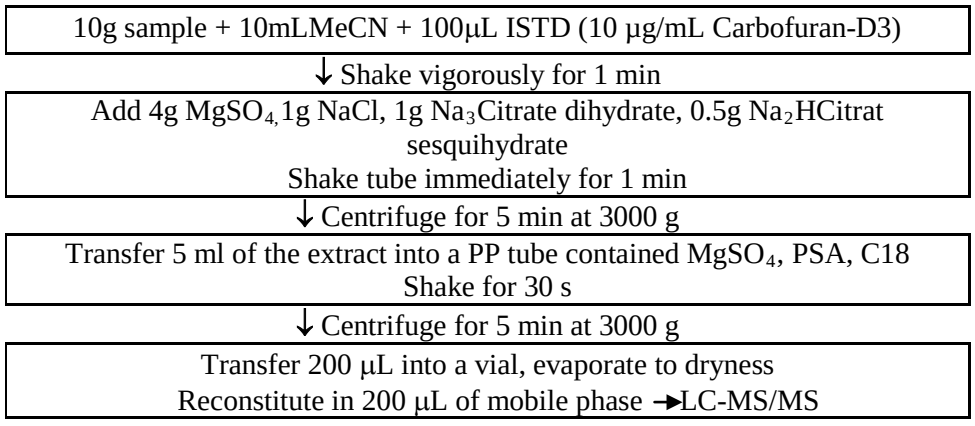
Chemicals: The analitical fungicide standards were manufactured by Dr. Ehrenstorfer GmbH, Germany. As an internal standard carbofuran-D3 (99.7%) was purchased from Pestanal, Fluka (Germany) and was used in the concentration of 1.0 mg/mL of the basic standard in acetonitrile with the dilution up to 1.0 μg/mL. The stock standard solutions were prepared by dissolving an analytical standard in acetonitrile while the working solution i.e. the mixture of the studied pesticides was obtained by mixing and diluting the stock standards with acetonitrile resulting in the final mass concentration of 10 μg/mL.

Aparature: For LC analysis, an Agilent 1200 (Agilent Technologies, USA) HPLC system th a binary pump was used. This was equipped with a reversed-phase C8 analytical column of 150×4.6mm and 5 μm particle size (Agilent Zorbax Eclipse XDB). The mobile phase was methanol and Milli-Q water with 0.1% formic acid in gradient mode, with the flow rate 0.6 mL/min. For the mass spectrometric analysis, an Agilent 6410 Triple-Quad LC/MS system was applied. Agilent Mass Hunter Data Acquisition, Qualitative Analysis and Quantitative Analysis software were used for method development and data acquisition.

Validation: Within the validation the recoveries of extraction, detection limits (LOD), quantification limits (LOQ) and linearity with carbofuran-D3 addition as an internal standard (IS) were determined according to SANCO/12571/2013. The LOD was determined as the lowest concentration giving a response of three times the average baseline. The ratio signal/noise in the obtained chromatograms for the LOD was calculated by MassHunter Qualitative Software. The linearity was checked using matrix matched standards (MMS) at the concentrations of 5.0, 10.0, 25.0, 50.0 and 100.0 ng/mL. The recovery was checked by enriching 10 g of a blank sample with the mixture of pesticide standard of 10 μg/mL in the amount of 100 and 50 μL (final mass concentration 0.10 and 0.05 mg/kg) and with the mixture of pesticide standard of 1 μg/mL in the amount of 100 μl (final mass concentration 0.01 mg/kg) with the addition of the internal standard carbofuran-D3.

Samples and fungicide extraction: Brown hare were collected from the agricultural areas from Vojvodina region. Fatty tissue of fifteen animals was immediately collected and put in dark plastic bags. All the samples were put in the freezer until they were analyzed by QuEChERS method (Figure 1).

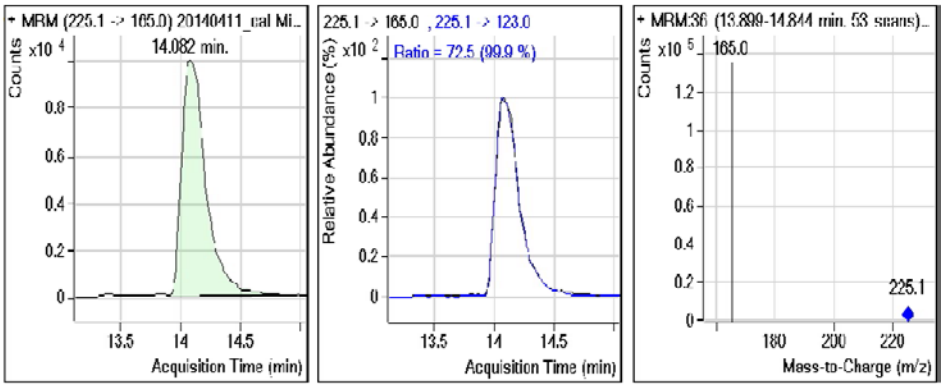
Figure 1. QuEChERS extraction of fungicide residues



RESULTS

The determination of the invested fungicides was done in monitoring mode of ion transfer (MRM) (Table 1).

Figure 2. MRM transitions of carbofuran-D3



Some basic validation parameters were given in Table 1. For all the studied pesticides the LOQ of 0.01 mg/kg, was reached while mathematically calculated LOD was 0.005 mg/kg.

Table 1. Retention times, MRM and basic validation parametersof studed pesticides

Pesticide	MRM transition (m/z)	t_R (min)	R^2	Recovery±RSD (%)
Azoxystrobin	404.1→372.1 404.1→344.1	16.33	0.9984	98.3±10.33
Carbendazim	192.1→160.1 192.1→132.0	8.05	0.9995	98.8±8.91
Cyproconazole	292.1→125 292.2→70.1	17.00	0.9978	81.0±1.39
Cyprodinil	226.1→108 226.1→93	17.40	0.9978	73.8±0.85
Dimethomorph	388.1→301.1 388.1→165	16.56	0.9995	93.5±4.15
Difenoconazol	330.1→121 330.1→101	17.34	0.9965	93.1±2.41
Epoxiconazole	330.1→121 330.1→101	17.34	0.9965	93.1±2.41
Fenpropimorph	304.3→147.1 304.3→130.1	15.17	0.9990	48.1±9.72

Flusilazole	316.1→247.1 316.1→165	17.30	0.9998	92.3±2.95
Flutriafol	302.1→123	15.18	0.9992	83.2±3.14
Metconazole	320→125 320→70	18.13	0.9973	77.3±2.85
Methalaxyl	280.2→220.1 280.2→192.1	15.47	0.9971	98.0±4.63
Oxadixyl	279.1→219.1 279.1→133.3	13.07	0.9902	102.3±2.38
Pencycuron	329.1→125.1 329.1→99.1	18.20	0.9986	48.1±3.18
Pyraclostrobin	388.1→194 388.1→163	17.98	0.9961	53.0±2.94
Propiconazole	342.1→159	17.92	0.9951	85.2±4.33
Spiroxamine	298.3→144.1 298.3→100.1	15.26	0.9981	47.3±6.51
Tebuconazole	203 →104 203 →175	18.01	0.9941	101.7±2.74
Thiophanate-methyl	343.1→151 343.1→93	14.11	0.9882	84.8±11.04
Triadimefon	202 →132 202 →96	18.77	0.9811	78.9±5.45
Trifloxystrobin	409.1→206.1 409.1→186.1	18.30	0.9885	103.0±5.97
Zoxamide	336→187 336→159	18.06	0.9953	91.3±1.78

DISCUSSION AND CONCLUSION

The fungicide residues of azoxystrobin, carbendazim, cyproconazole, cyprodinil, difenoconazol, pyrimethanil, tebuconazole and trifloxystrobin were detected in the analyzed brown hare adipose tissue. The most frequent fungicides were cyprodinil (6 samples) and difenoconazol (4 samples). Pyrimethanil, tebuconazole and trifloxystrobin were detected in three samples. The regulated MRLs values which are within the EU Pesticide database, supervised by the European Commission (Regulation (EC) No 396/2005), do not comprise pesticide residues in fatty brown hare tissue. But for most animals the MRLs were set and they are the same for different animals. Taking into account the EU MRLs, all the detections of cyprodinil were above the MRL of 0.02 mg/kg and they were in the range of 0.023 to 0.027 mg/kg. In two samples the concentrations of difenoconazol and trifloxystrobin were above the MRLs of 0.05 and 0.06 mg/kg, respectively.

The high concentration of tebuconazole was detected in one sample and it was 1.500 mg/kg, while the MRL for this pesticide is 0.1 mg/kg.

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DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRIC METHOD FOR THE DETERMINATION OF T-2 AND HT-2 TOXIN

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Abstract: Among the type A trichothecenes the most attention is paid to T-2 and HT-2 toxins due to their higher prevalence in crops. The European Food Safety Authority (EFSA), the Panel on Contaminants in the Food Chain established a group of tolerable daily intake (TDI) of 100 ng/kg b.w. for the sum of T-2 and HT-2 toxins. The estimates of chronic human dietary exposure to the sum of T-2 and HT-2 toxins based on the available occurrence data are below the TDI for populations of all age groups, and thus not a health concern. *Fusarium langsethiae*, *F. poae*, and *F. sporotrichioides* are the predominant species that invade cereal crops and produce T2 and HT2 under cool and moist conditions in the field. Similar to most trichothecenes, T2 and HT2 not only inhibit protein synthesis and cell proliferation in plants, but also cause acute or chronic intoxication of humans and animals. The LC-MS/MS method was optimized and validated for the simultaneous determination of T2 and HT2 mycotoxins. The MRM transitions for T2 were from 484.2 to 185.1 and from 484.3 to 215.2, with the retention time of 5.854 min. The retention time of HT2 toxin was 4.250 min, with the MRM from 447.4 to 345.1 and from 447.4 to 263.1.The extraction was performed with acetonitrile/water (80/20, V/V). After the filtration the extract was cleaned up using MycoSep (Romer Labs) push-through columns.The mobile phase consisted of a mixture of 0.1% formic acid, the flow 0.4 ml/min in gradient programme from 90% water to 5% water for 20 min. The recovery is about 70–80% at two levels 10 and 100 µg/kg. The limit of quantification (LOQ) obtained by chromatographic parameters optimization, for T2 and HT2 were 5 and 3 µg/kg. The good linearity for T2 and HT2, in the range of 2.5 to 500ng/mL (3–625 µg/kg) was obtained with the R^2 of 0.9980 and 0.9972, respectively. The obtained method is applicable to the analyses of real samples.

Key words: *T-2, HT-2, method validation, LC-MS/MS*

INTRODUCTION

Mycotoxins are secondary metabolites produced by a wide range of fungi known to contaminate a variety of food and agricultural commodities worldwide. Their occurrence in food, beverages and feed has been recognized as potential threat to human and animals (Vuković et al. 2014).

T-2 toxin (T2) and HT-2 toxin (HT2) are type A trichothecene mycotoxins produced by several *Fusarium* species in cereal grains mainly *Fusarium sporotrichioides*, *Fusarium poae* and *Fusarium langsethiae*. The occurrence of T2 and HT2 is generally restricted to small grain cereals and maize. The results of recent surveys on cereals across Europe have shown that HT2 and T2 are common contaminants, particularly in oats and oats products. Low to moderate concentrations occur in wheat, barley, maize and rye (Lattanzio et. al 2012). T2 is rapidly metabolized *in vivo* to HT2 that also induces adverse effects similar to T2, with no remarkable difference in terms of potency (Canady et al. 2001). Therefore, a combined provisional maximum tolerable daily intake was set at (TDI) of 100 ng/kg b.w for the sum of T-2 and HT-2 toxins. Current EU legislation states that intake estimates indicate that the presence of T2 and HT2 can be of concern for public health, and maximum permitted levels for the sum of T2 and HT2 are actually under discussion. To reduce the levels of biogenic toxins, the European authorities are currently discussing further regulations on mycotoxins. Within the European Union (EU), harmonized legislation is setting maximum limits for aflatoxins and ochratoxin A in cereals and cereal products. Limits for *Fusarium* toxins (DON, ZEA, HT2, and T2) are currently being drafted in EU member states (Biselli et al. 2005).

Therefore, the development and validation of reliable method like liquid chromatography with tandem mass spectrometry (LC-MS/MS), for the determination of these mycotoxins will be the first step towards more investigation/research on the presence of these toxins in cereals and cereal products which is necessary and of 'high priority'.

MATERIAL AND METHODS

Instrumentation and chromatographic conditions for LC-MS/MS

LC was performed with an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump, a G1367D autosampler and a G1316B column oven (Agilent Technologies, USA). Chromatography separation was achieved by Zorbax Ecllipse XDB C18 column (50 x 4.6 mm, 1.8 μm) (Agilent, USA) maintained at 30 °C. The analytical separation was performed using gradient program starting from 90% water to 5% water for 15 min, with methanol as mobile phase A, and as water mobile phase B, both containing 0.1% formic acid (70:30, v:v).

The flow rate was maintained at 0.5 mL/min. The mass analysis was carried out with an Agilent 6410B Triple Quadrupole mass spectrometer equipped with multi-mode ion source (MMI, Agilent Technologies, Palo Alto, CA, USA) (Turner et al. 2004). The data acquisition and quantification was conducted using MassHunter Workstation software B.04.01 (Agilent Technologies 2010). The following ionization conditions were used: electrospray ionisation (+ESI) positive ion mode, drying gas (nitrogen) temperature 325 °C, drying gas flow rate 5 L/min, nebulizer pressure 50 psi and capillary voltage 3000 V. The dwell time was 100 ms. External standard method was used for quantification of T2 and HT2.

Chemicals and reagents

T2, 100µg/mL in acetonitrile, was purchased from Sigma-Aldrich (Germany) and HT-2, 100 µg ml^{-1} in acetonitrile was purchased from Sigma-Aldrich (Germany).Test material 2291, animal feed purchased from FAPAS (York, UK), were concentration of T-2 292 µg/kg and HT-2 329 µg/kg. Methanol and acetonitrile (for HPLC, Gradient Grade), were purchased from J. T. Backer (United Stated). Formic acid (98/100%, laboratory reagent grade) was from Fischer Scientific (Loughborough, UK). Pure water was obtained from Purelab® ELGA water purification system (Vivendi Water Systems Ltd UK). Glass microfiber filters (GF/A) were from Whatman, Cat. No 6880-2504 (Maidstone, UK). Econofilters regenerated cellulose (0.45µm) were from Agilent, Germany. MycoSep 227 Trich+Multifuctional columns (Recorder: COCMY2227) were from Romer Labs (Austria).

Preparation of standard solutions and reagents

The working mix standard solution containing both compounds (1000 ng/mL in acetonitrile) was obtained by the further dilution of stock individual solutions with acetonitrile and stored at -20°C. Calibration solution from 25–500 ng/mL was prepared in mixture methanol/water (50/50, V/V) containing 0.1%HCOOH.

RESULTS

Optimization of the chromatographic separation for T2 and HT2

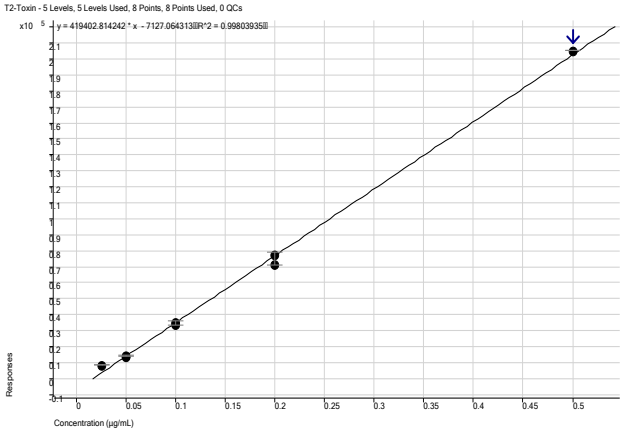
For the quantification the one with the best signal sensitivity (Q) was preferred and for the confirmation the second transition (q) and the ratio of abundances between both ion transitions (Q/q) was used. The cone voltages were selected according to the sensitivity of the precursor ions and the collision energies were chosen to give the maximum intensity of the fragment ions obtained. Table 1 lists the mass spectrometer parameters as precursor and product ions as well as the optimized cone voltages and collision energies used. The production spectra obtained on triple

quadrupole instrument generally provide fragments which are of diagnostic value for structural elucidation and confirmation.

The MRM transitions for T2 were from 484.2 to 185.1 and from 484.3 to 215.2, with the retention time of 5.854 min. The retention time of HT2 toxin was 4.250 min, with the MRM from 447.4 to 345.1 and from 447.4 to 263.1.

Method validation

The calibration curves, in the range of 2.5 to 500 ng/mL (3–625 µg/kg), were linear in the studied working range with the correlation coefficients greater than 0.99 (T2y=419402x-7127, R^2=0.9980; HT2y=2978372x-39405, R^2=0.9972) (Figure 1). The recovery was 79.2% for T-2 and 74.6% for HT-2 obtained use by FAPAS T2291QC. The chromatogram obtained from QC material of animal feed is depicted in Figure 2. demonstrating good chromatographic separation and satisfactory sensitivity.



a

b

Figure 1. Calibration curve of T2 (a) and HT2 (b)

The precision of the method in terms of repeatability (r) (intra-day precision) and reproducibility (R) (inter-day precision) was evaluated calculating the relative standard deviation (%RSD) of five samples of reference material also analyzed in triplicate on different days (Table 3). The limit of detection was calculated by MassHunter Software, for those concentrations that provide a signal to noise ratio of 3:1. These values of the LODs are 0.1µg/kg for the T2 and 2.5µg/kg for HT2. The limits of quantifications (LOQs) estimated as those concentrations of analytes which calculated at least 10xLOD, were 1.0 µg/kg for T2 and 25.0µg/kg for HT2**.**

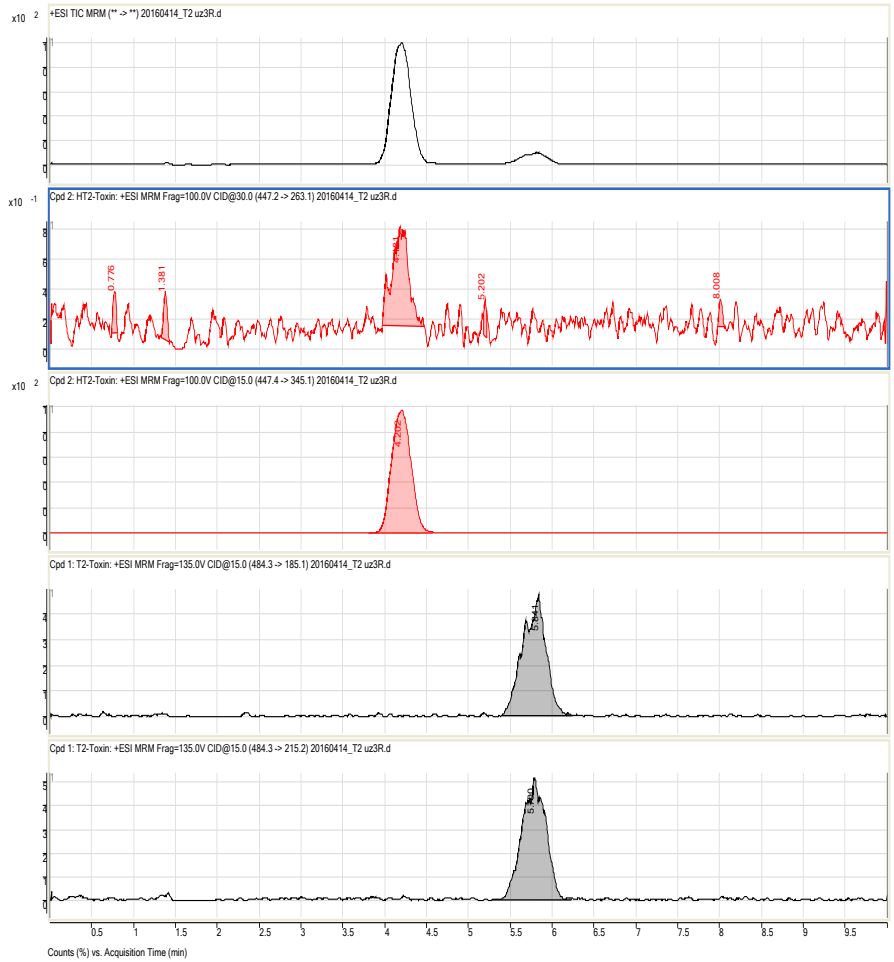


Figure 2. LC-MS/MS chromatogram of QC material (FAPAS T2291QC) (C_{T-2}=231µg/kg, C_{HT-2}=245µg/kg)

DISCUSSION AND CONCLUSION

The validated liquid chromatography tandem mass spectrometry method (LC-MS/MS) using MycoSep 227 Trich+Multifuctional columns for the mycotoxins extraction, provides a very high sensitivity, good reproducibility, appropriate linearity and can be applied with a high reliability to the analysis of the T2 and HT2 content in real samples.

The basic validated parameters were: the recovery is about 70–80% at two levels 10.0 and 100.0 µg/kg. The limit of quantification obtained by chromatographic parameters optimization, for T2 and HT2 were 5.0 and 3.0 µg/kg. The good linearity for T2 and HT2 in the range of 2.5 to 500.0ng/mL (3.0−625.0µg/kg) was obtained with the R^2 of 0.9980 and 0.9972, respectively was obtained.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of the Ministry of Education and Science, Republic of Serbia, Project Ref. TR31018.

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QuEChERS PESTICIDES EXTRACTION USING EMR SORBENT

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Abstract: For the efficient removal of lipids from a wide range of matrices during the QuEChERS extraction and purification the different sorbents can be used. The novel sorbent mixture, which is primarily present as a sorbent for fatty matrices, containing C18 and some special kind of polymers, known as EMR – Lipid (Enhanced Matrix Removal – Lipid) was applied and tested on non lipid matrices. Our work deals with a highly colored extract such as an extract of sour cherries. The validation of five pesticides, which are registered in the Republic of Serbia for use in sour cherries protection, the liquid chromatography tandem mass spectrometry detection (LC/MS-MS) was used. LC-MS/MS was equipped with a reversed-phase C18 analytical column of 50×4.6 mm and 1.8 μm particle size (Agilent Zorbax Eclipse). The mobile phase was methanol and Milli-Q water with 0.1% formic acid in gradient mode, with the flow rate of 0.4 ml/min. For the mass spectrometric analysis, an Agilent 6410B Triple-Quad LC/MS system was applied. Agilent Mass Hunter software was used for method development, data acquisition and quantification. Carbofuran-D3 was used as an internal standard. The validation comprised the basic validation parameters such as LOQ, recovery, precision and linearity. For all investigated pesticides (boscalid, tebuconazole, pyraclostrobin, propiconazole and chlorpiryfos), the recovery values for 0.1 and 0.01 mg/kg spiking levels were in accordance with SANTE/11945/2015 Document and ranged from 73.5% (pyraclostrobin) to 116.0% (chlorpyrifos) with %RSD less than 20%. The LOQ for all investigated pesticides were set on 0.01 mg/kg. The good linearity was obtained for all pesticides with the R^2 over 0.99.

Key words: *QuEChERS, EMR-Lipid, pesticide residues, sour cherries, LC-MS/MS*

INTRODUCTION

The guidance document on analytical quality control and validation procedures for pesticide residue analysis in food and feed (SANTE/11945/2015), indicates that the matrix effects are known to occur frequently in LC method and should be assessed at the initial method validation stage (Bursić 2015 – Slovačka). The growing concern over the environmental protection led to the decrease in the application of toxic solvents and development of extraction method on solid phase (SPE) in order to avoid liquid-liquid extraction (LLP) as a purification procedure.The further decrease in solvent application as well as in sample preparation time resulted in the development of a number of alternative extraction procedures: matrix solid phase dispersion (MSPD), supercritical fluid extraction (SFE), solid phase microexstraction (SPME) (Bursić et al. 2011).

Anastasiades et al. (2003) developed a quick, essential, cheap, efficient, robust and safe method (QuEChERS) so as to overcome the limitations of the existing preparation methods. The QuEChERS method uses acetonitrile, with the application of adequate combination of salts, sodium citrate dibasic sesquihydrate, anhydrous magnesium sulfate and sodium chloride with the different purification procedures. The most frequently used sorbents in the purification step are PSA (primary secondary amine), AC (active carbon), GCB (graphitized black carbon sorbent), C_{18} sorbent, Z-Sep sorbent (silicon dioxide coated by zirconium) and as the latest EMR-Lipid (Bursić et al. 2016). The innovative Enhanced Matrix Removal – Lipid (EMR – Lipid) technology is designed for efficient removal of lipids from a wide range of samples using a single sorbent material, but the exploration of these sorbent properties should not be stop on this.

On our market the boscalid, tebuconazole, pyraclostrobin, propiconazole and chlorpiryfos are registered for the use in cherry protection (Savčić-Petrić 2015). So, we decided to implement the EMR – Lipid on colored extract such as sour cherries extract full of planar molecules and to check its properties during the validation of five pesticides, which are registered in the Republic of Serbia for use in sour cherries protection. In our work the liquid chromatography tandem mass spectrometry detection (LC/MS-MS) was used.

MATERIAL AND METHODS

Chemicals and apparatus

The analitical standards manifactured by Dr. Ehrenstorfer GmbH, Germany which were used in the research work are chlorphyrifos (99.5%), tebuconazole (99.0%), pyraclostrobin (97.5%), boscalid (99.0%) and

propiconazole (99.9%). As an internal standardcarbofuran-D3 (Fluka>99%) was used in the concentration of 1 mg/mL of the stock standard in acetonitrile with the dilution up to 10.0 μg/mL. The stock standard solutions were prepared by dissolving an analytical standard in acetonitrile while the working solution i.e. the mixture of the studied pesticides was obtained by mixing and diluting the stock standards with acetonitrile resulting in the final mass concentration of 10 μg/mL.

LC-MS/MS conditions

HPLC	Agilent 1200
Column	Zorbax Ecllipse XDB C18 (50x4.6mm, 1.8μm)
Column Tem.	30°C
Mobile phase	A=0.1% HCOOH in MeOH B=0.1% HCOOH in water
Flow rate	0.4 ml/min, gradient
MS	Agilent 6410B
Nebulizer gas	40 psi
Dry gas	5 L/min at 325°C
Vaporiser	235°C
Ionisation	ESI +
Scan Tipe	dMRM
MassHunter Workstation Software B.04.00	

Validation parameters

The validation comprised the basic validation parameters such as LOQ, recovery, precision and linearity. During the validation of the multi-residue method for the determination of pesticide residues in sour cherries by LC-MS/MS according to SANTE/11945/2015, the linearity was checked at the concentrations of 0.02, 0.05., 0.1, 0.15 and 0.2μg/mL with the addition of the internal standard carbofuran-D3 (final concentration of 0.1μg/mL). The recovery values were checked for 0.1 and 0.01 mg/kg spiking levels.

Pesticide extraction

Scheme 1 shows the procedure of sour cherries extract purification by use of EMR-Lipid sorbent.

10 g (±0.1) sample + 10 mL MeCN + 100 µL ISTD (10 µg/mL Carbofuran-D3)
↓ Shake vigorously for 30 min
Add 4g $MgSO_4$, 1g NaCl, 1g Na_3-citrate dihydrate, 0.5 g Na_2H-citrat sesquihydrate Shake tube immediately for 2 min
↓ Centrifuge for 5 min at 3000 rpm
Add 5 mL water to a 15 mL EMR-lipid dSPE tube Transfer 5 ml of supernatant to EMR-lipid dSPE tube Shake for 60 s
↓ Centifuge for 5 min at 5000 rpm
Transfer 5 mL of supernatant to 15 EMR-lipid polish tube (2 g NaCl/$MgSO_4$, ¼) Shake for 60 s
↓ Centifuge for 5 min at 5000 rpm
0.5 mL supernatant + 10 µL HCOOH (1%) LC-MS/MS

Scheme 1. QuEChERS extraction

RESULTS

Before the calibration and quantification of pesticides it is necessary to set the acquisition parameters of mass spectrometer to determine the reactions for ion monitoring (MRM), to find the energy of collision cell (CE) and the fragmentation energy (Frag) where the response of the studied pesticide will be the highest for the given conditions (Table 1). The recovery of the studied pesticides was checked for the levels of 0.01 and 0.1 µg/kg. The control of the detector response linearity was carried out for a series of mass concentrations: 0.02, 0.05., 0.1, 0.15 and 0.2 µg/mL.

The average values of the recovery from sour cherry matrix as well as the correlation coefficients (R^2) are shown in Table 1.

Table 1. Pesticides analyzed by LC-MS/MS and some of their MS and analytical performance characteristics

Pesticides	MRM transitions (m/z)			Frag (V)	CE (V)	t_R (min)	Aver. Rec. (EMR) %	%RSD (EMR)	R^2
Boscalid	343.0	→	307.1 (271)	160	15 (35)	17.13	105.9	9.22	0.9987
Chlorpyrifos	349.9	→	97 (197.9)	13	41 (15)	20.67	116.4	2.79	0.9968
Pyraclostrobin	388.1	→	194 (163)	100	10 (10)	18.7	73.5	14.86	0.9987
Pyrimethanil	200.1	→	107.1 (82.1)	136	26 (30)	15.67	106.6	10.33	0.9987
Tebuconazol	308.1	→	125 (70)	100	25 (25)	18.45	83.1	10.79	0.9994

The LC-MS/MS chromatogram of spike sample (0.1 mg/kg) with investigated pesticides (boscalid, tebuconazole, pyraclostrobin, propiconazole and chlorpiryfos) was shown in Figure 1.

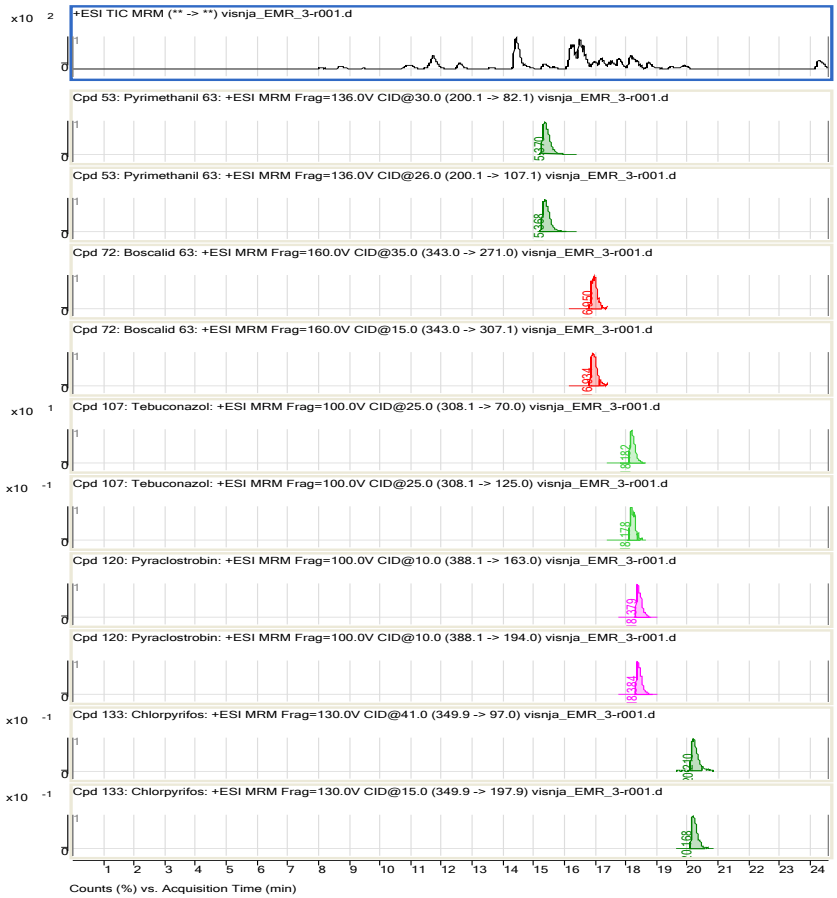

Figure 1. LC-MS/MS chromatogram of spike sample

DISCUSSION AND CONCLUSION

The validated QuEChERS with EMR – Lipid method which uses a liquid chromatography tandem mass spectrometry (LC-MS/MS) provides a very high sensitivity, good reproducibility, appropriate linearity and can be applied with a high reliability to the analysis of the boscalid, tebuconazole, pyraclostrobin, propiconazole and chlorpiryfos residues in sour cherries, in real samples.

For all investigated pesticides, the recovery values for 0.1 and 0.01 mg/kg spiking levels were in accordance with SANTE/11945/2015 Document and ranged from 73.5% (pyraclostrobin) to 116.0% (chlorpyrifos) with %RSD less than 20%. The LOQ for all investigated pesticides were set to 0.01 mg/kg. The good linearity was obtained for all pesticides with the R^2 over 0.99. All the validated parameters confirm that the method is suitable for the determination of boscalid, tebuconazole, pyraclostrobin, propiconazole and chlorpiryfos residues in real sour cherries samples according to the regulations of the Serbian and EU MRLs (Off. Gazzete RS No 29/2014 regulates the maximum limits to the amount of pesticide residues in agricultural products whereby the Regulations are in compliance with the MRLs as regulated in the European Union, i.e. by Regulation 396/2005).

ACKNOWLEDGMENTS

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QUALITY MEAT OF GOATS

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Abstract: In the world, especially in China, India, Pakistan and Nigeria goat meat represents an important food in human diet. At us, it is more talked about goats as harmful to the forest and somewhere to erosion, than emphasized their usefulness. It was not attempted to improve conditions and change the method of breeding, and in 1954. enacted „The Law of prohibition of goats keeping", which is unique in the world. The goat meat is increasingly consumed because of its characteristic taste and desired chemical composition, and as the food of animal origin is rich in proteins, vitamins, minerals, and the amount of fat, especially cholesterol is low. The growing and consumption of goat meat beside this qualitative composition are determined by religion, traditions, custom market as well as consumer habits. The aim of study was to contribute to wider use of this type of meat in human nutrition. For testing are used ten meat samples of race „Balkan goat", which average carcasses weight during slaughtering was 47.75 kg at age two years. The goats were fed with meadow hay and were grazing. During the year as supplement to the meals, they received concentrate (clean groats). We tested content of moisture by SRPS ISO 1442:1998, total ash by SRPS ISO 936:1999, total fat by SRPS ISO 1443:1992, total protein by SRPS ISO 937:1992 and total phosphorus by SRPS ISO 13730:1999. Test results were: average moisture content 67.0%, average total ash content 0.8%, average total fat content 17.3%, average total protein content 14.6% and average total phosphorus content 2.8 g/kg. If compare our results with the chemical composition of sheep meat (water content 64.0%, protein 18.0% and fat 18.0%), it is clear why many countries focus their efforts from low to highly productive goat breeds and intensive way of growing. The research was done within the project TR 31053, „Implementation of new biotechnological solutions in breeding of cattle, sheep and goats for the purpose of obtaining biologically valuable and safe food, funded by the Ministry of Education and Science of the Republic of Serbia".

Key words: *goats, meat, quality, composition*

GROWTH OF HEAT-RESISTANT MICROORGANISMS ISOLATED FROM MILK AT STORAGE TEMPERATURES FOR DAIRY PRODUCTS

Milijana Babić, Radoslava Savić Radovanović, Aleksandra Nikolić, Silvana Stajković

Abstract: In the dairy industry temeratures of pasteurisation are applied to destroy pathogen microorganisms and reduce the number of saprophytic microorganisms. Among saprophyte microoganisms there are microorganisms that survive pasteurization temperatures and it can be spore-forming, or non-sporeforming gram-positive bacteria. Heat-resistant microorganisms are defined as microorganisms that survive temperature of law pasterization (at 63–65^{o}C for 30 minutes). Some mesophilic microorganisms possess heat-resistant spores that survive high heat regimes, and after completed heat treatment there is possibility for spore germination, which than may propagate under conditions of room temperature or in conditions for cheese ripening.

The aim of this study was to determine the ability of heat-resistant microorganisms to grow at storage temperatures for milk and cheese ripening.

Material represented 20 isolates of heat-resistant microorganisms from milk. The isolates of heat-resistant microorganisms from slant were seeded into nutrient broth, which was incubated at 30^{o}C for 24h, and than from prepared serial decimal solutions inoculated 1 ml from corresponding decimal dilution into sterile Petri-dish, which was overflowed with nutrient agar. Inoculated Petri-dishes were incubated at 30^{o}C/72h, than at rooom temeprature (20±2^{o}C/72h), because these are conditions for storage of products such as sterilized milk, whereas temperature of 10^{o}C±1^{o}C/10 days, because it is temperature for storage of pasteurized milk, and temperature for cheese ripening as well. Standard SRPS ISO 4883 was used for the enumeration of microorganisms.

The results showed that 20 isolates growed at 30^{o}C/72h and room temeperature (20±2^{o}C/72h), and there is no deviations between avarage values for number of heat-resistant microorganisms ($\log_{10}$ cfu/mL), but it was observed that interval between minimal and maximal number ($\log_{10}$ cfu/mL) was highrer for isoaltes incubated at room temperature. At temperature for cheese ripening 10^{o}C±1^{o}C, 9 isoaltes of heat-resistant microorganisms did not show growth and remaining 11 isolates growed and the number ranged from 6,32 to 4,10 $\log_{10}$ cfu/mL ($\bar{x}$=5,26).

Key words: *heat-resistant microorganisms, growth, storage temepartures*

SENSORY AND PHYSICOCHEMICAL PROPERTIES OF COMMERCIAL SAMPLES OF HONEY

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Abstract: An analysis of fifty different honey samples collected from various regions in Serbia was done as part of regular testing. Sensory and physicochemical properties of four honey types, blossom, clover, acacia, and honeydew, were studied. Methods established by the International Honey Commission as well as the methods specified by the Rulebook on the Quality of Honey and Other Bee Products and Quality Control Methods (*OG SFRY*, No. 4/85) and the AOAC official method 958.09 were used. High Performance Liquid Chromatography was used to determine the sugar content. Sensory analysis was carried out using quantitative descriptive analysis method.

The ranges of physicochemical properties were: moisture 13.1–16.7%, reducing sugars 65.0–84.60%, sucrose 0.4–4.68%, total acidity 8.0–30.2 meq/kg, ash content 0.05–0.52% and water insoluble solids content 0.01–0.03%. Also, electrical conductivity, diastase activity and content of 5-hydromethylfurfural (HMF) were measured. HMF was detected in all samples and its concentrations ranged between 0.46 and 12.06 mg/kg, which is below the maximum concentration allowed by the legislation. The presence of HMF which resulted from heating or prolonged storage was also discussed and it provided insight into the reasons why common methods of honey processing may compromise its quality.

This paper gives an overview of current knowledge on the quality criteria and the methods used for their determination. The aim was to compare the results of our analysis with reference values taken from the Rulebook on the Quality and Other Requirements for Honey, Other Bee Products, Products Based on Honey and Other Bee Products (*OG SM*, No. 45/03) and the current Rulebook of January 1, 2016 on the Quality of Honey and Other Bee Products (*OG RS*, No. 101/15). Furthermore, parameters and reference values from the Rulebooks governing quality and other requirements for honey valid until 2016 are compared with those from the current Rulebook. Different aspects of such analysis are dealt with in detail, including the methods of choice, calibration strategies, sample treatments and preparation procedures. It has been observed that the honey produced in Serbia is of good quality.

Key words: *quality honey, HMF, sensory analysis, bee products*

INTRODUCTION

The Codex Alimentarius defines honey as „the 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". Honey is produced primarily from floral nectars, and fructose and glucose are the major components (Rizelio et al. 2012). Honey samples that are available commercially, differ in quality on account of various factors like geographical, seasonal and processing conditions, floral source, packaging and storage period (Sulieman et a. 2013). The specificity of honey products, *i.e.*, flavor, aroma, color and texture, depends predominantly on the type of flowers, or plants, from where bees take the nectar, or the honeydew, to produce honey (Iftikhar et al. 2014, Sebeho 2015). Some other factors have a lesser influence on the properties of honey, i.e., the physiology of bees and their foraging habits, the climatic conditions (temperature, humidity) and the post-collection processing. In this light, honey is a highly complex substrate to analyze, since it contains many volatile components with different chemical structures of low concentration in a sugar matrix where polar substances are the major components.

The consumtion of honey in the human diet is related not just to the need for sugar sources, also of particular importance is a sort of „hedonistic" choice that leads consumers to choose a well-identified kind of honey. Customer choose is linked to organoleptic characteristics, and these depends on the boatanical origin of the product.

Nevertheless, the composition of honey is tightly associated to its botanical source and also to the geographical area from where it originated, because soil and weather determines melliferous flora. With regard to their origin, honey could be classified as floral when it is derived from the nectar of flowering plant or non-floral (honeydew) when it is derived from sweet deposits secreted by living parts of plants or excreted onto them by sap-sucking insects (Manyi-Loh et al. 2012). Consequently, there is disparity in the chemical composition between floral and honeydew honey. Physicochemical characteristics such as pH, acidity, ash content, color, and electrical conductivity have been considered to be useful characteristics for differentiating floral honeys from honeydew honeys.

Another important issue related to honey quality is the determination of transformation products. The compound created during the decomposition of monosaccharides (mainly fructose) is 5-hydroxymethylfurfural (HMF). It is usually absent in fresh honey but its concentration tends to increase during honey conditioning and storage and in excessive heat.

The aim of present study was to evaluate and compare the physico-chemical properties of some commercial honey samples from Serbia. International honey standards are specified in a European Honey Directive and in the Codex Alimentarius Standard for Honey, both of which are presently under revision. In this article, present knowledge on the different quality criteria of honey and other requirements for honey are compared.

In addition, the identity and quality standards were described, such as sugars, moisture, acidity, ash and electrical conductivity, HMF and diastase activity, along with the minimum and maximum limits established by the current Rulebook of January 1, 2016 on the Quality of Honey and Other Bee Products (*OG RS*, No. 101/15). This study would be helpful to understand local honey properties and very important towards the commercialization of regional honey.

MATERIALS AND METHODS

All reagents were of analytical grade, solvents were of chromatographic purity and the water was purified by deionization (Milli-Q system, Millipore, Bedford, MA, USA). Fifty commercial samples of Serbian honey were studied for sensory and physico-chemical properties. The samples were stored at room temperature in a fresh and dark place before analysis. Sensory analysis was carried out using quantitative descriptive analysis method.

The HPLC analyses of HMF were carried out with Waters system, consisting of a auto sampler and UV detector (1525 Binary HPLC Pump, 2487 Dual λ Absorbance Detector, 717 Plus Autosampler). The chromatographic separation was performed with the Zorbax EclipsePlus C18 (3.5μm, 3.6mmx 150 mm) chromatographic column. In the case of HMF, the mobile phase was deionized water-methanol (90:10), at a flow rate of 1 mL/min.

The methods that were used to determine the physicochemical parameters are various. The determination of water was based on refractometry. Titration using sodium hydroxide was used to determine the free acid, while the electrical conductivity is defined as the conductivity of a 20% aqueous solution of honey at 20°C. The electrical conductivity was measured on the Model 4330 Conductivity meter. Electrical conductivity of honey is defined as the conductivity of a 20% aqueous solution of honey at 20°C, where 20% refers to the dry weight of honey. The results are expressed in mS/cm, according to the method prescribed by the International Honey Commission (IHC).

Ash content of honey was determined by calcinations overnight of approximately 5 g of honey sample at 550°C in the furnace to constant weight.

The diastasis was proven using the hydrolysis method. The method for the determination of diastase activity in honey is based on the hydrolysis

of 1% solution of starch by the enzyme from 1g of honey during one hour at a temperature of 40°C. HPLC Waters system with silica-based amino columns and refractometric detection were used for determination contents of fructose, glucose and sucrose.

RESULTS

Table I Results of determining quality parametrs of different honey samples

Honey Sample	Moisture %	Acidity, meq/kg	HMF, mg/kg	Diastase No.	Ash
Blossom	16.20 ± 4.96	17.01 ± 8.52	0.28 ± 4.78	21.56 ± 6.45	0.39 ± 0.25
Clover	16.89 ± 3.69	14.60 ± 5.87	0.80 ± 11.90	35.45 ± 10.50	0.52 ± 0.30
Accacia	15.32 ± 2.67	28.60 ± 4.20	1.50 ± 8.56	32.45 ± 7.45	0.46 ± 0.23
Honeydew	14.70 ± 2.56	29.23 ± 5.23	1.78 ± 5.64	42.9 ± 8.75	0.35 ± 0.15
Reference value (OG SM, No 45/03)	max 20	max 40	max 40	min 8	max 0.5 max 1.0 for clover max 1.2 honeydrew
Reference value valid until 2016	max 20	max 50	max 40	min 8	

Table II Distribution of fructose, glucose and sucrose in honey samples

Honey Sample	Fructose, %	Glucose, %	Sucrose, %	Reducing sugars
Blossom	35.25 ± 4.85	28.54 ± 5.24	0.4 ± 1.95	69.86 ± 3.28
Clover	33.25 ± 5.75	29.45 ± 4.98	0.4 ± 3.24	73.83 ± 3.54
Accacia	35.79 ± 7.45	27.56 ± 6.23	0.4 ± 4.68	80.95 ± 3.65
Honeydew	36.23 ± 5.23	29.54 ± 6.23	0.4 ± 2.39	67.17 ± 2.17
Reference value (OG SM, No 45/03)	-*	-	max 5 for all honey max honeydrew	max 65
Reference value valid until 2016	-	-	max 5 for all honey max 10 for accacia	max 60 for blossom and max 45 for honeydew

*not regulated

The range of electrical conductivity were: for the bloosom honeys: 0.300–0.350 mS/cm; for the clover honeys: 0.256–0.301 mS/cm; for the acacia: 0.091–0.230 mS/cm and for the honeydew: 0.240–0.468 mS/cm. Water insoluble solids content were between 0.01–0.03 % for all honeys.

DISCUSSION

The most important criteria when buying honey are taste, colour and quality. The present study was undertaken to determine the physico-chemical parameters of honey samples obtained from various regions in Serbia.

The determinatation of moisture by refractomertry is very simple and reproducible method, used successfully in routine honey control. The moisture percentage or water content of honey was observed between 13.1 to 16.7 percent. Honey moisture content, a critical variable influencing product quality, granulation and texture, is significantly affected by conditions under which honey is stored following its extraction from the hive. Higher water content could lead to undesirable honey fermentation during storage. The water content depends upon the environmental factors during production such as weather and humidity inside the hive, but also on nectar conditions and treatment of honey during extraction and storage. It can be reduced before or after extraction by special techniques. Honey having high water content is more likely to ferment. A maximum value of 20g/100g (20%) is suggested in the draft for an old and new standard. In routine honey control carried out by method from the Rulebook all honey samples had a water content of less than 20%

The enzymes are very sensitive to overheating (above 35°C) or storage at too high a temperature. Because they are destroyed by heating, a low enzyme level may mean that honey has been heated.

The ash content in honey is generally small and depends on nectar composition of predominant plants in their formation. The ash content in the honey samples varied between 0.05–0.52 % (Table I), which is in the acceptable range. The ash content is associated with botanical and geographical origins of honey samples.

The HMF content is widely recognized as a parameter in evaluating the freshness of honey. This is a byproduct of fructose decay and formed during storage or during heating (Iftikhar et al. 2014). Thus, its presence is considered the main indicator of honey deterioration. The excessive value of HMF indicates overheating during processing, prolonged storage or adulteration with invert sugars. In present investigation the HMF values were recorded between 0.46 and 12.06 (Table I). Maillard reaction, a non-enzymic browning reaction occurs between sugars and free amino acids during thermal treatment of honey. It is believed that Maillard reaction products are acting as antioxidants. HMF is one of the major intermediate products of Maillard reaction which is formed from the condensation of

sugars in the presence of an amino acid during processing, prolonged storage or excessive heating of honey. Its maximum concentration is 15mg/kg in honey and is regulated by law.

Concentration of HMF, as the major honey quality factor is an indicator of honey freshness and overheating. In fresh honeys there is practically no HMF, but it increases upon storage, depending on the pH of honey and the storage temperature. HMF (5-hydroxymethylfurfural) is essential to evaluate the conformity of honey to the current legislation. Elevated concentrations of HMF in honey provide an indication of overheating, storage in poor conditions or age of the honey. Both the Codex Alimentarius Commission (Alinorm 01/25, 2000) and the European Union (Directive 110/2001) established that its concentration in honey usually should not exceed 80 or 40 mg/kg, respectively.

This method for detmining mineral content (ash) is now very often replaced by the faster and easier electrical conductivity measurement. The ash content is a quality criteria for honey botanical origin, the blossom honey having a lower ash content than honeydrew honeys (Gairola et al. 2013). At present, this measurement is generally replaced by the measurement of electical conductivity. The ash content could be kept as a quality factor during a transition period, until coductivity is accepted as a world-wide standard. That is the new change in the new Rulebook, in the old Rulebook, the parameter called mineral content in honey is thrown out, and the new one is electrical conductivity.

Electrical conductivity depends on the ash and acid content of honey: the higher their content, the higher the resulting conductivity.

Glucose, fructose and sucrose are the major constituents of honey. The glucose, fructose and sucrose contents ranged from 29.50% to 43.24%, 27.56% to 35.77% and 0.4% to 4.68% respectively (Table II). The sum of the fructose and glucose content is very close to the sum of all reducing sugars, as frucose and glucose represent mostly more than 90% of all reducing sugars.That is the differnce between old and new Rulebook, in the old Rulebook content of frucose and glucose is identify with reducing sugars, but in the new Rulebook of January 1, 2016 on the Quality of Honey and Other Bee Products (*OG RS*, No. 101/15), it is just content of fructose and glucose.

The „apparent reducing sugars‟ correspond roughly to the sum of the main honey sugars fructose and glucose and some minor reducting disaccahides, mainly maltose. Many honeydrew honeys have high amount of non-reducing oligosaccharides such as melezitose and raffinose (Tomasini et al. 2012). Because of these finding, the standard for apparent sugars has been modified compered with the previous one: a minimumof 45% has been proposed, compered to the old standard with a minimum of 65 %. Honeydew honeys are darker and also have a higher pH and greater acidity which could be attributed to the characteristic high acetic acid concentration.

Diastase catalyzes the transformation of starch to maltose. Honey diastase activity is a quality factor, influenced by honey storage and heating and thus an indicator of honey freshness and overheating. Although there is a large natural variation of diastase, the old and new standard propisuju vrednost of a minimum DN value of 8. When interpreting diastase results one should take into consideration that certain unifloral honeys have a naturally low diastatic activity.

Honey insoluble matter includes pollen, honey-comb debris, bee and filth particles and is thus a criterion of honey cleanness. It is measured by filtration of a honey solution on a glass crucible. Water insoluble solids content for all honey samples were below 0.1%. The mean value for the total acidity was between 8.00 and 32.00 meq/kg. None of the tested samples collected from all the tested areas exceeded the limit of 40 meq/kg established indicating absence of undesirable fermentation. The variation in acidity among diffrent honey types may be attributed to either variation in the contents of organic and inorganic acids due to harvest season or floral types.

The old standard fixed a maximum acidit yin honey of 40 milliequivalents/kg, which has been increased to 50 milliequivalents/kg in the new one, as there are some honey, which have a higher natural acidity.

CONCLUSION

All the tested commercial samples satisfy the prescribed criteria listed in the Rulebook on the Quality of Honey and Other Bee Products regarding the physicochemical parameters:

- the moisture percentage or water content of honey ranges on average from 13.1 to 16.7%;
- the mean values of total acidity in this research range from 8.00 to 30.2 meq/kg;
- the mean values of electrical conductivity in the samples used in this research range from 0.092 to 0.468 mS/cm;
- the sum of glucose and fructose, i.e. of reducing sugars and the values obtained in this research range from 65.00 to 84.6%;
- the sucrose share ranges from 0.4 to 4.68%;
- the obtained averages of the diastase activity range from 15.11 to 51.65 DN;
- the HMF share ranges from 0.46 to 12.06 mg/kg.

It has been observed that the honey produced in Serbia is of good quality. When the process is controlled carefully, the key to the quality of the end products lies in the good quality of the raw material. Therefore, adequate training in the good beekeeping practices is vital to obtain the product that consumer expects and legislation requires.

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WATER INSOLUBLE SOLIDS CONTENT IN HONEY ORIGINATING FROM VOJVODINA REGION

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Abstract: Honey insoluble matter includes wax, pollen, honey-comb debris, bee and filth particles and is thus a criterion of honey cleanness. The measurement of water insoluble solids is important to detect honey impurities that are, in some samples, higher than the permitted maxima.

To the purpose of determining the water insoluble solids, 40 samples of different honeys originating from Vojvodina region were collected. The investigated samples included meadow honey, acacia honey, linden honey, multiflower honey and sunflower honey.

Water insoluble solids content wasdetermined as residue mass obtained by filtering honey solution through sintered glass and drying the residue for 1 h. Water insoluble solids of 31 out of 40 honey samples (77.5%) analysed in this study completely correspond with the national Regulation No. 101/2015 and Codex Standard for Honey (2001), indicating adequate processing and good quality. Nine samples (22.5%) did not meet characteristics established in the Regulation No. 101/2015 and Codex Standard for Honey (2001). The result of water insoluble solids of five samples acacia honey, three samples oflinden honey, as well as one sample sunflower honey exceeded the maximum level permitted by the Regulation (0.1%). Contrary to these results, water insoluble solids in twelve samples of meadow honeyand seven samples ofmultiflower honey were in accordance with Regulation.

Increased content of water insoluble solids in honey can be attributed to insufficient knowledge of beekeepers and lack of adequate equipment. Better compliance of honey quality with the relevant Regulation, i.e., the adequate level of water insoluble solids in honey, can be accomplished by regular practical training of beekeepers offering relevant knowledge and skills on the production process, storage, package and distribution of honey.

Key words: *water insoluble solid, honey*

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TECHNOLOGICAL QUALITY OF BEEF DURING THE TWENTY-ONE DAYS OF STORAGE IN A VACUUM

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Abstract: The changes in the chemical, technological and sensory characteristics of vacuum preserved pieces of beef (*M. longissimus dorsi*) obtained from the Simmental breed are presented in the paper. Pieces of meat of approximately the same size and weight were vacuumed and maintained at a temperature of 2–4ºC refrigerated up to 21 days. Every seven days chemical, technological and sensory properties of the meat were investigated. According to the survey it was found that the water content reduced, while the content of fat, protein and ash increased. Roasting loss, drip loss, tenderness and water holding capacity were better during storage, while the meat colour was darker. Sensory properties (tenderness and juiciness) were better and the smell/odour and the taste were slightly inferior.

Key words: *M. longissimus dorsi, vacuum, quality*

INTRODUCTION

The meat is considered as one of the most common group of foods in the human diet, so the researchers from around the world daily perform various experiments with the aim of finding the most efficient techniques to extend the sustainability and hygienic quality of meat. Because of its specific chemical composition and high water content, the meat is among the most perishable foods. The spoilage can be defined as any change from the sensory standpoint deeming the foodstuff unacceptable to the consumer (Gram et al. 2002). The occurrence of unpleasant smell and taste may occur as a result of the growth of microorganisms, wherein the intensity of sensory changes is associated with the decomposition of nutrients in meat and the formation of undesirable volatile metabolites (Babić et al. 2012).

Consumers require fresh meat of acceptable sensory properties on the offer. This trend has caused the development of the vacuum packaging that provides long term sustainability of meat and preservation of the basic parameters of freshness (Babić et al. 2014). In order to obtain a high quality product in regard to the sensory properties, in the industrial processing agricultural products undergo numerous processes and operations (Popov

2007). At the present time there is a market demand for foods that are minimally processed without added preservatives and additives (Kocić-Tanackov et al. 2012), so that the food in vacuum-packaging is increasingly present in retail. Vacuum packaging can be defined as the removal of air from the package and the purpose of this technology is to extend the sustainability of food. Meat packaging protects sensory, physical-chemical and microbiological properties of these products during the manipulation by the producers to consumers and is very dynamic area of meat industry (Kuzelov et al. 2011). Meat and meat products, today, need to be packaged attractively, as consumer demand is constantly growing (Philips et al. 2001).

Special attention should be directed to packaging and handling of meat, since any disruption of vacuum consistency (punctured wrapping) will lead to spoiling of meat due to activity of aerobic micro organisms.

Many authors have examined the impact of sustainability on the vacuum packaging of beef meat (Bell and Garout 1994, Lawrence et al. 2003, Stetzer et al. 2008), examined the sustainability of vacuum packed meat pieces cooling at different temperatures and they found that the meat cuts that have been kept at lower temperature had better sensory characteristics of meat cuts than those that have been kept at relatively highertemperatures.

Vacuum packaging is the preservation method withmost advantages since it uses the least space; allows the improvement of tenderness, due to aging, without evaporation losses; and has a longer microbial shelf-life than aerobic systems (Oliete et al. 2006).

The objective of the present study was to evaluate the effect of storagetime at 2–4°C on chemical, technological and sensory characteristics of one beef skeletal muscles of Simmental breed, which is the most common cattle breed in Serbia.

MATERIALS AND METHODS

Slaughtering and primary processing of 20 carcasses, cutting of carcass sides and dissection of the three rib cut were carried out in the experimental slaughterhouse and technological and sensory properties analysed in the laboratory of the Institute for Animal Husbandry (Belgrade, Serbia). Three rib cut (9-10-11 rib) was separated from the left chilled carcass side, cut at the cranial edge of the 9th and 11th rib and cut parallel to the spinal column. The samples were vacuum bagged in a vacuum packing machine (VAC STAR, S 223 L) and stored in a refrigerator at 2–4°C for 21 days.

The analysis and determination of the chemical composition of the sample of *M. longissimus dorsi* (the water content – the method of drying the sample at 103±2°C (SRPS ISO 1442, 1998), fat content – extraction method according to Soxhlet (SRPS ISO 1444, 1998), the amount of

mineral substances (ash) – by annealing the sample at 550±25°C (SRPS ISO 936, 1999) and protein content – according to method by Kjeldahl (SRPS ISO 937, 1992), were performed.

Technological and sensory properties were monitored in the sample of *M. longissimus dorsi* after 1, 7, 14 and 21 days, namely: cooking loss – determined on the basis of the difference in weight of the pieces of meat (size: 3 x 4 x 1.5 cm and weight about 70 g) before and after cooking in distilled water (wherein the ratio of meat and water was 1:2) in a closed glass vessel (at 100°C for 10 minutes) and expressed as the percentage relative to the weight of the sample prior to cooking (Official Gazette of SFRY, no. 2/85, 12/85 and 24/86); roasting loss – determined on the basis of the difference in weight of the pieces of meat before and after roasting. A cut of *M. longissimus dorsi* muscle, transversely cut to the direction of the muscle fibres, weighing 150±1 g was wrapped in aluminium foil and baked for 25 minutes at 250°C, upon completion of roasting, it was extracted from the foil and immediately measured; drip loss – a piece of meat of approximately the same weight of about 200±2 g was vacuumed and kept in a refrigerator at 2–4°C for a number of days (7, 14, and 21 days). After the expiry of the specified number of days, the bag was opened, the piece of meat extracted and measured. Drip loss is calculated as the difference in weight of the pieces of meat during vacuum packaging and after removing it from the bag. The softness (tenderness) of meat is determined using the consistency-meter according to Volodkevich (1938), by cutting pieces of meat transverse to the direction of the muscle fibres. PH value of meat was measured using the pH meter with combined puncture electrode Hanna HI 83141 (Hanna Instruments, USA). Water holding capacity (WHC) of meat was determined by the method according to Grau and Hamm (1953), where the value of WHC is expressed in cm^2. Instrumental colour measurement was done using the Chroma Meter CR-400 (Minolta, Japan), which was previously calibrated in relation to a standard white surface (the illumination of D65, viewing angle of 2° and aperture probe 8 mm) on the fresh meat samples (24 hours post mortem). Samples of meat were cut and left 30 min in air to stabilize the colour. Colour values are represented in the CIE L*a*b* system (CIE 1976), where L* is a measure indicating lightness of meat, a* relative share of red and b* the relative share of yellow colour. For each sample of meat three readings were done and their average value was used for statistical data processing.

The scores of taste, aroma, juiciness/succulence and tenderness of the meat were determined after cooking and after roasting. Seven semi trained assessors were included in sensory evaluation. The quantitative descriptive scale of 5 points was used for each evaluated parameter: marbling: 1 – very bad marbling, 2 – bad marbling, 3 – neither good nor bad marbling, 4 – good marbling, 5 – very good marbling; taste and odour: 1 – very bad, 2 – bad, 3 – neither good nor bad, 4 – good, 5 – very good; Softness/tenderness: 1 – very firm, 2 – firm, 3 – neither firm nor soft, 4 – soft, 5 – very soft;

juiciness/succulence: 1 – very dry, 2 – dry, 3 – neither dry nor succulent, 4 – succulent, 5 – very succulent.

The obtained data were analysed using analysis of variance in single factorial experiment (One-way ANOVA) by SPSS Statistics 20. The statistical significance of differences between mean values was determined by t-test.

RESULTS AND DISCUSSION

The chemical composition of *M. longissimus dorsi* is presented in Table 1. There were no statistically significant differences. The water content reduced during storage, while the content of fat, protein and ash increased. Similar results were obtained by Kuzelov et al. (2011).

Table 1. Chemical composition of *M. longissimus dorsi* in %

Item	0	7	14	21	t-test
Water	75,03 ± 1,00	74,88 ± 1,05	74,51 ± 1,15	74,03 ± 1,09	ns
Fat	1,45 ± 0,64	1,47 ± 0,71	1,49 ± 0,69	1,55 ± 0,77	ns
Protein	22,38 ± 0,72	22,42 ± 0,70	22,50 ± 0,76	22,70 ± 0,69	ns
Ash	1,11 ± 0,04	1,18 ± 0,04	1,45 ± 0,04	1,62 ± 0,04	ns

ns – not significant

Table 2 shows the technological properties of *M. longissimus dorsi*. Roasting loss was statistically ($p<0,01$) significantly lower at 14 and 21 days, while drip loss statistically ($p<0,001$) was significantly higher after 14 and 21 days. The meat was the most tender at 21 days. Similar results are presented by Miščević et al. (2007). The pH value increased statistically ($p<0,05$) significantly during storage time of vacuum-packed meat. Statistically ($p<0,05$) significant differences were obtained in water holding capacity which improved in storage. The meat was statistically ($p<0,001$) significantly darker during storage.

The initial pH was 5.52–5.69. It was the same with otherstudies (De Santos et al. 2007, Seyfert et al. 2007). Luy et al. (2016) state that the pH value significantly decreased significantly decreased at the beginning of storage.

The length of time meat is kept in chilled storage has an effect on the rate of colour change (Stanišić et al. 2012). Oliete et al. (2006) found that lengthening of the vacuum storage time of *M. longissimus thoracis* muscle (1^{st}, 7^{th} and 14^{th} day) resulted in a rise of the a* and b* parameters. Dark red colour that comes from reduced myoglobin is not acceptable for consumers (Mancini and Hunt 2005).

Feldhusen et al. (1995) showed that there were clear colour changes in beef *M. longissimus dorsi* during cold storage for up to 5 days at +5°C. The author stated that the degree of lightness (L*), percentage of red colour

(a*) and percentage of yellow colour (b*) all increased during storage, which was confirmed by results obtained in this trial.

Table 2. Technological properties of *M. longissimus dorsi*

Item	1	7	14	21	t-test
Cooking loss, %	42,64 ± 1,96	43,18 ± 1,30	42,84 ± 2,53	42,60 ± 0,95	ns
Roasting loss, %	42,53 ± 1,80	41,54 ± 1,46	40,00 ± 1,43	40,86 ± 0,67	**
Drip loss, %	-	2,13 ± 1,52	4,89 ± 1,22	4,85 ± 1,21	***
Tenderness	13,33 ± 3,33	7,67 ± 1,85	5,57 ± 2,06	4,86 ± 1,93	***
pH	5,52 ± 0,06	5,58 ± 0,07	5,64 ± 0,07	5,69 ± 0,05	*
WHC, cm2	13,05 ± 1,94	13,47 ± 0,92	12,93 ± 0,68	12,80 ± 0,31	*
Colour					
L*	39,17 ± 2,48	38,54 ± 1,49	38,21 ± 1,65	36,95 ± 1,70	***
a*	19,87 ± 0,93	20,24 ± 2,00	19,93 ± 1,69	19,65 ± 0,95	ns
b*	6,68 ± 1,40	8,42 ± 2,17	6,68 ± 2,86	5,34 ± 2,59	***

ns not significant
*** significant at the level of ($p<0,001$)
** significant at the level of ($p<0,01$)
* significant at the level of ($p<0,05$)

Consumers commonly rely on the color and odor when making decisions regarding the safely and acceptability of meat on retail display (Brewer et al. 1994). The effect of vacuum treatment, storage time and their interaction on the sensory attributes' significant difference was analyzed by theirpvalues. Sensory characteristics of cooked meat are shown in Table 3. Tenderness and flavour have been identified as the most important attributes that determine eating quality of Europeans (Glitsch 2000). Tenderness is one of the major criteria that contributes most to eating satisfaction and consumers are willing to pay more for tender beef (Chambaz et al. 2003).

Based on the results of sensory evaluation of *M. longissimus dorsi*, tenderness and succulence of meat statistically ($p<0,001$) were significantly better after prolonged holding in a vacuum. Tenderness decreased with storage time (Oliete et al. 2006). Differences in sensory properties can be attributed to the different content of intramuscular fat (Christensen et al. 2011). Statistically ($p<0,05$) significant difference was determined in smell/odour and flavours that were slightly inferior during storage in a vacuum. Similar results are reported by Miščević et al. (2007) and Williamson et al. (2013). With the increase of storage time, the aminesubstance was produced due to the microorganism metabolism (Nychas et al. 2008, Lyu et al. 2016) making the beef had a bad smell.

Table 3. Sensoryproperties of *M. longissimus dorsi*

Item	0	7	14	21	t-test
Cooked meat					
Smell/odour	5,00 ± 0,00	4,50 ± 0,50	4,59 ± 0,49	4,41 ± 0,54	*
Taste/aroma	4,86 ± 0,23	4,54 ± 0,35	4,36 ± 0,45	4,63 ± 0,39	*
Tenderness	3,00 ± 0,59	4,09 ± 0,54	4,41 ± 0,54	4,59 ± 0,54	***
Succulence	3,45 ± 0,42	4,36 ± 0,45	4,54 ± 0,42	4,50 ± 0,54	***

*** significant at the level of ($p<0,001$)
* significant at the level of ($p<0,05$)

CONCLUSION

Based on the results of the test, the following can be concluded:

- The water content reduced while the content of fat, protein and ash increased during vacuum storage;
- During storage, roasting loss and drip loss of vacuum-packed meat decreased;
- Meat tenderness and water holding capacity were improved;
- Meat colour was darker;
- Sensory evaluation (tenderness and succulence) improved while the smell/odour and taste were slightly inferior.

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TEXTURAL AND COLOUR CHANGES THROUGH THE ONE YEAR STORAGE OF DRY FERMENTED SREMSKA SAUSAGE MANUFACTURED WITH DIFFERENT PORK FAT LEVELS

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Abstract: Traditionally processed dry fermented Sremska sausage was produced with three different meat/fat ratio: LF – low fat (85/15), MF – medium fat (80/20) and HF – high fat (75/25). The effect of pork fat reduction on the instrumental colour and texture profile parameters during 360 days of vacuum storage was studied. Results showed that sausages with reduced fat (LF) had lower L*, a* and b* values compared with other two groups. In relation to storage time, a decrease in L* and a* values after 90 days was observed in all sausage groups. Hardnesses, gumminess and chewiness values were highest in LF group and lowest in HF group, while MF sausages were intermediate. During the storage these three TPA parameters significantly increased in all three groups of sausages ($p<0.05$). From the results obtained it can be concluded that the effect of fat content on colour and TPA parameters of sausages was highly significant between groups and throughout the storage.

Key words: *Sremska sausage, fat level, vacuum storage, instrumental colour, TPA parameters*

INTRODUCTION

Over the past decades, increased concerns about the potential health risks associated with the consumption of high fat foods or healthier fatty acid compositions has led that the amount of fat in meat products is reduced (Colmenero 2000, Mendoza et al. 2001). However, some traditional meat products, such as dry fermented sausages, remain high in fat. Fat is one of the most important raw material derived from slaughtered pigs and one of the main ingredients for the production of quality meat products (Stanišić et al. 2012) and it is important as a source of energy and essential fatty acids, and as carriers of fat soluble vitamins and it also improves flavor, texture,

tenderness and juiciness (Choi et al. 2010). While the numberof reduced-fat meat products has increased, consumer acceptance of these products has been slow because consequently, the reduction of fat can affect the acceptability of these products (Giese 1996). To be successful, reduced-fat products must be sensory, nutritionally and functionally acceptable to consumers (Muguerza et al. 2003).

Fermented sausages are defined as ground meat mixed with salt and curing agents, stuffed into casings and subjected to a fermentation process in which microorganisms play a crucial role (Lücke 1994). There is a great variety of dry fermented sausages, depending on kind of raw material and manufacturing processes and each European country has at least a few of its own typical types of sausages (Casaburi et al. 2007). Sremska sausage is a dry fermented sausage, produced in the north-western part of Serbia (Srem region) and is characterized by specific hot taste, aromatic and spicy flavour, dark red colour and hard consistency (Stanišić et al. 2012). According to a standard recipe, Sremska sausage have approx. 30% fat content after manufacture, but as a result of drying, these values rise to about 40–50% at the end of production process.

Thus, the aim of the present study was to investigate the effect of fat content on textural and colour changes through the one year storage of dry fermented Sremska sausage.

MATERIALS AND METHODS

Sremska sausages were prepared in a meat processing plant of the Institute for Animal Husbandry (Belgrade, Serbia) in the period January–February 2016, from the meat of Swedish Landrace pig breed. For the production of Sremska sausage, meat from shoulder was used and back fat and three groups of sausages were made according to meat/fat ratio: LF – low fat (85/15), MF – medium fat (80/20) and HF – high fat (75/25). Meat and fat were chopped and minced to 8 mm particle size and mixed with in a cutter, whereupon they were transferred to a mixer and the same amounts of ingredients were added: 2.2% NaCl, 0.3% glucose, 0.17% garlic (powder), 0.55% hot red paprika (powder) and 0.5% sweet red paprika (powder). No starter culture was added, thus fermentation was spontaneous.

The sausage mixture was stuffed into natural casings (pig small intestines) of around 32 mm diameter and sizes of approximately 700–800g. After stuffing (day 0) the sausages were drained in a cold store (4±2°C) for 12 h, for the surface to dry, after which they were hung to dry in a traditional smoking house. The ripening was as follows: the first stage lasted 14 days in a traditional smoking house at 10–15°C with 75–90% relative humidity (RH), where they were smoked for 6 h each day; last 7 days (from day 15 to day 21) sausages were processed in a drying room at 14–16°C with about 75% RH, to reach about 35.0% moisture content. The total processing time was 21 day.

Sampling of sausages was carried out at the end of production (day 0) and after 90, 180 and 360 days of vacuum storage at 4±2°C. All samples were analysed after removing the outer casing and grounding in the mixer (Ultra Turrax T18, IKA, Germany). Each analysis was done in duplicate.

Each sausage was cut and the colour was measured three times using Chromameter CR-400 (Minolta Co. Ltd, Tokyo, Japan), configured with the following parameters: D65 light source, 10° observer, and 8 mm aperture size and calibrated using a white ceramic tile. The measurements were done according to CIE L*a*b* system: lightness (L*), redness (a*) and yellowness (b*) (CIE, 1976). The colour measurements were performed at room temperature (20±2°C) immediately after the samples were cut.

Texture analyses were done using a universal testing machine (Instron model 4301, Instron Ltd., England) and nine readings were taken for each variant of sausage. The samples were held for equilibration to room temperature and compressed twice to their original height, with a compression aluminium platen of 75 mm (P/75) and a 250 kg load cell. Pre-test speed was 3 mm/s, test speed was 1 mm/s and post-test speed was 1 mm/s. The following parameters were obtained: hardness, adhesiveness, springiness, cohesiveness, and chewiness.

An analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SPSS 20.0 software (IBM SPSS Statistics, Version 20, IBM Corp, USA) was performed for all variables considered. If the effect of main factor (group or time) was found significant, Tukey test was used to evaluate the significance of difference at $p<0.05$. The interactions were not statistically significant ($p>0.05$) and were dropped from the models. All the data in the tables are expressed as means ± standard deviation.

RESULTS

Fat content and storage time significantly affected the CIEL*a*b* colour parameters of three groups of Sremska sausages (Table 1). Although MF and HF sausages had different fat content, there were no significant differences in L* values between these groups throughout the storage period. As expected, LF sausages, with lowest fat content, had the lowest L* values compared with other two groups. In relation to storage time, a decrease in L* values after 90 days was observed in all sausage groups, which remains the same till the end of storage. With respect to a* values, LF group had the lowest values compared with other two groups, whilst MF and HF sausages had similar a* values. Additionaly, a significantly decrease in redness was observed during the storage period: for LF sausages after 90 days, for MF sausages after 360 days and for HF sausages after 180 days, compared with day 0.

The degree of yelowness (b*) was the lowest in LF group, where it remains the same during the storage period ($p=0.826$). MF and HF sausages

had similar b* values, with the exception at 180 days of storage where it was higher in MF group ($p<0.001$). Similarly to LF, b* values of sausages from HF group remains the same during storage ($p=0.434$), whilst in MF group b* values significantly differ between days 90 and 180, with no differences between the beginning and the end of storage.

Table 1. Instrumental colour changes of three groups of Sremska sausages through the one year of vacuum storage

Properties	Group	Day				p
		0	90	180	360	
L*	LF	36.10 ± 4.82^{Aa}	31.05 ± 1.99^{Ab}	32.44 ± 2.03^{Abc}	34.48 ± 3.80^{ac}	0.001
	MF	39.94 ± 2.25^{Ba}	37.26 ± 2.19^{Bb}	35.89 ± 2.07^{Bb}	35.68 ± 3.25^{b}	0.009
	HF	40.27 ± 3.67^{Ba}	36.56 ± 2.87^{Bb}	35.97 ± 2.17^{Bb}	35.36 ± 2.13^{b}	0.001
	p	0.004	<0.001	0.005	0.623	
a*	LF	17.88 ± 2.41^{Aa}	15.98 ± 1.92^{Ab}	15.85 ± 0.92^{Ab}	15.38 ± 1.79^{Ab}	0.006
	MF	20.69 ± 0.65^{Ba}	19.67 ± 2.10^{Ba}	19.37 ± 0.87^{Ba}	16.20 ± 1.60^{ABb}	<0.001
	HF	20.10 ± 2.43^{Ba}	18.93 ± 1.21^{Ba}	16.51 ± 1.30^{Ab}	17.21 ± 1.16^{Bb}	<0.001
	p	0.001	<0.001	<0.001	0.045	
b*	LF	14.76 ± 2.56^{A}	13.91 ± 3.35^{A}	14.08 ± 2.13^{A}	14.74 ± 2.81^{A}	0.826
	MF	20.42 ± 1.70^{Bab}	21.01 ± 2.29^{Ba}	20.59 ± 2.66^{Bab}	18.52 ± 2.35^{Bb}	0.142
	HF	18.50 ± 2.04^{B}	19.64 ± 3.70^{B}	17.86 ± 2.39^{C}	18.14 ± 2.14^{B}	0.434
	p	<0.001	<0.001	<0.001	0.002	

[a-c] Different letters within the same row denote significant differences between means ($p<0.05$).
[A-C] Different letters within the same column denote significant differences between means ($p<0.05$).

Results from the TPA analysis showed many differences between groups of sausages (Table 2). Hardnesses, gumminess and chewiness values were highest in LF group and lowest in HF group, while MF sausages were intermediate. During the storage these three indicators significantly increased in all three groups of sausages ($p<0.05$). Springiness values were the lowest in LF group, but only at the beginning of storage (day 0) and at the end (day 360), while MF and HF sausages differ only at day 0

($p<0.001$). Springiness values of sausages significantly decreased after 90 days of storage and then increased at the end of storage, for all analysed groups.

LF sausages had the lowest cohesiveness values, which increased during storage time ($p<0.001$). Contrary to LF group, cohesiveness values of MF sausages decreased after 360 days of storage ($p<0.001$), whilst in HF group the significant differences were established only between days 90 and 180, with no differences between day 0 and 360 ($p=0.006$).

Table 2. Textural characteristics of three groups of Sremska sausages through the one year of vacuum storage

Properties	Group	Day				p
		0	90	180	360	
Hardness (N)	LF	162.79 ± 14.17^{Aa}	189.10 ± 20.91^{Aa}	296.38 ± 19.91^{Ab}	277.35 ± 49.88^{Ab}	<0.001
	MF	76.41 ± 9.33^{Ba}	107.39 ± 3.60^{Bb}	105.94 ± 8.13^{Bb}	133.26 ± 12.56^{Bc}	0.002
	HF	57.45 ± 4.04^{Ba}	65.86 ± 13.90^{Ca}	77.27 ± 6.60^{Cab}	96.72 ± 7.27^{Cb}	0.019
	p	<0.001	<0.001	<0.001	<0.001	
Springiness (mm)	LF	0.42 ± 0.05^{Aa}	0.37 ± 0.01^{b}	0.42 ± 0.02^{a}	0.45 ± 0.04^{Ac}	<0.001
	MF	0.51 ± 0.01^{Ba}	0.36 ± 0.01^{b}	0.44 ± 0.02^{c}	0.50 ± 0.02^{Ba}	<0.001
	HF	0.46 ± 0.03^{Ca}	0.36 ± 0.02^{b}	0.42 ± 0.02^{c}	0.49 ± 0.02^{Bd}	<0.001
	p	<0.001	0.926	0.101	0.001	
Cohesiveness	LF	0.29 ± 0.02^{Aa}	0.32 ± 0.01^{Aab}	0.37 ± 0.02^{Ac}	0.34 ± 0.02^{Ab}	<0.001
	MF	0.46 ± 0.06^{Ba}	0.45 ± 0.02^{Ba}	0.44 ± 0.01^{Ba}	0.37 ± 0.02^{Bb}	<0.001
	HF	0.42 ± 0.05^{Bab}	0.44 ± 0.01^{Ba}	0.39 ± 0.02^{Ab}	0.42 ± 0.01^{Cab}	0.006
	p	<0.001	<0.001	<0.001	<0.001	
Gumminess (N)	LF	57.18 ± 11.90^{Aa}	94.18 ± 4.17^{Ab}	71.44 ± 10.75^{Ac}	94.00 ± 15.46^{Ab}	<0.001
	MF	34.69 ± 0.69^{Ba}	47.78 ± 2.20^{Bb}	46.87 ± 3.58^{Bb}	48.98 ± 3.00^{Bb}	0.035
	HF	24.23 ± 1.66^{Ba}	28.91 ± 5.70^{Ca}	30.32 ± 3.34^{Ca}	40.23 ± 2.67^{Cb}	0.008
	p	<0.001	<0.001	<0.001	<0.001	
Chewiness (N/mm)	LF	23.81 ± 4.91^{Aa}	34.38 ± 1.96^{Ab}	29.93 ± 5.09^{Ac}	42.42 ± 7.27^{Ad}	<0.001
	MF	17.76 ± 0.77^{Ba}	17.28 ± 0.87^{Ba}	20.82 ± 1.47^{Bab}	24.47 ± 2.21^{Bb}	0.003
	HF	11.12 ± 1.46^{Ca}	10.43 ± 2.27^{Ca}	12.83 ± 1.83^{Ca}	19.79 ± 1.95^{Cb}	<0.001
	p	<0.001	<0.001	<0.001	<0.001	

$^{a-d}$ Different letters within the same row denote significant differences between means ($p<0.05$).
$^{A-C}$ Different letters within the same column denote significant differences between means ($p<0.05$).

DISCUSSION

The content of fat particles has an influence on instrumental colour parameters. Hand et al. (1987) observed that when fat content was reduced, L* values decreased, which is also reported by Papadima and Bloukas (1999), Soyer et al. (2005), Bozkurt and Bayram (2006) and Olivares et al. (2010) and confirmed in this study. The decrease in L* values during storage, represented by formation ofdark color, is probably due to browning reaction (Lorenzo and Franco 2012), which was more pronounced in HF sausages ($p=0.001$) compared with other two groups.

According to Candogan and Kolsarici (2003), meat products with reduced fat content are expected to be redder due to greater lean meat contents. Soyer and Ertas (2007) concluded that sausages containing low fat had the lowest yellowness butthe highest redness values. However, in this study LF sausages, with lowest fat content, had the lowest a* values, probably due to more darker colour of meat after fermentation and rippening, whilst MF and HF sausages had similar values of redness. MF and HF sausages showed significantly higher b* values compared to LF ones, probably because of their higher lipid content (Soyeret al. 2005), as also reported by Olivares et al. (2010) and Muguerza et al. (2002).

A reduction in fat content is usually associated with an increase in the hardness and chewiness of fermented sausages (Mendoza et al. 2001, Muguerza et al. 2002, Salazar et al. 2009). In this experiment, hardness and chewiness values were the lowest in HF sausages ($p<0.001$), probably due to the higher fat content (Gómez and Lorenzo 2013). The highest hardness values were in LF sausages, and they were almost 2–3 times higher than in other groups. Low values of chewinessof HF groups indicate that it takes less effort to prepare food forswallowing compared to LF and MF sausages (Stajić et al. 2014). Lower cohesiveness of LF sausages indicates that meat pieces were less firmly bound. Springiness values, which were lowest in LF sausages, indicate that the elasticity of those sausages is lower compared with other two groups. The stability of the texture parameters during storage is desirable because it allows products to remain fresh looking after their manufacture. The storage period affect changes in texture for all investigated TPA parameters, but to a greater degree for hardnesses, gumminess and chewiness for all groups of sausage.

The findings of this study showed that it is possible to reduce fat content of dry fermented Sremska sausages, but considerably affecting the products instrumental colour and texture quality after the end of processing and during storage.

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INDICATORS OF EFFICIENCY OF PURIFIERS AS PART OF SLAUGHTERHOUSE INDUSTRY

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Abstract: The increasing growth of world population and rapid industrialization, increased pollution of freshwater due to inadequate water discharges, which is especially important in developing countries and which represents an increasingly serious global problem today. Continuing decreasing availability of freshwater resources has set the goal of wastewater treatment from disposal to reuse and recycling. In order to achieve this it is necessary to provide high efficiency purifiers.

The aim of this paper is to present basic parameters of efficiency of filters as part of the slaughter industry. Water samples were taken before the treatment process, after the execution of water treatment and in the fishpond, where the fishpond presents bioindicator of environmental pollution.

Chemical parameters of water were carried out standard operating methods in the laboratory for chemical analysis Scientific Veterinary Institute „Novi Sad", Novi Sad.

The efficiency of filters was demonstrated by measuring the levels of chemical oxygen demand (COD) and biological oxygen demand (BOD) pre treatment process and after treatment of waste water, which we have expressed in percentages.

Based on these results, we can conclude that the efficiency of slaughterhouse wastewater treatment is very high at around 98%.

Key words: *Slaughterhouse wastewater, wastewater treatment, slaughterhouse industry, environmental protection, COD, BOD.*

RECYCLING OF ANIMAL BY-PRODUCTS WITHIN THE FOOD CHAIN

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Abstract: Animal by-products (ABPs) are entire animal bodies, parts of animals, products of animal origin or other products obtained from animals that are not fit or intended for human consumption. They must be dealt with in accordance with strict regulations designed to prevent harm to people, animals and the environment. ABPs are categorised by the risks they pose and the methods used to deal with them. Category 3 are parts of slaughtered animals, which are fit for human consumption but are not intended for human consumption, that can be rendered into processed animal protein (PAP) and re-used as an ingredient in animal feed. From the economic and ecological point of view, as well as for the sustainability discussion, this approach becomes increasingly important.

Key words: *animal by products, feed, regulation*

INTRODUCTION

In most developed countries, slaughtering is a centralized activity. The consumers in these countries have a preference for lean meat and a few selected offal only, such as brain, kidney, sweetbread, tongue, etc. For this reason, the carcass is often deboned at the slaughterhouse and cooled before being sent to retail outlets. As a result, large quantities of by-products (bones, lungs, spleen, oesophagus etc.) are left behind at the slaughterhouse. They fall in the category of inedible offal. For economic and environmental considerations, these need to be suitably processed and utilized. Clean fatty tissues may be processed into edible fat. Other tissues may be used to produce protein meals or individual products like bone-meal, meat-meal and blood-meal. In principle all by-products can be processed in rendering plants for petfood and feed, incineration plants that only incinerate ABPs, biogas plants, composting plants, intermediate and storage plants and put to further use.

Animal by-products (ABPs) are animal carcases, parts of carcases and other animal by-products that are *not intended for human consumption*.

They are a potential source of risks to public and animal health. For example improper use of animal by-products has resulted in outbreaks of serious diseases such as foot and mouth disease, classical swine fever, avian influenza and the spread of bovine spongiform encephalopathy (BSE) (OIE Terrestrial Animal Health Code 2010, Aquatic Animal Health Code 2010). Legislation has been in place for many years to control these risks by setting out the rules for collection, storage, transport, treatment, use and disposal of animal by-products. One of the preventive measures within the Europe was a ban on the processed animal proteins (PAP), including meat and bone meal (MBM), as feed ingredient intended for food-producing animals. It was followed by extensive official control activities (Nesic et al. 2014).

PROCESSED ANIMAL PROTEINS IN FEED

After the outbreak of bovine spogiformne encephalopathy (BSE), commonly known as „mad cow disease", diagnosed in the UK in 1986, it was found that it spreaded by feed, through the infectious ruminant proteins processed into meat and bone meal (MBM). Eradication started immediately and one of the most important measures was to establish the regulations to avoid entry of these nutrients in the food chain. In the European Union, according to Regulation 999/2001 (European Commission 2001) and 1234/2003 (European Commission 2003) the use of processed animal proteins, including different types of MBM, was prohibited for all farm animals entering the food chain, except fishmeal for non-ruminants. Thus rigorous measurement was made in accordance with the fact that these feedingstuffs represent a potential source of prion infection, not only for animals, but also indirectly through food of animal origin, for humans. Due to this approach epidemiological situation in Europe is much better today (Paisley et al. 2008).

On the other hand, materials that are excellent source of essential amino acids and valuable proteins have been rejected or destroyed over the years, thereby requiring special measures of safe disposal. Therefore, an intensive research is done for a model to get by-products of animal origin back in use in farmed animals' nutrition, but in a safe and controlled manner. Regulation 1774/2002 (European Commission 2002), replaced by Regulation (EC) no 1069/2009 (European Commission 2009) and Regulation (EU) No 142/2011 (European Commission 2011), provided such possibility, but with the restriction of the use of protein derived from the same species, so-called „intraspecies recycling". The European Commission, in its plan from 2005 predicted modification measures in line with the latest technological and scientific knowledge (European Commission 2005) and European association of fat and rendering industry EFPRA supported this kind of proposal (EFPRA 2006). Since the half of 2013 the use of non-ruminant meat and bone meal is again permitted in

aquaculture in the European Union by Regulation 51/2013 (European Commission 2013). However, the ban has been partially lifted so far, but this is only the first step in alleviating measures.

CATEGORISATION OF ANIMAL BY-PRODUCTS

As animal by-products can present a risk to human and animal health, in particular in relation to Transmissible Spongiform Encephalopathies (TSEs), dioxin contamination, and exotic diseases such as Classical Swine Fever and Foot and Mouth Disease, they are divided into three categories according to their level of risk and prescribed by Regulation (EC) no 1069/2009 (European Commission, 2009) and Regulation (EU) No 142/2011 (European Commission, 2011). Those products posing a high risk should only be used for purposes outside the feed chain, while their use posing a lower risk should be permitted under safe conditions. Such triage of animal materials is also accepted in other countries outside the European Union, as well as in Serbia.

Category 1

Category 1 material is defined in Article 8 of Regulation (EC) no 1069/2009 (European Commission 2009). That is the highest risk material, and consists principally of material that is considered a TSE risk, such as Specified Risk Material (those parts of an animal considered most likely to harbour a disease such as BSE, e.g. bovine spinal cord). Pet animals, zoo and circus animals and experimental animals are also classified as category 1 material due to the level of veterinary drugs and residues they are likely to contain and due to the fact that adequate diagnoses of the exact cause of death of exotic animals can be difficult to achieve. Several are known to harbour TSEs and may carry other diseases. Likewise, wild animals may be classified as category 1 material when they are suspected of carrying a disease communicable to humans or animals. Catering waste from means of international transport is also category 1.

Category 2

Category 2 material is also high risk material and is defined in Article 9 of Regulation (EC) no 1069/2009 (European Commission 2009). It includes fallen stock, manure and digestive content. Category 2 is also the default status of any animal by-product not defined in Regulation (EC) 1069/2009 as either category 1 or category 3 material.

Category 3

Article 10 of Regulation (EC) 1069/2009 defines category 3 animal by-products (European Commission 2009). These are low risk materials which include parts of animals that has been passed fit for human consumption in a slaughterhouse but which are not intended for

consumption, either because they are not parts of animals that we normally eat (hides, hair, feathers, bones etc) or for commercial reasons. Category 3 material also includes former foodstuffs (waste from food factories and retail premises such as butchers and supermarkets). Catering waste, including domestic kitchen waste is category 3 material, though it is only in the scope of the Regulations in certain situations, to prevent it from being fed to livestock (which is banned under the Regulation) or such as when it is intended for composting or anaerobic digestion.

DISPOSAL AND USE OF ANIMAL BY-PRODUCTS

Category 1

Article 12 of Regulation (EC) 1069/2009 (European Commission 2009) sets out the disposal routes for category 1 material. As the highest risk material, generally speaking this material must be destroyed by incineration, or by rendering followed by incineration. These are the only options for TSE suspects. Other category 1 material is also permitted to be pressure-rendered and disposed of in an authorised landfill site. International catering waste may be disposed directly in an authorised landfill site. In principle the Regulation allows for category 1 material to be used as a fuel for combustion. Category 1 material can also be used for the manufacture of derived products as specified by the Regulation, such as medical devices.

Category 2

Article 13 of Regulation (EC) 1069/2009 (European Commission 2009) sets out the disposal routes for category 2 material. The basic options of incineration and rendering followed by incineration are permitted, as with category 1 material. All category 2 material can also be pressure-rendered and disposed of in an authorised landfill site. Use as fuel for combustion is foreseen as well. Category 2 material can also be used for the manufacture of derived products as specified by the Regulation, such as medical devices. However there are also other uses for category 2 material which do not apply to category 1 material. Category 2 material can be pressure-rendered and then used for the production of organic fertilisers and soil improvers. It can also be pressure-rendered and used in an approved composting or anaerobic digestion plant. A very limited number of category 2 materials (manure, digestive tract content, milk and milkbased products and colostrum) may be applied directly to land without processing provided there is no risk of transmitting a disease. There is also an option for ensiling of category 2 fish under national rules.

Category 3

Article 14 of Regulation (EC) 1069/2009 (European Commission 2009) sets out the use and disposal routes for category 3 material. As low-risk material, there are a much wider range of options for use and disposal of category 3 material compared to higher risk material. Category 3 material can like category 1 and category 2 material be incinerated, or rendered followed by incineration. Category 3 material can also be rendered followed by disposal in an authorised landfill (unlike higher category material this does not have to be pressure rendering). Use as fuel for combustion is foreseen in the Regulation as well. Category 3 material can also be used for the manufacture of derived products as specified by the Regulation, such as medical devices. Category 3 material can be rendered for the production of petfood and organic fertilisers or soil improvers. Rendered category 3 material can also be used in the production of animal feedingstuffs, though TSE related restrictions on the feeding of processed animal protein severely restrict this. Category 3 material can be used directly in approved composting or anaerobic digestion plants, and in the production of raw petfood. Certain category 3 material (raw milk, colostrum and products derived from these) can be applied directly to land provided there is no risk of transmitting a disease. Shells from shellfish and eggshells may be applied to land in accordance with national rules. There is also an option for ensiling of category 3 fish under national rules.

FEEDING AND FEED CONTROL

Article 11 of Regulation (EC) 1069/2009 (European Commission 2009) bans intra-species recycling (feeding material derived from a species to a creature of the same species) and the feeding of catering waste to farmed animals. Article 11 also prohibits the feeding to farmed animals of herbage from land to which organic fertilisers or soil improvers have been applied within unless grazing restrictions have been observed.

Classical light microscopy was the only official method for the detection of constituents of animal origin in compound feed in the EU for years, but this method offered very limited species differentiation properties (Van Raamsdonk et al. 2007). Therefore, research teams focused on the development of analytical systems that will enable the detection of animal species from which feed ingredients are derived (Fumiere et al. 2012) and will provide re-introduction of these feedstuffs into animal feed with caution and under strict control. Success of such projects determines the pace and the speed at which the changes occur. For now, the first step has been made by re-use of meat and bone meal of non-ruminant origine in feed for aquaculture and by formalization of polymerase chain reaction (PCR) as official laboratory method for this purpose in addition to the classical light microscopy in the Regulation 51/2013 (European Commission 2013).

CONCLUSION

From the economic and ecological point of view, as well as for the sustainability discussion, recycling of animal by-products within the feed chain becomes increasingly important. In addition to the mandatory triage of animal material, adequate treatment of it and appropriate control procedures make the health risks negligible and the benefit immeasurably large. By manufacturing of feed from sanitary safe raw materials (animal by-products belonging to Category 3 materials) added value is created, with assurance of the rational development of livestock production and of protection of the environment. Thus the use of feeding stuff of animal origin has growing significance.

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