FOLIA VETERINARIA

The scientific journal of the
UNIVERSITY OF VETERINARY MEDICINE AND
PHARMACY IN KOŠICE — Slovakia

ISSN 0015-5748
eISSN 2453-7837

3

LXI • 2017

FOLIA VETERINARIA is a scientific journal issued by the University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia.
The journal is published quaterly in English (numbers 1—4) and distributed worldwide.

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Electronic Publisher: De Gruyter Open, Bogumila Zuga 32A str. 01-811
Warsaw, Poland

ISSN 2453-7837 on-line
ISSN 0015-5748 print
EV 3485/09

Publisher's identification number: IČO 00397474

September 2017

FOLIA VETERINARIA

PUBLISHED BY
THE UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE
SLOVAKIA

Folia Veterinaria
Vol. 61, 2017

VYDÁVA
UNIVERZITA VETERINÁRSKEHO LEKÁRSTVA A FARMÁCIE V KOŠICIACH
2017

FOLIA VETERINARIA, 61, 3, 2017

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OPEN

DOI: 10.1515/fv-2017-0021

FOLIA VETERINARIA, 61, 3: 5—11, 2017

PREVALENCE AND DETECTION OF FLAVIVIRUSES OCCURRING IN SLOVAKIA

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ABSTRACT

The tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) are arboviruses of the genus *Flavivirus* in the family *Flaviviridae*. Their hosts are vertebratesof which rodents are the reservoirs of TBEV and birds are the reservoirs of WNV. Both viruses are transmitted from reservoirs to mammals by vectors. TBEV is transmitted by ticks (mostly *Ixodes* spp.) and WNV by mosquitoes (mostly *Culex* spp.). Both viruses are capable of infecting mammals, including man. TBEV and WNV are neurotropic, however infection is, in most cases, subclinical or accompanied by only moderate general signs. However, in some cases they can cause serious disturbances of the CNS. Our study focused on the detection of the genomes of TBEV and WNV in vectors by means of the reverse-transcription polymerase chain reaction (RT-PCR). The flavivirus genome was detected by means of oligonucleotides delineating the sequence in NS5 gene that encodes viral RNA-dependent RNA-polymerase. For the detection of TBEV, we used the oligonucleotide pair detecting the structural envelope protein. The positive samples were subjected to the sequence and phylogenetic analysis. The WNV was not detected in any of the pooled samples prepared from 616 mosquitoes captured in the vicinity of the village Drienovec, district Košice-surroundings. The investigation of 676 ticks demonstrated the presence of one strain of TBEV. One blood-fed *I. ricinus* female was obtained from a goat grazing in a pasture in the Dúbrava area close to Prešov. The genetic analysis revealed the presence of a strain close to the endemic strainsof TBEV Hypr and Neudörfl. The results of our study can become a motivation for additional studies in model locations oriented on ecology and circulation of these important zoonotic flaviviruses.

Key words: arbovirus;tick-borne encephalitis virus TBEV; West Nile virus WNV

*both authors contributed equally

INTRODUCTION

The family *Flaviviridae* includes pathogenic viruses of humans and other animals. It comprises the genera *Flavivirus*, *Pestivirus*, *Hepacivirus* and *Pegivirus*. Many representatives of the genus *Flavivirus* are arboviruses transmitted by vectors — ticks or mosquitoes [8]. The WNV was first described in Uganda in 1937 in a woman with signs of CNS disorders and later it was also demonstrated in mosquitoes, birds and horses. Although it was originally described in the tropics, it was later detected in other regions where it became endemic. The first information about the occurrence in Slovakia dates back to the 1960's, when antibodies to the virus were found in human serum. In the period of 1960—1978, WNV was isolated from *Aedescantans* mosquitoes and neutralization antibodies were detected in 5.4 % of the birds, 5.3 % of the small mammals, 1 % of the hares and 4.4 % of free living ungulates [5]. During the years 2008 and 2011,neutralization antibodies were detected in 8.3 % of non-vaccinated horses [7]. Currently, we lack sufficient information on the prevalence and transmission of WNV in Slovakia. The presence of WNV was confirmed in oral and cloacal swabs and in samples from the brain of birds [3]. Although no case of West Nile fever has been recorded in the Slovak territory, the latest research confirmed the transmission of the WNV among birds and indicated the risk of potential transfer of infection to horses and humans [3].

The tick-borne encephalitis virus (TBEV) is one of the most important tick transmitted arboviruses in Europe. In addition to direct tick transmission, it can spread also by the consumption of non-pasteurised milk products. The first mention of TBEV in Slovakia dates back to 1951, when an extensive outbreak occurred in the district of Rožňava associated with the consumption of raw goat milk [12]. Since then,the cases of tick-borne encephalitis have been reported every year. While in 2013 there were reported 163 cases of tick-borne encephalitis in humans, in 2014 the number of cases decreased to 117 and in 2015 to 88 (a decrease of 28 % in comparison to the 5-year average).

Tick-borne encephalitis occurs in all age categories, from infants up to old people. The course of the disease differs, from oligosymptomatic up to life threatening encephalomyelitis or encephalomyeloradiculitis[11].

Currently, flaviviruses have become a relevant topic, not only due to progressing global warming and climate changes, but also because of the new emerging diseases caused by these agents. Because of the increasing importance of diseases caused by flaviviruses in Slovakia, we devoted our study to the occurrence of these viruses in their vertebrate vectors.

MATERIALS AND METHODS

Ticks and mosquitoes

For the determination of the prevalence of TBEV, we used 676 adults, larvae and nymphs of various species of ticks (Table 1) collected in the Slovak districts of: Žilina, Prešov, Bardejov, Košice-surroundings, Ružomberok, Stará Ľubovňa and Námestovo. They were collected by flagging, or directly from animals, including people.

Table 1. Division of the examined ticks according to species and gender

Species	Gender and developmental stage	Total number
Ixodes ricinus	F	202
	M	105
	N	272
	L	80
Dermacentor marginatus	F	5
	M	7
D. reticulatus	F	3
	M	1
Haemaphysalis	F	1
Total		**676**

F — adult female; M — adult male; N — nymph; L — larva

For the examination of the prevalence of WNV,we obtained 616 mosquitoes (Table 2) from Drienovec within the district of Košice. They were captured in May and July 2016 by means of CDC light traps developed by Centres for Disease Control (CDC) using dry ice as an attractant. Individual pools comprised no more than 25 mosquitoes of the same species.

Table 2. Division of the investigated mosquitoes according to species

Species	Number
Aedes cinereus	57
Ae. rossicus	2
Ae. vexans	166
Anopheles claviger	14
Culex pipiens	70
Culiseta annulata	1
Ochlerotatus cantans/annulipes	75
Oc. caspius	8
Oc. cataphylla	6
Oc. geniculatus	1
Oc. leucomelus	14
Oc. punctor	155
Oc. sticticus	47
Total	**616**

Isolation of nucleic acids from ticks and mosquitoes

The vectors were washed in 400 µl 70 % ethanol for 10 min. Then the ethanol was removed and the vectors were washed twice in water free of nucleases. After mechanical homogenization employing either an apparatus Precellys (Bertin, France) or Tissue Lyser (Qiagen, Germany), the total DNA or RNA was extracted using a commercial QIAamp cador Pathogen Mini Kit (Qiagen, Germany), according to manufacturer's instructions.

Reverse-transcription polymerase chain reaction (RT-PCR)

Complementary DNA was prepared by means of reverse transcriptase (Revert Aid H Minus Reverse Transcriptase, Thermo Scientific, Germany) using random hexamers (Thermo Scientific). For PCR (PCR Dream Taq Green PCR Master Mix, Thermo Scientific) we used primers (Table 3) delineating sequences in TBEV envelope protein and RNA-dependent RNA-polymerase of flaviviruses under the following thermal conditions: initial denaturation 95 °C/1 min; 30 cycles 95 °C/30 sec; 50 °C/30 sec; and 72 °C/40 sec. The final extension took place at 72 °C and lasted 5 min.

Table 3. Oligonucleotides used for detection of TBEV on the basis of RNA-polymerase and envelope protein

Primer	Sequence [5´→3´]	PCR product [bp]
TBEV-Eprot-F	GTTCCTGTGGCRCAYATTG	326
TBEV-Eprot-R	CCTGGRGGYARCTGCATYTCTATG	
PanFlavi-NS5-F	WTRGCMATGACWGAYACHAC	599
cFD2*	GTGTCCCAGCCGGCGGTGTCATCAGC	

Source: Scaramozzino et al. [13]

Sequence analysis

After agarose electrophoresis, DNA fragments of presumed length were excised and used for sequence analysis using software Geneious (Biomatters, New Zealand). The partial sequences were compared with the world database by means of BLAST (Basic Local Alignment Search Tool) analysis. Subsequently,the genetic similarity with the TBEV nucleotide sequence was determined using an algorithm Clusta lW, and a phylogenetic tree was produced.

RESULTS

The WNV was not recorded in any mosquito sample. Of the total number of 675 tick samples, a DNA fragment of length of approximately 350 bp was recorded in several cases, however, subsequent re-amplification confirmed the positivity in only one case (Fig. 1). It concerned a blood-fed female of the species *I. ricinus*, collected from a goat in April 2016 in Prešov-Dúbrava. BLAST analysis confirmed the TBEV. This fragment was named TBEV E prot 389C Dúbrava/PO/2016. The sequence of nucleotides agreed to the highest percentage with European subtypes of TBEV (Table 4). The highest agreement was observed with strains Hypr (99 %) and Neudörfl (98 %) and then with strains K23 and Salem (97 %), which was also reflected in the phylogenetic tree (Fig. 2).

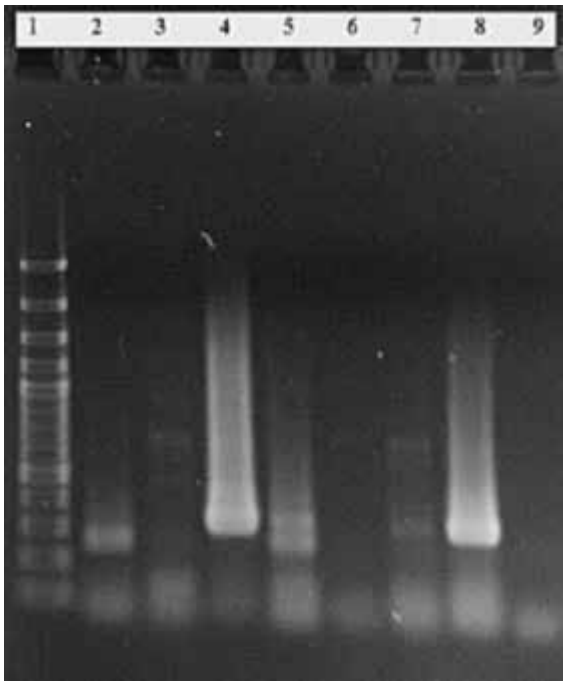

Fig. 1. Result of PCR for identification of TBEV in ticks

Line 1 — 100 bp DNA standard; lines 2—7 — tick samples; line 8 — positive isolation control; line 9 — negative isolation control. Sample in line 4 is unambiguously positive for the presence of TBEV fragment. Samples in lines 2, 3, 5 and 7 were negative after re-amplification

DISCUSSION

Tick-borne encephalitis is a viral zoonotic disease caused by the tick-borne encephalitis virus (TBEV). It is the most important human pathogenicity virus in Eurasia. In the years 1964—1999,the research that was conducted in Slovakia focused on the genus proportion of ticks found in Slovakia. The relevant samples were examined also for the presence of TBEV. This research showed that of 77 000 ticks, 90 % were *I. ricinus*. The TBEV virus was detected only in 0.14 % of the vector [4]. The prevalence of TBEV in ticks in Slovakia has been rarely investigated. The present study examined ticks by the molecular methods. We examined ticks from various regions of Slovakia. No information was available whether the virus was found in these areas in the past. TBEV was detected in one engorged *I. ricinus* female, which is the typical vector. The observed prevalence of TBEV (0.14 %) was similar as that in the study by Grešíková and Nosek [4]. In another study conducted in the Tribeč mountains in the spring of 1964, TBEV was detected in 0.2 % of the 2153 ticks exam-

Table 4. Percentage expression of nucleotide sequence similarity for the partial sequence of envelope protein of the TBEV strains

	TEU39292	Dúbrava	FJ572210	AM600965	TEU27495	AF069066	JN003206	AF527415	JN003205	AB062064	AB062063	NC_005062
TEU39292 TBEV Hypr (EU)		99	99	99	100	85	85	85	87	88	87	86
TBEV Eprot_389C_Dúbrava/PO/2016	99		97	97	98	85	85	84	87	88	87	85
FJ572210 TBEV Salem (EU)	99	97		98	99	85	85	85	86	87	85	86
AM600965 TBEV K23 (EU)	99	97	98		98	85	85	85	86	87	85	86
TEU27495 TBEV Neudoerfl (EU)	100	98	99	98		85	85	84	87	88	87	85
AF069066 TBEV Vasilchenko (SIB)	85	85	85	85	85		100	95	86	86	84	79
JN003206 TBEV Aina (SIB)	85	85	85	85	85	100		95	86	86	84	79
AF527415 TBEV Zausaev (SIB)	85	84	85	85	84	95	95		85	85	85	79
JN003205 TBEV Irkutsk-1861 (FES)	87	87	86	86	87	86	86	85		98	97	81
AB062064 TBEV Sofjin-HO (FES)	88	88	87	87	88	86	86	85	98		97	82
AB062063 TBEV Oshima 5-10 (FES)	87	87	85	85	87	84	84	85	97	97		79
NC_005062 OHFV	86	85	86	86	85	79	79	79	81	82	79	

Similarity of nucleotide sequence of the TBEV strains determined by an algorithm ClustalW. Values in boxes are expressed in per cent: (EU) — European subtype; (SIB) — Siberian subtype; (FES) — Far East subtype; OHFV — Omsk haemorrhagic fever virus

Similarity of nucleotide sequence of the TBEV strains determined by an algorithm Clusta lW. Values in boxes are expressed in per cent: (EU) — European subtype; (SIB) — Siberian subtype; (FES) — Far East subtype; OHFV — Omsk haemorrhagic fever virus.

This phylogenetic tree was produced on the basis of Neighbor-Joining method and the Tamura-Niei model; bootstrap of 1000 replicates; illustrated are only credibility values above 75 %.

ined. In the primary foci in Topoľčianky and Jelenec, the prevalence was 0.5 % and 0.9 %, respectively [4]. In arecent study conducted in Poland, there were examined 471 adult ticks *D. reticulatus* collected in the national park Biebrza, Bialowiezsky forest and Masurian region in North-West Poland. The RT-PCR revealed the prevalence of the virus ranging from 0.99 % to 12.5 % depending on the location and the mean prevalence reached 2.1 % [2]. In another survey investigators examined 87 *I. ricinus* ticks and 148 *D. re-*

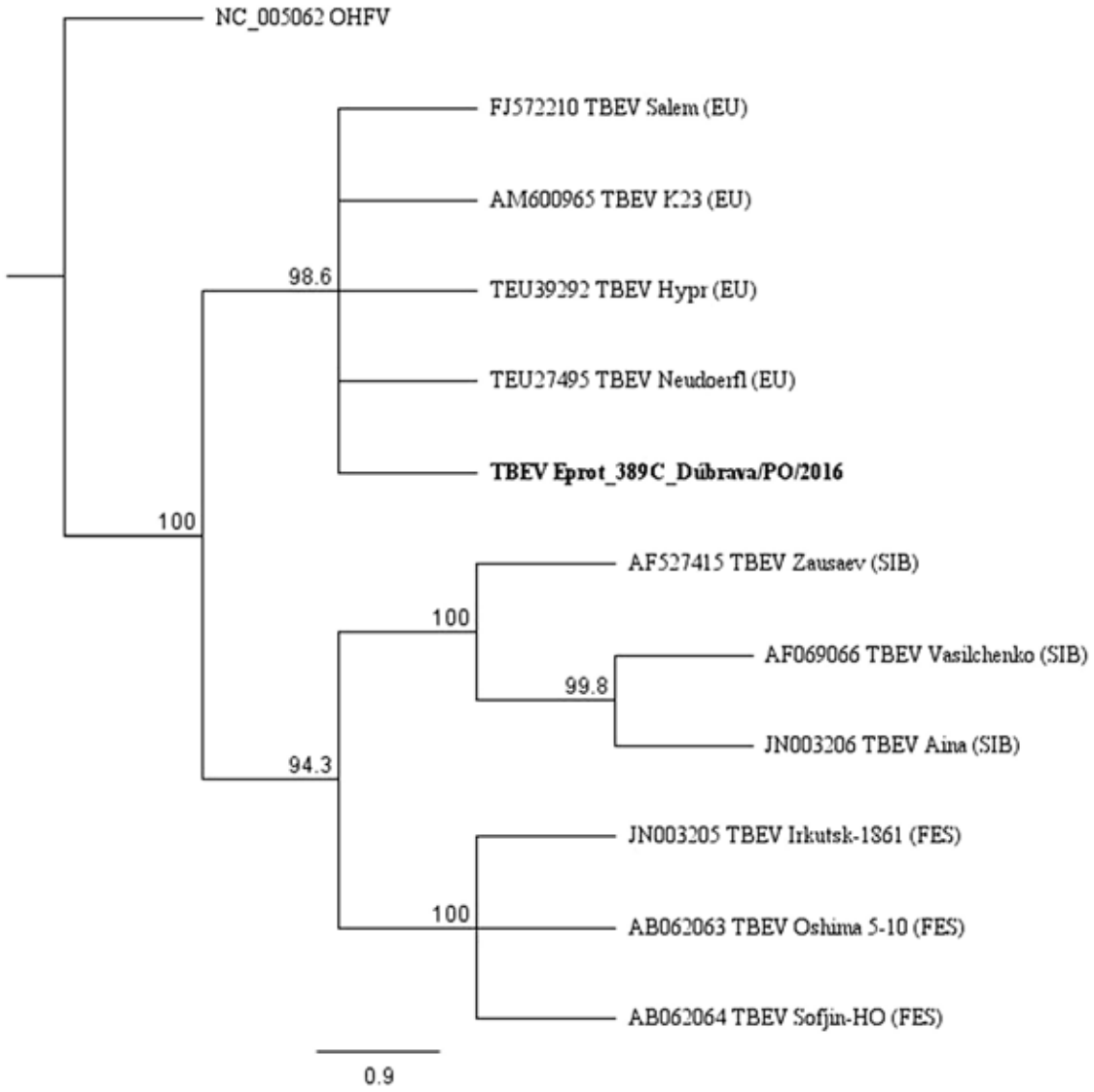

Fig. 2. Phylogenetic tree of the strain TBEV produced on the basis of partial sequence of the envelope protein

ticulatus ticks collected in the period from April to September 2008 and 2009 in the district of Lublin in East Poland. By means of RT- PCR,the virus was detected in 1.6 % of the *I. ricinus* and in 10.8 % of the *D. reticulatus*. The prevalence was lower in males and nymphs than in females. In some locations the virus was not detected[14].

The prevalence of a disease is related to the prevalence of the vector. The spread of *D. reticulatus* can be divided into two areas, western and eastern Europe. Western Europe covers the territory from France up to eastern Germany and eastern Europe spreads from east Poland to Siberia. Since the 1990's, these ticks have been found in areas that were previously free from them[9]. The vector occurred also along the Danube and Morava rivers and in eastern Slovakia, close to Latorica, Bodrog and Tisa rivers. In the recent period, it expanded to the north and occurred along the Laborec river up to the Vihorlat mountains, but also in lower regions of the Váh, Hron and Ipeľ rivers.

I. ricinus occurs throughout Europe, from Ireland to the Urals and from northern Sweden down to northern Africa. In Slovakia, it is spread in the whole territory up to 600—800 m above sea level. Due to global warming, the upper boundary of its occurrence has been shifting gradually to more than 1000 m above sea level [10].

In the recent years, we recorded the continuous spreading of ticks to new areas, particularly into north-east Europe [2]. The positive detection of TBEV RNA in a *I. ricinus* female in Dúbrava in the Prešov district indicates the pres-

ence of a natural focus. In order to confirm its presence, it is necessary to examine also its reservoir, namely small mammals. A positive tick was collected from a grazing goat. Its owner reported the production of cheese from the goat milk. Because information about the occurrence of TBEV in reservoirs in Dúbrava and its potential spreading via alimentary path is absent, additional studies appear desirable.

West Nile fever is a viral zoonotic disease transmitted by mosquitoes. West Nile virus was isolated from 43 mosquito species, particularly of the genus Culex. The principal vectors in Europe are *Cx. pipiens*, *Cx. modestus and Coquillettidia richiardii*. Virus isolates from haematophagous arthropods have been occasionally reported. West Nile fever has been known in Europe for several decades. Cases were reported mostly in the Mediterrean region and south-east Europe, but also in Italy, Romania, France, Hungary and Austria [7, 15].

In 2008—2009 research was carried out in Hungary and Austria on 19 pools from Hungary, each consisting of 2–25 mosquitoes, and 4983 mosquitoes from Austria, of the species *Cx. pipiens*. The investigated mosquitoes were collected in south-east Hungary and from the vicinity of Vienna, upper and lower Austria, Burgenland and Steiermark in September and October of 2008. The WNV genome was detected in 7 pools by the RT-CPR method. All positive mosquitoes were *Cx. pipiens* females collected in lower Austria, close to the location associated with the mortality of birds caused by WNV. Data from the preliminary surveillance of mosquitoes indicated a similar degree of infection in mosquitoes involving lineage 2 (approx. 5 %) in comparison with lineage 1 in the endemic regions. Because this research focused mainly on mosquitoes *Cx. pipiens* and only a few other mosquito species were captured, we lack information on the role of other mosquito species in the transmission of lineage 2 WNV [1].

Our study was a follow up of the previous study that took place in the Drienov wetland and recorded WNV genome in oral and cloacal swabs of free living birds [3].The results of our study were likely affected by a low number of examined vectors, so the presence of WNV in the respective locations cannot be excluded. Due to global warming, one can assume that an increase in the temperature and humidity will support the spreading of the vector. This indicates the need for surveillance of West Nile fever in the sensitive regions.

CONCLUSIONS

The positive finding of TBEV in the Dúbrava region indicates the occurrence of a natural focus. However, further studies in this location involving vectors and reservoirs are needed to confirm this hypothesis. The negative finding of WNV in the investigated area could be affected by several factors. The important ones include the influence of the climate on the population of mosquitoes in the relevant season and low number of examined vectors. Thus, one cannot guarantee the absence of this agent in the locations investigated, which opens a space for further investigation of the occurrence of WNV in mosquitoes in Slovakia.

ACKNOWLEDGEMENT

This study was supported by projects VEGA 1/0729/16 and INFEKTZOON (ITMS: 26220120002).

REFERENCES

1. **Bakonyi, T. E.,Ferenczi, K., Erdelyi, O., Kutasi, T., Csorgo, B., Seidel, H., et al., 2013:** Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. *Vet. Microbiol.*, 165, 61—70.
2. **Biernat, B., Karbowiak, G., Werszko, J., Stanczak, J., 2014:** Prevalence of tick-borne encephalitis virus (TBEV) RNA in *Dermacentor reticulatus* ticks from natural and urban environment, Poland. *Exp. Appl. Acarol.*, 64, 543—551.
3. **Csank, T., Bhide, K., Bencurova, E., Dolinska, S., Drzewniokova, P., Major, P., et al., 2016:** Detection of West Nile virus and tick-borne encephalitis virus in birds in Slovakia, using a universal primer set. *Arch. Virol.*, 161, 1679—1683.
4. **Grešíková, M., Nosek, J., 1967:** Isolation of tick-borne encephalitis virus from *Ixodes ricinus* ticks in the Tribeč region. *Bull. World Health Organ.*, 36, 67—71.
5. **Grešíková, M., Nosek, J., 1981:** *Arboviruses in Czechoslovakia* (In Slovak). 1st edn., Veda, Bratislava, 181 pp.
6. **Hubalek, Z., Halouzka, J., 1999:** West Nile fever – a reemerging mosquito-borne viral disease in Europe. *Emerg. Infect. Diseases*, 5, 643—650.
7. **Hubalek, Z., Ludvikova, E., Jahn, P., Treml, F., Rudolf, I., Svobodova, P., et al., 2013:** West Nile Virus equine serosur-

vey in the Czech and Slovak Republics. *Vector Borne Zoonotic Dis.*, 13, 733—738.

8. **ICTV, 2017:** *The ICTV Online (10th) Report on Virus Taxonomy*. Classification and Nomenclature of Viruses.
9. **Karbowiak, G., 2014:** The occurrence of the *Dermacentor reticulatus* tick, its expansion to new areas and possible causes. *Ann. Parasitol.*, 60, 37—47.
10. **Kerlik, J., Avdičová, M., Musilová, M., Dobler, G., Molčányi, T., Csank,T., et al., 2016:** Presence of tick-borne encephalitis virus in ticks from selected locations in Slovakia. In *Proceedings of the 5th scientific congress: Zoonoses — Protection of Public and Animal Health*, October 18—20, Slovak Medical University, Bratislava. 322 pp.
11. **Kunze, U., 2008:** Is there a need for travel vaccination against tick-borne encephalitis? *Travel Med. Infect. Dis.*, 6, 380—383.
12. **Rajčáni, J., Čiampor, F., 2006:** *Family Flaviviridae. Medical Virology* (In Slovak). Bratislava, Slovak Academy of Sciences, 436—451.
13. **Scaramozzino, N. J., Crance, M., Jouan, A., DeBriel, D. A., Stoll, F. O., Garin, D., 2001:** Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested Reverse Transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J. Clin. Microbiol.*, 39, 1922—1927.
14. **Wójcik-Fatla, A., Cisak, E., Zajac, V., Zwoliński, J., Dutkiewicz, J., 2011:** Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* and *Dermacentor reticulatus* ticks collected from the Lublin region (eastern Poland). *Ticks and Tick-borne Diseases*, 2, 16—19.
15. **Zeller, H. G., Schuffenecker, I., 2004:** West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. *Eur. J. Clin. Microbiol. Infect. Dis.*, 23, 147—156.

Received May 10, 2017
Accepted June 19, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0022

FOLIA VETERINARIA, 61, 3: 12—18, 2017

SERO SURVEY OF FOOT AND MOUTH DISEASE VIRUS INFECTION IN CATTLE CROSSING SOME MAJOR BORDER STATES IN NORTHWESTERN NIGERIA

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ABSTRACT

Foot and mouth disease (FMD) poses a major constraint to international trade in animals and animal products in sub-Saharan Africa. A retrospective and serological survey was conducted in two major Border States of Sokoto and Kebbi in north-western Nigeria. This study was aimed at determining the sero-prevalence of FMD virus (FMDV) antibodies in cattle at international animal control posts and to examine cattle population movement across the border area for a period of one year (January to December 2014) from the available records. Eight hundred and eighty (880) sera samples were collected and screened for the presence of antibodies to FMDV using the competitive enzyme linked immunosorbent assay (ELISA) technique. The data were subjected to chi-square and relative risk to check for independence and association. An overall seropositive rate was found to be 55.2% (486/880). A 79.9% (359/450) sero-positive rate was obtained from the Kamba border, while 29.5% (127/430) was found at the Illela border. Kamba showed a statistically significant ($P < 0.05$) higher sero-prevalence when compared with cattle that are crossing the Illela border (Relative Risk 2.70; 95% Confidence Interval 2.317—3.149). Retrospective data from the control posts revealed that an average number of 2019 and 2747 of cattle, respectively, crossed the Kamba and Illela international borders monthly. The highest influx of animals from the Niger Republic through the Illela international border was encountered between the month of March and April 2014. The magnitude of the presence of FMDV Non-structural protein (NSP) antibodies in the study areas is an indication of the infection and the presence of the virus in the study areas and the neighbouring countries.

Key words: border; foot and mouth disease virus; Nigeria; retrospective survey; sero-prevalence

INTRODUCTION

Foot and mouth disease (FMD), is a highly contagious viral disease of both domestic and cloven hoofed animals characterized by high morbidity and decreased livestock productivity, with affected countries being excluded from international animal trade [3]. FMD is caused by a virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of the virus namely: A, O, C, SAT-1, SAT-2, SAT-3 and Asia 1. Infection with one serotype does not confer immune protection against another. Within serotypes many subtypes can be identified by biochemical and immunological tests [10]. Also, there are topotypic differences between one location and another. Within serotypes, many subtypes can be identified by biochemical and immunological tests [11].

FMD is endemic to most of sub-Saharan Africa, except in a few countries in Southern Africa, where the disease is controlled by the separation of infected wildlife from susceptible livestock as well as by vaccination [6]. In most parts of Africa, FMD outbreaks are often under reported either because of its endemicity as well as the fact that it is not associated with high mortalities in adult susceptible animals, as such it is not perceived as an important livestock disease among herdsmen [7]. Studies conducted by Lazarus et al. [7] in some selected states in Nigeria showed high level of antibodies to the virus in cattle (Yobe State; 82 %, Plateau; 80 %, Ogun; 77.77 %, Taraba; 73.50 %, Adamawa; 68 %; Borno; 67 %).

It is an important constraint to international trade in animal and animal products. It especially restricts world trade in the South-North direction. The endemically or sporadically infected countries, generally face total embargoes on the export of their live animals and fresh meat to many other countries of the world [4]. FMD is a transboundary animal disease and the consequences of FMD outbreaks include death, economic losses in terms of international trade, cost of control measures and other indirect losses.

This study determined the seroprevalence of FMD virus antibodies in cattle at animal control posts in Illela and Kamba International border of Kebbi and Sokoto States of Nigeria. It also examined retrospectively the cattle population movement across the border area for a period of one year (January to December 2014). The information obtained on the presence and current status of FMDV infection in cattle in the study area shall serve as baseline information for further epidemiological studies and control of FMD in livestock in Sokoto and Kebbi States.

MATERIALS AND METHODS

Study area

This study was conducted in two border towns of Illela in Sokoto State and Kamba in Kebbi State, Northwest Nigeria. Illela shares a border with Birnin Konni of Niger Republic to the North. It is located on latitude 13° 43' N and longitude 5° 18' E; it has an area of about 1,246 km^2 and a population of 150,489 as of the 2006 population census. Kamba is located in North-Western part of Kebbi State. It shares a border with the Republic of Benin and Niger republic to the West, and lies on latitude 12° 41' N and Longitude 8° 40' E. It has a total land mass of 2,003 km^2 and a population of 144, 273 as of the 2006 population census [8].

The Nigeria international livestock control posts are located within these border towns that serve the purpose of quarantine services. The major occupational activities of the people in these areas include mixed farming, livestock rearing, mining and trading. These borders serve as major route for pastoralist herds that transverse countries mainly within the African sub region including Niger and Benin Republic.

Ethical clearance

Ethical clearance was obtained from the Ministry of Animal Health and Fisheries Development of Sokoto and Kebbi State.

Study design

The two Border States were conveniently selected from the six north-western states of Nigeria. The study area was selected based on their geographical location, ruminant population density, movement pattern, as well as cattle trek route and international boundaries. A pilot study was conducted to determine the average number of cattle that passed through the border on a monthly bases. The study was a cross-sectional serological survey to detect antibodies to FMD virus in cattle using the competitive ELISA technique.

The available records of the number of cattle that crossed through the borders on a monthly basis between

January and December 2014 were obtained from the office of the international livestock control posts at both locations.

Sampling method

A systematic sampling approach was adopted after seeking the consent of the cattle owners for the collection of blood sample from representative animals as they moved through a chute across the border. The systematic sampling involved selection of units at equal intervals, the first animal being selected randomly. This sampling technique did not require knowledge of the total size of the study population and samples collected by this technique tends to be evenly spread in the population [14]. A total of 880 blood samples were collected from the two border towns for a period of 5 months (June to November, 2015).

Sample collection

Cattle congregated at the Niger Republic side of the border at Illela and Kamba. They were allowed to cross over to Nigeria through a chute. The chute is a fenced structure constructed of metal bars with a wide area at the entrance and a narrow area in front to allow passage of a single animal at a time. Within the chute, the age was determined by teeth eruptions [2], the sex by external genitalia and breed types by body type and head characteristics. Five millilitres of blood each was collected, from the jugular vein using a 10 ml syringe, into sterile vacutainer tubes. The blood sample was then allowed to clot and later centrifuged at 10,000 rpm for 15 minutes at the General Hospital Kamba and a primary health care facility at Illela to obtain the sera. The sera were then transported in ice pack to the Central Research Laboratory of the Faculty of Veterinary Medicine Usmanu Danfodiyo University Sokoto and stored at –20 °C until further processing.

Serological analysis

Serum samples were processed using ID Screen® FMD NSP (Foot and mouth disease non-structural protein) Competitive ELISA (Innovative Diagnostic, IDVet® France). The test is a competitive ELISA for the detection of anti-FMDV non-structural protein (NSP) antibodies in serum and plasma of bovine, ovine, caprine and porcine.

The principle of this technique is based on the formation of a colourless reaction following interaction of a positive test serum in a well, coated with FMDV NSP antigen after addition of a chromogenic substrate. Optical density was read using an ELISA reader (Optic System IVYMEN® 210 °C, USA) at 450 nm.

Data analysis

The data obtained were analysed using SPSS® package version 16, categorical variables (location, age, breed and sex) were evaluated using chi-square to check for associations, relative risk at 95 % confidence interval was used to measure strength of association between variables and sero-prevalence of FMD virus. Values of $P < 0.05$ were considered significant.

RESULTS

Retrospective data from the two control posts showed that an average number of 2019 and 2747 of cattle (respectively) crossed the Kamba and Illela international border monthly from January to December 2014 (Figure 1). From the available records, there are inadequate field officers to monitor; trade animal movement, disease outbreaks and vaccination status at the international livestock control posts. More so, no report on FMD or other transboundary animal diseases were recorded within the stipulated period.

From this study, an overall prevalence of 55.2 % (486/880) of antibodies to FMD NSP was obtained from cattle crossing the two major borders in the north-western Nigeria. However, Kamba shows a statistically higher prevalence (79. 9 %: 359/450) of FMD NSP antibodies as compared to Illela (29.5 %: 127/430), as indicated in Table 1. Based on sex specific prevalence, cows showed a higher statistically significance prevalence (60.5 %, $P < 0.05$) of FMD virus as compared to bulls crossing Kamba and Illela borders as showed in Table 2. Red Bororo breed of cattle showed a higher prevalence (65.6 %) of the virus when compared to Sokoto Gudali and White Fulani (Table 3). Young cattle less than two years of age showed a statistically higher level of circulating antibodies to FMD virus than older cattle crossing the two borders (Table 4).

DISCUSSION

From the retrospective records, no outbreaks of FMD or other diseases were reported from January to December,

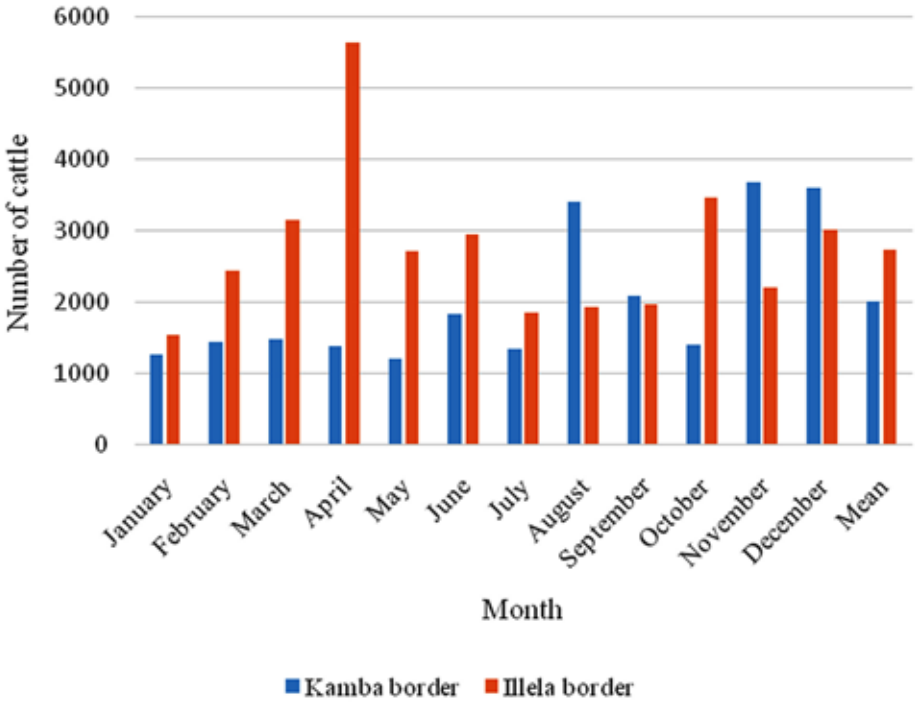

Fig. 1. Retrospective data on number of cattle that crossed Kamba and Illela borders in north-western, Nigeria between January and December 2014
Source: Federal Ministry of Livestock and Pest Control, Kebbi and Sokoto State

Table 1. Sero-prevalence of antibodies to FMDV at Kamba and Illela border towns in north-western Nigeria between January and December 2015

Location	Number of sample	Number of positive	Prevalence [%]	Relative risk	95% CI
Kamba	450	359	79.9	2.70	2.317—3.149
Illela	430	127	29.5	1.00	
Total	**880**	**486**	**55.2**		

P < 0.005; CI – confidence interval; $\chi 2 = 222.45$

Table 2. Sex specific sero-prevalence of antibodies to FMDV at Kamba and Illela border towns in north-western Nigeria

Sex	Number of sample		Number of positive sample		Prevalence [%]		Total		
	Kamba	Illela	Kamba	Illela	Kamba	Illela	**Sample**	**Positive**	**Prevalence [%]**
Male	171	197	126	50	73.7	25.4	368	176	47.8
Female	279	233	233	77	83.5	33.0	512	310	60.5
Total	**450**	**430**	**359**	**127**	**79.9**	**29.5**	**880**	**486**	**55.2**

P = 0.0002; $\chi 2 = 13.502$

Table 3. Breed specific sero-prevalence of antibodies to FMD virus at Kamba and Illela border towns in north-western Nigeria

Breed	Number of sample		Number of positive sample		Prevalence [%]		Total		
	Kamba	Illela	Kamba	Illela	Kamba	Illela	**Sample**	**Positive**	**Prevalence** [%]
Red Bororo	263	138	225	38	85.5	27.5	401	263	65.6
Sokoto gudali	85	138	59	40	69.4	30.0	223	99	44.4
White fulani	102	154	75	49	73.5	31.8	256	124	48.4
Total	**450**	**430**	**359**	**127**	**79.9**	**29.5**	**880**	**486**	**55.2**

$P < 0.05$; $\chi 2 = 32.758$

Table 4. Age specific sero-prevalence of antibodies to FMDV at Kamba and Illela border towns in north-western Nigeria

Age	Number of sample		Number of positive sample		Prevalence [%]		Total		
	Kamba	Illela	Kamba	Illela	Kamba	Illela	**Sample**	**Positive**	**Prevalence** [%]
≤ 2 years	296	197	264	67	89.1	22.6	493	331	67.1
>2 years	154	233	95	60	61.7	25.8	387	155	40.1
Total	**450**	**430**	**359**	**127**	**79.9**	**29.5**	**880**	**486**	**55.2**

$P < 0.05$; $\chi 2 = 63.248$

2014. This might be due to inadequate field officers, lack of diagnostic facilities and virtually non-existing quarantine station at the border posts. Highest influx of animals from Niger Republic through the Illela international border was encountered between the month of March and April 2014. These months coincided with the peak of the hot dry season. Animals therefore move southward en-mass for pasture/feed, and may lead to increase in the off-take to reduce the cost of feeding and general maintenance of the herds [9].

The present serological study revealed the presence of circulating antibodies to FMDV at the two international control posts. An overall prevalence of 55.2 % is an indication of a moderately high presence of the virus in the study population. Similar studies conducted by Lazarus et al. [7] in some selected states (Yobe State; 82 %, Plateau; 80 %, Ogun; 77.77 %, Taraba; 73.50 %, Adamawa; 68 %, and Borno; 67 %) in Nigeria has shown consistency in high level of antibodies to the virus in cattle. This poses a threat to international animal trade between Nigeria and neighbouring countries. The high prevalence obtained in this study may be associated with an extensive movement of livestock, high rate of contact between animals at markets while on transit, common grazing places, as well as watering points along cattle routes [1], especially as these study areas are common points of entry for animals coming from Niger, and Benin Republic before onward distribution to other parts of Nigeria. The Kamba border shows a higher prevalence (79.9 %; 359/450) of FMDV as compared with the Illela border (29.5 % 127/430). This partly may be due to sampling ratio and largely due to the fact that the Kamba border serve as the first portal of entry for trade cattle from Niger, Benin Republic, Burkina Fasso and Mali into Nigeria.

Sex specific prevalence indicated that the seropositiv-

ity was higher amongst female 60.5 % (310/512) than male 47.8 % (176/368). There was a significant association between FMDV antibodies and sex. This is in agreement with Olabode et al. [13] who also recorded a higher prevalence of FMDV antibodies in females than in males. This may be attributed to the fact that older females are more likely to have been infected through longer association with other animals [12] as they are kept for longer for breeding purposes. It may also be attributed to immunosuppression associated with pregnancy which may predispose to infections.

The distribution of seropositive FMD carriers according to breed in this study showed that 65.6 % of the Red bororo were positive which indicated the highest occurrence compared to the other breeds of cattle such as White fulani (48.4 %) and Sokoto Gudali (44.4 %). This finding could be associated with the predominance of Red bororo breeds over the other breeds in the study area at the time of sampling.

Sero-positivity of FMD according to age showed that young cattle at the age below 2 years had the higher occurrence (67.1 %) compared to the adult group of more than 2 years (40.1 %) with a significant association. This is in agreement with [5] and [12] who asserted that young cattle were more poised for trade as their heavy availability in the market could be associated with affordability by prospective buyers. This affordability and high turnover may also fast track migration and contact with other FMD exposed cattle that suffered infection from multiple serotypes thus producing antibodies against all serotypes of FMD. Low immunity in young animals can equally increase susceptibility to infectious agent such as the FMD virus [5]

CONCLUSIONS

The magnitude of the presence of FMDV NSP antibodies in the study area is an indication of infection and the presence of the virus in the study area and neighbouring countries. Sex, age and breed are factors associated with FMD infection. Adequate standard laboratories, clinics and quarantine units should be provided in the border posts to enable screening for FMDV infection and other transboundary animal diseases.

ACKNOWLEDGEMENTS

The authors appreciate the cooperation of the Federal Ministry of Livestock and Pest Control Services, Kebbi and Sokoto State offices and their staff at both border posts, laboratory staff of health facilities used as well as staff of Central Research Laboratory of Faculty of Veterinary Medicine, Usmanu Danfodiyo University for their technical supports.

REFERENCES

1. **Abegunde, A. A., Ezeokoli, C. D., Umoh, J. U., Addo, P. B., 1988:** Epidemiology of foot and mouth disease in Nigerian cattle viral diseases of animals in Africa. *OAU/STRC Scientific publication Lagos Nigeria*, 203—212.
2. **De-Lahunta, A., Harbel, R. E., 1986:** *Applied Veterinary Anatomy.* W. B. Sounders Company, 4—16.
3. **Domingo, E., Baranowski, E., Escarmís, C., Sobrino, F., 2002:** Foot-and-mouth disease virus. *Comp. Immunol. Microbiol. Infect. Dis.*, 25, 297—308.
4. **FAO 1997:** *Prevention and Control of Transboundary Animal Disease*. Production and Health Paper. Food and Agricultural Organization of United Nations (FAO/UN), Rome, Italy, 133, 59—60.
5. **Ishola, O. O., Wungak, Y. S., Olugasa, B. O., David, L. D., Ekong, P. S., 2011:** Serological survey of foot and mouth disease in cattle in Jos south Local Government Area of Plateu state. *Vom Journal of Veterinary Science*, 8, 16—21.
6. **Lazarus, D. D, Adamu, S. S., Shamaki, D., Fasina, F. O., 2010:** Foot-and mouth disease virus situation in Nigeria. In *Book of Abstracts EuFMD Week 2010*. European Commission for the Control of Foot-and Mouth Disease, Research Group of the Standing Technical Committee, Vienna-Austria, Sept. 28th—Oct. 1st., 139—140.
7. **Lazarus, D. D., Schienlens, W. J. G., Wungak, Y., Kwange, D., Fasina, F. O., 2012:** Seroepidermiology of foot and mouth disease in some border states of Nigeria. *African Journal of Microbiology Research*, 6, 1756—1761.
8. **National Bureau of Statistics 2007:** *Office Gazette* (FGP 71/52007/2,500 (OL24). Legal notice on publication of the details of the national and state provisional totals 2006 Census. http://www.nigeriastat.gov.ng.Connections/Pop2006.pdf. Accessed on May 30th, 2008.

9. **Ogundipe, G. A. T., 2002:** Role of veterinary quarantine services in monitoring animal movements and disease prevention in Nigeria. *Nigerian Veterinary Journal*, 23, 1—15.
10. **OIE 2004:** *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds, bees)*. 5th edn., OIE, Paris, France, 111—128.
11. **OIE 2009:** Foot-and-Mouth Disease, Chapter 2.1.5. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees)*, Web Edition. Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.05_FMD.pdf. Accessed on 27th June, 2011
12. **Olabode, H. O., Kazeem, H. M., Raji, M. A., Ibrahim, N. D., 2013:** Sero-prevalence of foot and mouth disease virus antibodies in trade cattle (*Bos Indicus*) in Kwara state-Nigeria. *Veterinary World*, 6, 828—832.
13. **Olabode, H. O. K., 2011:** *Molecular Studies of Foot and Mouth Disease Virus in Kwara State, North-Central Nigeria*. PhD. Thesis, Ahmadu Bello University, Zaria, Nigeria, 201 pp.
14. **Thrusfield, M., 2005:** *Veterinary Epidemiology*. 3rd edn., Blackwell Science, Oxford, 7—13.

Received May 18, 2017
Accepted June 30, 2017

DE GRUYTER
OPEN

DOI: 10.1515/fv-2017-0023

FOLIA VETERINARIA, 61, 3: 19—22, 2017

PREVALENCE OF CLAW DISEASES IN DAIRY COWS WITH DIFFERENT BODY CONDITION

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ABSTRACT

The aim of this study was to examine the relationship between body condition and claw diseases in dairy cows. The data used in this study were obtained during two sessions of routine orthopaedic treatment and hoof trimming on a dairy farm in Eastern Slovakia. In the spring and autumn terms, 482 dairy cows were examined for claw diseases and their body conditions were recorded. Out of the 482 dairy cows, 56 % were affected by one or more claw diseases in one or more legs. Digital dermatitis and sole ulcers were the most often detected claw diseases found in this study. The dairy cows with a low body condition score (BCS < 3) showed a higher prevalence of claw diseases (P < 0.05) than the cows with a body condition score greater than 3. However, this difference could not be found when infectious diseases of the claws (digital dermatitis, foot rot, and interdigital dermatitis) were analysed. The results of this study allowed us to conclude that the dairy cows with a lower grade of body condition suffered more from non-infectious claw diseases (sole ulcer, white line diseases).

Key words: body condition score; claw diseases; dairy cows; lameness

INTRODUCTION

In the recent decades, animal welfare has been receiving more and more attention in Europe as well as in other parts of the world. Lameness is one of the most important welfare issues occurring in the intensive dairy industry, and is also a source of substantial economic losses in the dairy industry [16]. Lameness is also considered one of the greatest sources of economic loss in the intensive dairy industry, in Western Europe and North America, second only to infertility and mastitis. While in the developing countries lameness is less important than infectious diseases and malnutrition [3]. The diseases of the claws account for up to 90 % of all bovine lameness cases [8].

When evaluating the cost of each case of lameness, many factors have to be considered, which can be divided into direct and indirect costs. Indirect losses are related to:

prolonged calving interval, culling losses, reduced milk yield, weight loss and replacement costs. The direct losses are considered to be the veterinary expenses and drugs. A quarter of all losses of lameness, are due to the reduced milk yield and the discarded antibiotic-treated milk during the therapy periods. Cows with a higher yield have an increased risk of lameness, and problems with lameness occur primarily in the early lactation period; one to three months postpartum. The fertility problems caused by lameness are the result of:

1. Failure of oestrus detection, because the cows are often unwilling/unable to mount other cows;
2. Anoestrus;
3. Poor body condition at the time of insemination and negative energy balance; and
4. Low grade metritis [15].

In the recent years, more focus has been directed into what role the body condition of cows and lipid mobilisation plays in the development of claw diseases. Research has indicated that body condition scores correlate positively with the thickness of the digital cushion, and that the digital cushion thickness correlates negatively with claw capsule lesions [1].

A fundamental part of controlling bovine claw diseases, as with any other disease, is a comprehensive understanding of the aetiology, risk factors, and pathophysiology of the development of the diseases. The purpose of this study was to examine the relationship between body condition score and claw diseases in dairy cows in order to form a better understanding of the development of claw diseases.

MATERIALS AND METHODS

The data used in this study were obtained during two sessions of routine orthopaedic and claw trimming visits (spring and autumn) to a dairy farm in Eastern Slovakia. In total, 482 dairy cows were included in the study. The cows at the farm were regularly trimmed twice a year and all of them were trimmed and examined at each of the two sessions from which the data were obtained. All of the cows were pure Holstein Friesian breed that were kept on manure solid bedding and fed a total mixed rations (TMR).

The Body condition score (BCS) and the following claw diseases were recorded during the examination: digital dermatitis, interdigital hyperplasia, sole ulcer, toe ulcer, acute/chronic/sub-clinical laminitis, foot rot, double sole, and white line disease [6].

The body condition of the cows was scored on a scale of 1—5 in 0.25 increments [5]. It was based on the examination of the transverse processes of the lumbar vertebrae, the ribs, ischial tuberosity, ligaments of the pelvis and surrounding fat.

The difference in the occurrence of claw diseases in groups with different BCS (3 groups: < 3; 3—4; > 4) was evaluated by the Chi-Square test for all main non-infectious (sole ulcer and white line diseases), and infectious diseases (digital dermatitis, interdigital dermatitis, and foot rot).

RESULTS

Out of the 482 dairy cows examined during spring and autumn terms, 270 (56 %) were affected by one or more claw diseases in one or more legs. The diseases were most predominately seen in the hind legs. The most commonly diagnosed diseases were digital dermatitis (36 %) and sole ulcers (13 %). Only one case of interdigital dermatitis was recorded and no cases of acute laminitis or foot rot were diagnosed.

The group of cows with BCS below 3 had a considerably higher prevalence ($P < 0.05$) of cows affected by claw diseases (66 %). The highest difference ($P < 0.01$) in the prevalence of claw diseases within the BCS groups could be

Table 1. Prevalence of claw diseases in cows with different BCS

Variable	BCS < 3	BCS 3—4	BCS > 4	X^2 test
Number of dairy cows	125 (100 %)	314 (100 %)	43 (100 %)	
All claw diseases	83 (66 %)[a]	165 (53 %)[b]	22 (51 %)[b]	P < 0.05
Non-infectious claw diseases	28 (22 %)[a]	37 (12 %)[b]	1 (2 %)[c]	P < 0.01
Infectious claw diseases	51 (41 %)	112 (36 %)	15 (35 %)	
Other claw diseases	4 (3 %)	16 (5 %)	6 (14 %)	

a, b, c — values with the different superscripts differ at P < 0.05; Non-infectious claw diseases: sole ulcer and white line diseases; Infectious claw diseases: digital dermatitis, interdigital dermatitis, and foot rot

seen for sole ulcer and white line disease (WLD) with the highest prevalence in the lowest BCS group. In contrast, no significant difference could be found in the prevalence of the infectious claw diseases (Table 1).

DISCUSSION AND CONCLUSIONS

Sole ulcers were a lot more frequent and were the second most commonly diagnosed disease in this study after digital dermatitis. Sole ulcers are one of the most common causes of lameness in dairy cows and the most important one [14]. Over the last few years, an extensive change has been in the supposed role of nutritional management in the control of lameness. In the past, the control of subacute ruminal acidosis (SARA) was believed to be a major factor in limiting claw horn lesions because of SARA's suggested role in the development of laminitis [4]. The control of SARA is still believed to be important for maintaining the health and production but it has now been proven to be of low priority in the control of lameness. If the results of recent studies prove to be correct, more focus should be put into minimizing condition loss during early lactation. Cows with low BCS in the periparturient period and early lactation were 3 to 9 times more likely to suffer from lameness than cows with higher BCS [7]. The prevalence of sole ulcers has been significantly associated with digital cushion thickness and BCS is positively correlated with the digital cushion thickness [1]. During early lactation, adipose tissue is mobilized and partitioned toward the mammary gland to support milk production [11]. Research suggests that adipose tissue is not only being mobilized from other parts of the body, such as intra-abdominal fat, subcutaneous fat, and muscle, but also from the digital cushion [1]. Moreover, older cows tend to have lower BCS, lower digital cushion thickness and increased levels of hoof damage [10]. Recently, the association between sole soft tissue thickness and claw lesion incidence was investigated in dairy cows. It could be found that cows which developed sole ulcers had a thinner digital cushion before the lesion occurrence [9]. However, they did not demonstrate a direct link between the decrease in the thickness of back fat and digital cushion after calving. This could possibly be due to a different rate of fat mobilization in the back area and in the claw. A thin digital cushion is believed to have a decreased ability to dampen compression of the corium by the third phalanx. Therefore, a reduction of the number of cows with low BCS at calving and decreasing condition loss in early lactation should most likely be a part of controlling claw horn lesions. The results of this study support these theories.

There was a significant difference in the prevalence of sole ulcers between the three groups of BCS. Only 2 % of the cows with BCS over 4 suffered from this disease, while 22 % of the cows with BCS lower than 3 were affected. As mentioned before, due to the limitation of the data used in this study, it is not possible to know for sure if cows with poor condition are more likely to develop white line disease and sole ulcer, or if cows with these diseases lose their condition as a consequence. To be able to differentiate between cause and effect the cows would have to be examined for these diseases and BCS evaluated on, for example, a weekly or monthly basis. However, some studies have demonstrated no dramatic drop in milk production in cows suffering from light grades of lameness [13]. Therefore, no significant increase in energy need should be expected in no severe forms of sole ulcer.

Digital dermatitis (DD) is an infectious disease and the most commonly found microbe is the bacteria *Treponema* spp. [2]. This infectious disease of cattle claws belongs to the most frequent causes of bovina lameness. Recently, a prevalence of 40 % for digital dermatitis was reported from dairy herds [12] which was similar to the observation in the present study. Known risk factors for cows to develop DD are increased amounts of moisture, urine and faeces in the pathways where the cows walk. A low BCS is not a known risk factor for digital dermatitis and there was no significant difference between the three BCS groups.

In conclusion, the results of this study indicated that there was an association between body condition score and the prevalence of non-infectious claw diseases. The cows with low body condition score are at risk of a higher prevalence of non-infectious claw diseases.

ACKNOWLEDGEMENT

This study was supported by the Slovak Research and Development Agency under the contract No. APVV-0701-11.

REFERENCES

1. **Bicalho, R. C., Machado, V. S., Caixeta, A. L. S., 2009:** Lameness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. *J. Dairy Sci.*, 92, 3175—3184.
2. **Capion, N., Boye, M., Ekstrom, C., Dupont, K., Jensen, T., 2013:** A study into digital dermatitis transmission and bacterial associated pathological changes involved in the disease. *Open Journal of Veterinary Medicine*, 3, 192—198.
3. **Collick, D. W., Ward, W. R., Dobson, H., 1989:** Associations between types of lameness and fertility. *Vet. Rec.*, 125, 103—106.
4. **Danscher, A. M., Toelboell, T. H., Wattle, O., 2010: B**iomechanics and histology of bovine claw suspensory tissue in early acute laminitis. *J. Dairy Sci.*, 93, 53—62.
5. **Edmonson, A. J., Lean, I. J., Weaver, L. D., Farver, T., Webster, G., 1989:** A body condition scoring chart for Holstein cows. *J. Dairy Sci.*, 72, 68—78.
6. **Egger-Danner, C., Nielsen, P., Fiedler, A., Müller, K., Fjeldaas, T., Döpfer, D., et al., 2015:** *ICAR Claw Health Atlas. Rome, ICAR*, 45 pp. http://www.icar.org/Documents/ ICAR_ Claw_Health_Atlas.pdf.
7. **Hoedemaker, M., Prange, D., Gundelach, Y., 2009:** Body condition change ante- and postpartum, health and reproductive performance in German Holstein Cows. *Reprod. Domestic Anim.*, 44, 167—173.
8. **Murray, R. D., Downham, D. Y., Clarkson, M. J., Faull, W. B., Hughes, J. W., Manson, F. J., et al., 1996:** Epidemiology of lameness in dairy cattle: description and analysis of foot lesions. *Vet. Rec.*, 138, 586—591.
9. **Newsome, R. F., Green, M. J., Bell, N. J., Bollard, N. J., Mason, C. S., Whay, H. R., Huxley, J. N., 2017:** A prospective cohort study of digital cushion and corium thickness. Part 1: Associations with body condition, lesion incidence, and proximity to calving. *J. Dairy Sci.*, 100, 4745—4758.
10. **Räber, M., Scheeder, M. R. L., Ossent, P., Lischer, Ch. J., Geyer, H., 2006**: The content and composition of lipids in the digital cushion of the bovine claw with respect to age and location — A preliminary report. *Vet. J.*, 172, 173—177.
11. **Rastani, R. R., Andrew, S. M., Zinn, S., Sniffen, C. J., 2001:** Body composition and estimated tissue energy balance in Jersey and Holstein cows during early lactation. *J. Dairy Sci.*, 84, 1201—1209.
12. **Solano, L., Barkema, H. W., Pickel, C., Orsel, K., 2017:** Effectiveness of a standardized footbath protocol for prevention of digital dermatitis. *J. Dairy Sci.*, 100, 1295—1307.
13. **Solano, L., Barkema, H. W., Pajor, E. A., Mason, S., Leblanc, S. J., Nash, C. G., et al., 2016:** Associations between lying behaviour and lameness in Canadian Holstein-Friesian cows housed in freestall barns. *J. Dairy Sci.*, 99, 2086—2101.
14. **Van Amstel, S. R., Shearer, J. K., 2006:** Review of Pododermatitis circumscripta (ulceration of the sole) in dairy cows. *J. Vet. Internal. Med.*, 20, 805—811.
15. **Weaver, A. D., Guy S. J., Steiner, A., 2007:** *Bovine Surgery and Lameness*. 2nd edn., Wiley-Blackwell Publishing Ltd., Oxford, 290 pp.
16. **Whay, H. R, Main, D. C. J., Webster, A. J. F., Green, L. E., 2003:** Assessment of the welfare of dairy cattle using animal-based measurements: direct observations and investigation of farm records. *Vet. Rec.*, 153, 197—202.

Received June 13, 2017

Accepted July 6, 2017

DE GRUYTER
OPEN

DOI: 10.1515/fv-2017-0024

FOLIA VETERINARIA, 61, 3: 23—31, 2017

THE INFLUENCE OF SUPPLEMENTATION OF FEED WITH *LACTOBACILLUS REUTERI* L2/6 BIOCENOL ON INTESTINAL MICROBIOTA OF CONVENTIONAL MICE

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ABSTRACT

FISH (fluorescence *in situ* hybridization) analysis of the intestinal tract of conventional mice, following 14-day supplementation of feed with host non-specific (porcine) strain *L. reuteri* L2/6, showed in the presence of complex microbiota, a significant increase in the counts of representatives of the genera *Lactobacillus* and *Bifidobacterium*, and a significant decrease in the representatives of the genera *Clostridium*, *Bacteroides* and *Enterobacteriaceae*. At the same time, the supplemented strain stimulated the population of caecal lactobacilli of the species *L. reuteri*. These results demonstrated that the *L. reuteri* L2/6 colonised the jejunum, ileum and caecum and modulated the investigated intestinal microbiota.

Key words: FISH analysis; intestinal microbiota; *Lactobacillus reuteri*; probiotics

INTRODUCTION

Harmonisation of autochthonous microbiota in the critical stages of life of the host is very important for intestinal health and reduction of the risk of infectious diseases. Stimulation of beneficial autochthonous microbiota of the gastrointestinal tract (GIT) by administration of preparations of biotechnological and natural character [3] appears to be one of the ways how to affect microbiocenosis of the intestinal tract and thus increase the resistance of an organism.

The preventive use of probiotics, products of biotechnological origin, has become a part of everyday life due to their generally positive effects on health. On the basis of the results of clinical studies, probiotics have been gradually incorporated also into treatment protocols [6]. Within the research of probiotics, considerable attention has been paid to representatives of the genus Lactobacillus which naturally occur in the GIT and are known for their beneficial effects on health. The assumed probiotic mechanism includes the influence of probiotic micro-organisms on composition, diversity and function of intestinal microbiota by means of

competition for nutrients, production of growth substrates and the modulation of intestinal immunity [24, 29]. It has been shown that diversity within a microbial population is related to its increased ecological stability [9].

The aim of this study was to investigate the influence of supplementation of the strain *Lactobacillus reuteri* on counts of selected microbiota in the jejunum, ileum, caecum and faeces of conventional mice.

MATERIALS AND METHODS

Bacterial strain

Our study was carried out using strain *Lactobacillus reuteri* L2/6 BIOCENOL CCM 8617 isolated from the GIT of pigs. A spontaneous rifampicin-resistant mutant of this strain was isolated by inoculation of a night culture of the strain onto MRS agar (BBL) containing 30 μg.ml^{-1} rifampicin — 10 serial passages (Sigma Chemical Co., Poole, Dorset, United Kingdom).

Experimental animals and administration of additives

The experiment was approved by the State Veterinary and Food Administration of SR under No. 1177/14-221. It was carried out on 40 clinically healthy, 28-day old, BALB/c line mice with a mean weight of 14.86 ± 0.13 grams, obtained from CRL (Charles River Laboratory, Germany). They were kept in an accredited facility with controlled microclimate of the Laboratory of gnotobiology of the University of Veterinary Medicine and Pharmacy in Košice. The animals were divided into four cages (10 mice per cage) that were placed in individual boxes to prevent cross-contamination.

The animals were fed ad libitum complex mixed feed for mice in barrier breeding systems Altromin 1311 (Altromin International, Germany) and had unlimited access to water kept in glass bottles. Cheddar cheese was used as a vehicle for the strain *Lactobacillus reuteri* L2/6 RIF. The strain was added to the milk during typical Cheddar cheese production. The cheese that was used as a control was a similar Cheddar cheese but without the *Lactobacillus reuteri* L2/6 RIF. The experiment consisted of two subsequent stages: stage of adaptation (4 days) and stage of administration of additives in the feed (14 days). During the administration period, 0.1 g per animal per day of 1-month-ripened Cheddar cheese containing *Lactobacillus reuteri* L2/6 RIF (1.2 × 109 CFU/g of cheese) was provided to the experimental group LR (20 mice). In addition, mice of the control group (20 mice) received 0.1 g per animal per day of the control cheese without *Lactobacillus reuteri* L2/6 RIF. The probiotic and control cheese were supplied to the mice once a day (in the morning) in the form of a grated cheese deposited on the surface of their feed. The animals ate the cheese immediately. Before administration of the first dose of cheese, no bacteria resistant to rifampicin were detected in the faeces of the mice.

Sampling and analysis of biological material

Samples of faeces were collected individually from each animal on days 1, 7 and 14 of the supplementation of the additives. After homogenisation of the samples (Stomacher Lab Blender 80), we prepared decimal dilutions in saline and 0.1ml of dilutions 10^{-1} to 10^{-9} were inoculated onto MRS agar containing 30 μg.ml^{-1} of rifampicin. The plates were incubated anaerobically (Gas Pak Plus, BBL) at 37 °C for 72 hours and the counts of *Lactobacillus reuteri* L2/6 RIF were determined (log CFU.g^{-1} faeces). Samples of faeces collected on days 1, 7 and 14 of supplementation of the additives were also processed for determination of the total lactobacilli using the fluorescence *in situ* hybridization (FISH) method [7]. After 14 days of supplementation of the additives, the mice were sacrificed by cervical dislocation after previous administration of sodium pentobarbital (Sigma-Aldrich, 86 mg.kg^{-1}). Immediately after killing, the GIT was removed from their abdominal cavities and 0.5 g samples of the content of each, jejunum, ileum, caecum, and faeces were collected and diluted with 500 μl of PBS solution. After dilution, cold 96 % ethanol was added (1 : 1) and all samples were stored at –20 °C. Counts of selected groups of bacteria were determined by the FISH method. Recalculation of bacterial counts was carried out as follows:

$$H = F \times E/G,$$

where H = bacterial count in one gram of faeces; F = mean bacterial count in one viewing field; E = number of viewing fields per total area of a filter; G = dilution factor.

FISH method

The basic FISH protocol designed by Czerwińsky et al. [7] was adjusted to our needs. The first step consisted of the fixation of the samples using 96 % ethanol (1:1). After fixation, decimal dilutions were prepared to obtain a concentration of 10^{-2}. In order to improve the adherence

of the bacteria to a polycarbonate filter, we applied 1 ml of 50 % Poly-L-lysine to the filter, allowed it to adhere for 3—5 min, and then remove it by a vacuum. From the diluted sample, a 100 µl aliquot was applied to the filter by means of a vacuum and the filter with the adhered bacteria was allowed to air dry. The filter was then dehydrated through ascending ethanol in steps of 50 %, 80 % and 90 %; each step lasting 3 min. After dehydration, the filter was allowed again to air dry for 3—5 min. The filter was then exposed to a fresh enzymatic solution (25 mM Tris pH 7.5; 10 mM EDTA pH 7.5; 585 mM saccharose; 5 mM $CaCl_2$; 2 mg.ml^{-1} lysozyme; 0.3 mg.ml^{-1} Na-taurocholate; 0.1 mg.ml^{-1} lipase) in a thermostat at 37 °C for 30 min to increase the permeability of the cellular wall. After the action of the enzymatic solution, the filter was again washed in a PBS solution and dehydrated with 50 %—80 %—96 % ethanol solutions for 3 min. Subsequently, the hybridization of the sample was performed overnight on a wells-containing slide in a thermostat: to one well we added 50 µl WB1 — hybridization buffer/washing solution (5 M NaCl; 1 M Tris pH 7.0; 10 % SDS) and relevant fluorescence-labeled probe (Sigma Aldrich) in a concentration of 100 mM and a volume of 2 µl. Hybridization temperatures were as follows: 50 °C for probes Lab 158 and Bif 164; 52 °C for probes Entbac and Bac 303; 54 °C for probes Chis 150 and Lbre. Characteristics of the probes used in the study are presented in Table 1.

Table 1. Characteristics of the probes used in the study

Probe	Sequence (5′→3′)	Microorganism/ Reference
Chis 150	TTATGCGGTATTAATCTYCCTTT	*Clostridium* sp., *C. cluster, C.tyrobutyricum, C.histolyticum* Franks et al. [10]
Lab 158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* sp./ *Enterococcus* sp. Harmsen et al. [15]
Bif 164	CATCCGGCATTACCACCC	*Bifidobacterium* sp. Langedijk et al. [19]
Bac 303	CCAATGTGGGGGACCTT	*Bacteroides, Prevotella* Apostolou et al. [1]
Entbac	CATGAATCACAAAGTGGTAAGCGC	*Enterobacteriaceae* Jansen et al. [18]
Lbre	ATCCATCGTCAATCAGG	*Lactobacillus reuteri* Quevedo et al. [25]

After hybridization, the filters were washed in PBS solution to remove unbound probe remnants. The samples were then washed in WB1 (washing solution) at 50 °C for 20 min and in PBS solution at 22 °C for 30 seconds, and incubated with 50 µl DAPI (6.3 µl.ml^{-1}) at 22 °C for 10 min to improve visualization and resolution of total bacteria. The filters were then washed in PBS solution and subsequently in 50 µl WB2 (washing solution) (5 M NaCl, 1 M Tris, H_2O), and incubated at 50 °C for 20 min, washed in PBS and allowed to dry at 22 °C for 10 min. Three drops of Vectashield solution (Vector Laboratories) were applied to a slide, covered by the filter and a cover slide was placed on the top. The bubbles were expelled and the slide was sealed and labelled.

Microscopic slides were evaluated at various spectres using a fluorescence microscope Carl Zeiss Axio Observer Z1 and software Axio Vision. Rel 4.8. A filter 38H with excitation 470 nm and emission of 525—550 nm (green colouring) was used for the slides labelled with 6-FAM (6-Carboxyfluorescein), while for the slides labelled with fluorochrome, Texas red (sulphorodamine 101 acid chloride) a filter 64HE with excitation 587 nm and emission 647—670 nm (red colouring) was used. The detection of the total bacteria was accomplished by means of DAPI (4',6-diamidino-2-phenylindole), using filter 49 with excitation 365 nm and emission 445–450 nm (blue colouring).

Statistical evaluation

The results are presented as means ± SD. The data were evaluated by GraphPad Prism 3.00 software using the unpaired t-test.

RESULTS

By day 14, a significant increase in counts of *Lactobacillus* Lab 158 ($P < 0.001$) and a significant decrease in counts of *Enterobacteriaceae* Entbac ($P < 0.001$; $P < 0.01$) were observed in the, jejunum, ileum, and faeces of mice from LR group in comparison with the control group (Figs. 1—3).

Similarly, in the caecum (Fig. 4) of the mice from the LR group, we observed a significant increase in *Lactobacillus* Lab 158 ($P < 0.01$) and a significant decrease in *Enterobacteriaceae* Entbac ($P < 0.001$) in comparison with the control mice. Moreover, in the caecum we could detect changes also in other investigated groups of bacteria in compari-

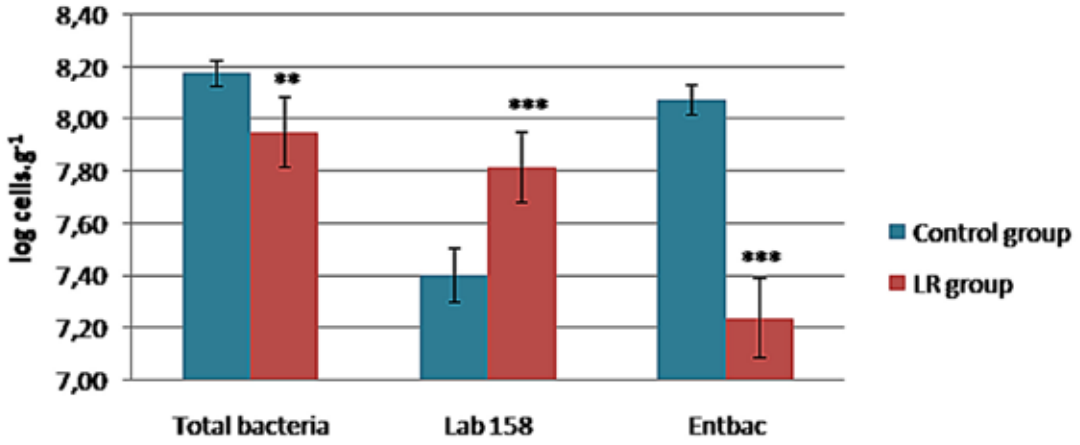

Fig. 1. Microbiological analysis of the jejunum of mice after 14-day supplementation of feed with *L. reuteri* L2/6 RIF
** P < 0.01; *** P < 0.001

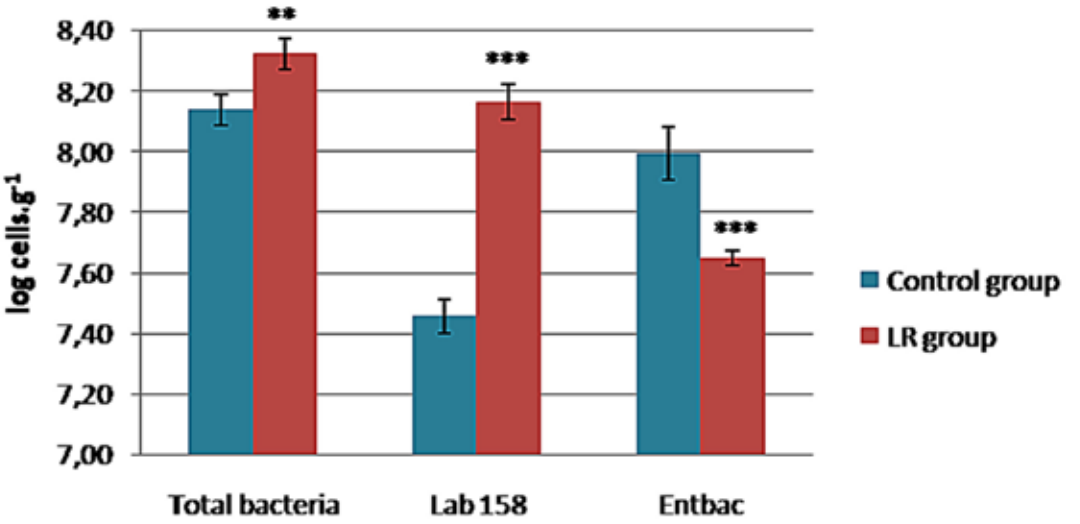

Fig. 2. Microbiological analysis of the ileum of mice after 14-day supplementation of feed with *L. reuteri* L2/6 RIF
** P < 0.01; *** P < 0.001

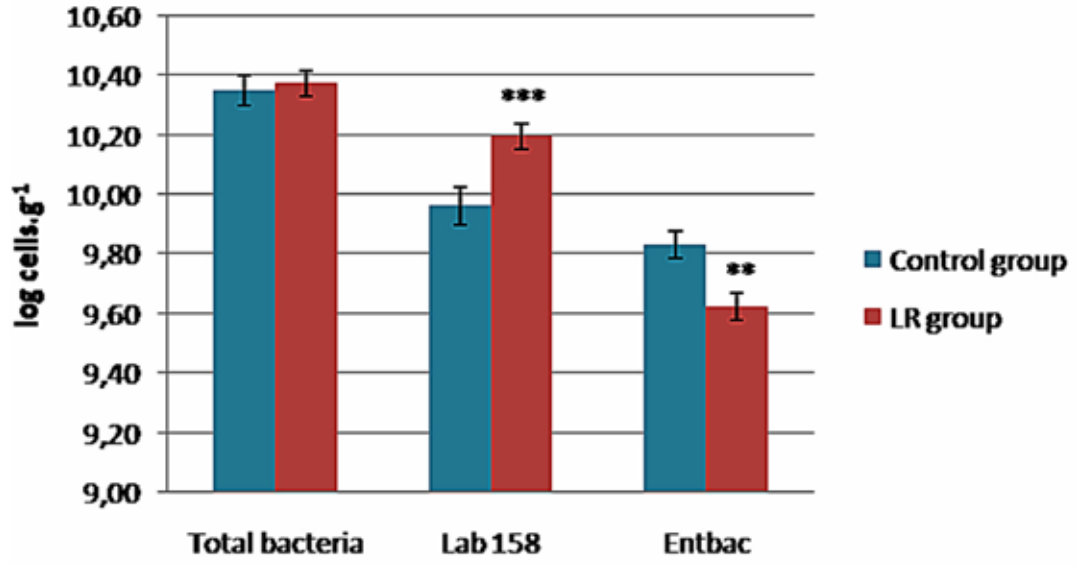

Fig. 3. Microbiological analysis of faeces of mice after 14-day supplementation of feed with *L. reuteri* L2/6 RIF
** P < 0.01; *** P < 0.001

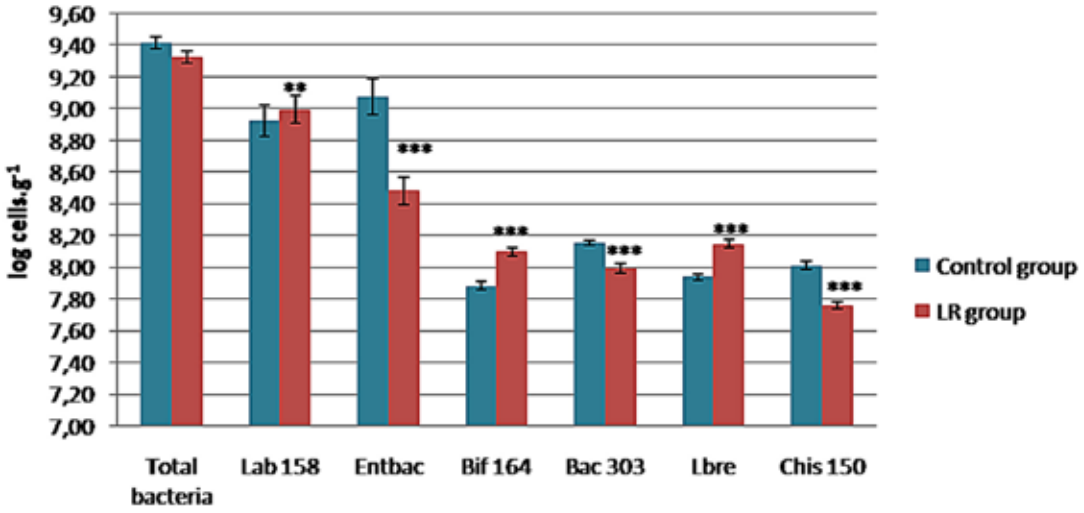

Fig. 4. Microbiological analysis of the caecum of mice after 14-day supplementation of *L. reuteri* L2/6 RIF
** P < 0.01; *** P < 0.001

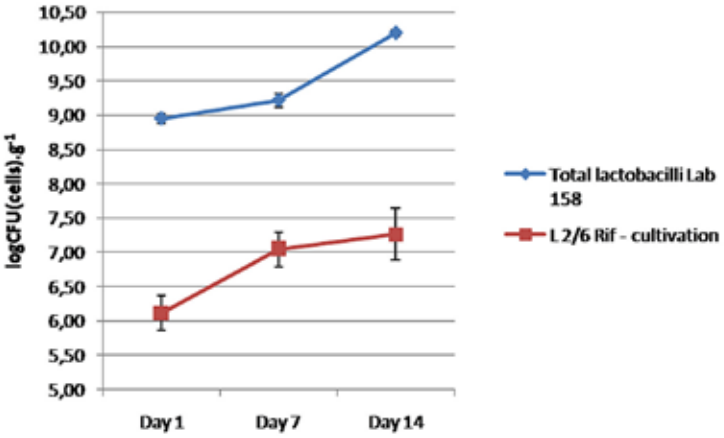

Fig. 5. Counts of *Lactobacillus reuteri* L2/6 RIF (log CFU.g^{-1}) in faeces of mice from the LR group during supplementation of feed, determined by the cultivation method in comparison with counts of the total lactobacilli (FISH, log cells. g^{-1})

son with the controls. In the caecum of the LR group, the counts of *Bifidobacterium* Bif 164 were significantly increased (P < 0.001) and the counts of Clostridium Chis 150 and Bacteroides Bac 303 were significantly decreased (P < 0.001) in comparison with the controls. The counts of the species *L. reuteri* Lbre were also positively affected (P < 0.001) in the group supplemented with *Lactobacillus reuteri* L2/6. The influence on the total bacterial count was more pronounced in the first two segments of the small intestine, the jejunum and ileum (P < 0.01; P < 0.01), in the LR group in comparison with the controls.

The counts of *Lactobacillus reuteri* L2/6 RIF in the faeces of the LR group during supplementation, determined by the cultivation method, showed an increasing tendency (Fig. 5). The counts of *Lactobacillus reuteri* L2/6 RIF, determined by the cultivation method in individual segments of the GIT of the LR group after 14-day supplementation of the strain were as follows: jejunum 6.55 ± 0.23 log CFU.g^{-1}; ileum 7.02 ± 0.25 log CFU.g^{-1}; caecum 7.20 ± 0.26 log CFU.g^{-1}; faeces 7.26 ± 0.38 log CFU.g^{-1}.

DISCUSSION AND CONCLUSIONS

Autochthonous physiological microbial flora of the GIT is considered one of the natural protective mechanisms of a macro-organisms. From the point of view of colonisation resistance, it helps for the host to resist the colonisation of

the GIT by pathogenic bacteria. Disturbance of its function results in insufficient protection against infectious agents [16]. One of the mechanisms by which probiotic bacteria may support colonisation resistance of natural microbiota is the production of antibacterial substances and an increase in the density and diversity of the beneficial components of the intestinal microbiota [2].

Supplementation of the porcine strains *L. reuteri* BSA131 and *L. fermentum* I5007, reduced the counts of potentially pathogenic *E. coli* and *clostridia* in the intestine of newborn piglets [4, 21]. Similarly, it was observed that a mixture of lactobacilli isolated from the GIT of piglets (*Lactobacillus gasseri*, *L. reuteri*, *L. acidophilus*, *L. fermentum*, *L. johnsonii* and *L. mucosae*) increased the counts of lactobacilli and bifidobacteria and reduced the counts of *E. coli* and anaerobic bacteria in the jejunum, ileum, caecum and colon of weaned piglets [5, 17]. It is well known that lactobacilli exhibit inhibitory activity against *E. coli* and enterobacteria-mediated production of organic acids, with a subsequent decrease in the pH and the production of H_2O_2 and lactoferin [20]. Increased concentrations of lactic and acetic acids and a related decrease in the pH in the ileum and colon following the administration of lactic bacteria were also confirmed in *in vivo* experiments on weaned piglets [14, 30]. Fuentes et al. [13] conducted a study that focused on the influence of the composition of microbiota in mice and observed that the administration of *L. casei* and *L. plantarum*, originating from milk products, resulted in changes in the proportions of lactobacilli (*L. helveticus*, *L. johnsonii* and *L. reuteri* predominated) in the faeces and individual segments of the intestine; however, the structure and counts of other representatives of microbiota were not affected, which indicated a different effectiveness of individual strains or species of lactobacilli. Preidis et al. [23] experimentally confirmed the transient increase in the phylogenetic diversity of representatives of the faecal microbiome of mice 24 hours after a single administration of *L. reuteri*. The above mentioned studies indicated that the administration of various species of lactobacilli may modulate intestinal microbiota profile and thus affect production of microbial metabolites that may improve intestinal health.

Our study investigated the influence of host non-specific strain *Lactobacillus reuteri* L2/6 isolated from the GIT of pigs. In previous *in vitro* studies, this strain demonstrated inhibitory activity against potential bacterial pathogens, and produced reuterin and lactic and acetic acids. It was capable of forming biofilms on abiotic surfaces. It also produced capsular exopolysaccharides and exhibited higher tolerance to both gastric and intestinal juices [26]. In our previous experiment [27] we observed the presence of a biofilm in the stomach, duodenum and caecum of germ-free mice following exogenous administration of this strain. The hybridization with a specific probe (Lab158) showed that the strain *L. reuteri* L2/6 formed a solid biofilm on a stratified squamous epithelium of the mice forestomach. In the duodenum and caecum, this strain occurred in the form of cells interspersed in mucus covering the mucous membrane, or in the form of small micro-colonies. The strain was capable of colonizing in high numbers individual parts of the intestinal lumen of germ-free mice.

Our microbiological analysis of the intestinal tract of mice after the 14-day supplementation of host non-specific strain *L. reuteri* L2/6 in the presence of complex microbiota, revealed that this strain was able of colonising the intestinal lumen and affect the counts of selected intestinal bacteria in the GIT of conventional mice. The FISH analysis of the caecal samples showed a significant increase in the counts of the genera *Lactobacillus* and *Bifidobacterium* and a significant reduction in the counts of the genera *Clostridium*, *Bacteroides*, and *representatives of the family Enterobacteriaceae*. At the same time, we were able to observe a positive effect on bacteria of *L. reuteri* species.

L. reuteri is an intestinal symbiont colonizing stratified squamous epithelium in the frontal part of the animal intestinal tract and is a stable part of the microbiota of the large intestine in humans [31]. *L. reuteri* is also a stable representative of sourdough used for making sourdough pastries [8]. Comparative genomics have revealed that the evolution of *L. reuteri* resulted in the development of host limited phylogenetic lines specialising in specific hosts [22]. The analyses indicated principally a different genomic evolution of the rat isolate *L. reuteri* 100-23 and human isolated *L. reuteri* F275 [11].

An extensive study conducted by Frese et al. [12] investigated the mechanisms implicated in the colonisation and formation of biofilms in specific strains of intestinal representatives of *L. reuteri*. Confocal microscopy showed adherence and formation of biofilms by rat strains on the forestomach epithelium of *Lactobacillus*-free mice, which was not observed in case of *L. reuteri* strains originating from other hosts, such as poultry, pigs or man. Contrary to

previous observations, strains originating from other hosts, except for rats, were capable of colonising the intestinal lumen of germ-free mice in high numbers in the absence of competitive microbiota [11]. Despite this colonisation, these strains were unable to adhere to forestomach epithelium and form biofilms. Frese et al. [12] reported that in a rat strain *L. reuteri* 100-23, a surface adhesin Lr70902, the so-called Fap1-like protein, played a principal role in the primary adhesion to the forestomach epithelium and thus was implicated in host specificity. Homologues of protein Lr70902 were found in *L. reuteri* isolated from rats and pigs and could play a key role in the exclusive binding to the epithelium of the relevant hosts. The authors theorized that a weak similarity of sequences of rat and pig strain's proteins can explain the observed host specificity.

Su et al. [28] reported that *L. reuteri* isolated from sourdough originated from the same phylogenetic line as the rat strains. Previous investigations showed that rat isolates of *L. reuteri* were able to persist for long periods of time in fermented food and *L. reuteri* LTH5448, isolated from sourdough, colonised the GIT of *Lactobacillus*-free mice and produced populations comparable with that produced by the rat strain *L. reuteri* 100-23 [32]. Genome hybridization showed that the genome composition of *L. reuteri* LTH5448 isolated from sourdough was very similar to the rat isolate *L. reuteri* 100-23. As *Lactobacillus*-free mice were effectively colonised only by *L. reuteri* strains originating from rats, the above observations provide obvious proof of intestinal (rat) origin of this isolate from sourdough. Transcription of proteins that ensure the competitiveness of *L. reuteri* in cereal fermentations occurred with high intensity also in biofilms, which supports the suggested model of a common intestinal origin of these isolates. With regard to the similarity of these two biotopes (i.e. availability of carbon source), *L. reuteri* may be capable of colonising two environmental niche — frontal segment of the intestine of cereals-consuming mammals and sourdough [28]. These observations stress the importance of bacterial adaptation to life in a certain type of habitat (biotope), for example when selecting beneficial strains for probiotic purposes.

The FISH analysis of the GIT of conventional mice showed that strain *L. reuteri* 2/6 was able to colonise their intestine in the presence of competitive microbiota. The supplemented strain stimulated the population of caecal lactobacilli of *L. reuteri* species and modulated the investigated intestinal microbiota. The above mentioned literary data allowed us to hypothesise that the tested porcine strain *L. reuteri* L2/6 can originate from cereals and shows characteristic features of the rat strain. However, additional genomic research of host specificity of this strain is needed to confirm this hypothesis.

ACKNOWLEDGEMENTS

This study was supported by the operation programme Research and Development, within the project: "INFEKTZOON — Centre of excellence for infections of animals and zoonoses", ITMS code: 26220120002, co-financed from resources of European fund for regional development.

REFERENCES

1. **Apostolou, E., Pelto, L., Kirjavainen, P. V., Isolauri, E., Salminen, S. J., Gibson, G. R., 2001:** Differences in the gut bacterial flora of healthy and milk-hypersensitive adults, as measured by fluorescence *in situ* hybridization. *FEMS Immunol. Med. Microbiol.*, 30, 217—221.
2. **Bermudez-Brito, M., Plaza-Díaz J., Muńoz-Quezada, S., Gómez-Llorente, C., Gil, A., 2012**: Probiotic mechanisms of action. *Ann. Nutr. Metab.*, 61, 160—174.
3. **Bomba, A., Nemcová, R., Gancarčíková, S., Herich, R., Guba, P., Mudroňová, D., 2002:** Improvement of the probiotic effect of microorganisms by their combination with maltodextrins, fructo-oligosacharides and polyunsatured fatty acids. *Br. J. Nutr.*, 88, 95—99.
4. **Chang, Y. H., Kim, J. K., Kim, H. J., Kim, W. Y., Kim, Y. B., Park, Y. H., 2001:** Selection of a potential probiotic *Lactobacillus* strain and subsequent *in vivo* studies. *Antonie Van Leeuwenhoek*, 80, 193—199.
5. **Chiang, M. L., Chen, H. C., Chen, K. N., Lin, Y. C., Lin, Y. T., Chen, M. J., 2015:** Optimizing production of two potential probiotic lactobacilli strains isolated from piglet faeces as feed additives for weaned piglets. *Asian Australas. J. Anim. Sci.*, 28, 1163—1170.
6. **Corcionivoschi, N., Drinceanu, D., Pop, I. M., Stack, D., Stef, L., Julean, C., Bourke, B., 2010:** The effect of probiotics on animal health. *Anim. Sci. Biotechnol.*, 43, 35—41.
7. **Czerwiński, J., Højberg, O., Smulikowska, S., Engberg, R. M., Mieczkowska, A., 2012:** Effects of sodium butyrate and salinomycin upon intestinal microbiota, mucosal mor-

phology and performance of broiler chickens. *Arch. Anim. Nutr.*, 66, 102—116.

8. **De Vuyst, L., Vrancken, G., Ravyts, F., Rimaux, F., Weckx, S., 2009:** Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. *Food Microbiol.*, 26, 666—675.
9. **Eisenhauer, N., Scheu, S., Jousset, A., 2012:** Bacterial diversity stabilizes community productivity. PLoS One. 7, e34517. doi: 10.1371/journal.pone.0034517.
10. **Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F., Welling, G. W., 1998:** Variations of bacterial populations in human faeces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microb.*, 64, 3336—3345.
11. **Frese, S. A., Benson, A. K., Tannock, G. W., Loach, D. M., Kim, J., Zhang, M., et al., 2011:** The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. *PLoS Genet.*, 7 (2. e1001314. doi: 10.1371/journal.pgen. 1001314.
12. **Frese, S. A., MacKenzie, D. A., Peterson, D. A., Schmaltz, R., Fangman, T., Zhou, Y., et al., 2013:** Molecular characterization of host-specific biofilm formation in a vertebrate gut symbiont. *PLoS Genet.*, 9, 1—13.
13. **Fuentes, S., Egert, M., Jiménez-Valera, M., Ramos-Cormenzana, A., Ruiz-Bravo, A., Smidt, H., Monteoliva-Sanchez, M., 2008:** Administration of *Lactobacillus casei* and *Lactobacillus plantarum* affects the diversity of murine intestinal lactobacilli, but not the overall bacterial community structure. *Res. Microbiol.*, 159, 237—243.
14. **Giang, H. H., Viet, T. Q., Ogle, B., Lindberg, J. E., 2010:** Growth performance digestibility gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. *Livest. Sci.*, 129, 95—103.
15. **Harmsen, H. J. M., Elfferich, P., Schut, F., Welling, G. W., 1999:** A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization. *Microbiol. Ecol. Health Dis.*, 11, 3—12.
16. **Hou, Ch., Zeng, X., Yang, F., Liu, H., Qiao, S., 2015:** Study and use of the probiotic *Lactobacillus reuteri* in pigs: a review. *Journal of Animal Science and Biotechnology*, 6:14 doi 10.1186/s40104-015-0014-3.
17. **Huang, C. H., Qiao, S. Y., Li, D. F., Piao, X. S., Ren, J. P., 2004:** Effects of *Lactobacilli* on the performance diarrhoea incidence VFA concentration and gastrointestinal microbial flora of weaning pigs. *Asian Aust. J. Anim. Sci.*, 17, 401—409.
18. **Jansen, G. J., Mooibroek, M., Idema, J., Harmsen, H. J., Welling, G. W., Degener, J. E., 2000:** Rapid identification of bacteria in blood cultures by using fluorescently labelled oligonucleotide probes. *J. Clin. Microbiol.*, 38, 814—817.
19. **Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. F., Welling, G. W., 1995:** Quantitative *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA targeted probes and its application in faecal samples. *Appl. Environ. Microbiol.*, 61, 3069—3075.
20. **Li, D., Ni, K., Pang, H., Wang, Y., Cai, Y., Jin, Q., 2015:** Identification and antimicrobial activity detection of lactic acid bacteria isolated from corn stover silage. *Asian Australas. J. Anim. Sci.*, 28, 620—631.
21. **Liu, H., Zhang, J., Zhang, S. H., Yang, F. J., Thacker, P. A., Zhang, G. L., et al., 2014:** Oral administration of *Lactobacillus fermentum* I5007 favours intestinal development and alters the intestinal microbiota in formula-fed piglets. *J. Agric. Food. Chem.*, 62, 860—866.
22. **Oh, P. L., Benson, A. K., Peterson, D. A., Patil, P. B., Moriyama, E. N., Roos, S., Walter, J., 2010:** Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. *ISME J.*, 4, 377—387.
23. **Preidis, G., Saulnier, D., Blutt, S., Mistretta, T., Riehle, K., Major, A., et al., 2012:** Probiotics stimulate enterocyte migration and microbial diversity in the neonatal mouse intestine. *FASEB J.*, 26, 1960—1969.
24. **Preidis, G., Versalovic, J., 2009:** Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterol.*, 136, 2015—2031.
25. **Quevedo, B., Giertsen, E., Zijnge, V., Lüthi-Schaller, H., Guggenheim, B., Thurnheer, T., Gmür, R., 2011:** Phylogenetic group- and species-specific oligonucleotide probes for single-cell detection of lactic acid bacteria in oral biofilms. *BMC Microbiol.*, 11:14. doi: 10.1186/1471-2180-11-4.
26. **Ryznerová, D., 2013:** *The Study of Properties of Probiotic Bacteria from the Point of View of their Biological Effects and Application* (In Slovak). Dissertation thesis, University of Veterinary Medicine and Pharmacy, Košice, Slovakia, 146 pp.
27. **Slížová, M., Nemcová, R., Maďar, M., Hádryová, J., Gancarčíková, S., Popper, M., Pistl, J., 2015:** Analysis of biofilm formation by intestinal lactobacilli. *Can. J. Microbiol.*, 61, 437—446.
28. **Su, M. S., Phaik, L. O., Walter, J., Gänzle, M. G., 2012:** Phylogenetic, genetic, and physiological analysis of sourdough isolates of *Lactobacillus reuteri*: food fermenting strains of

intestinal origin. *Appl. Environ. Microbiol.*, 78, 6777—6780.

29. **Thomas, C., Versalovic, J., 2010:** Probiotics-host communication: modulation of signalling pathways in the intestine. *Gut Microbes*, 1, 148—163.
30. **Thu, T. V., Loh, T. C., Foo, H. L., Yaakub, H., Bejo, M. H., 2011:** Effects of liquid metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faeces characteristics, intestinal morphology and diarrhoea incidence in post weaning piglets. *Trop. Anim. Health. Prod.*, 43, 69—75.
31. **Walter, J., 2008a:** Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. *Appl. Environ. Microbiol.*, 74, 4985—4996.
32. **Walter, J., Schwab, C., Loach, D. M., Gänzle, M. G., Tannock, G. W., 2008b:** Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, *in vitro* biofilm formation, and colonization of the mouse gastrointestinal tract. *Microbiol.*, 154, 72—80.

Received June 5, 2017
Accepted July 11, 2017

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DOI: 10.1515/fv-2017-0025

FOLIA VETERINARIA, 61, 3: 32—46, 2017

ORGANIC COLOURING AGENTS IN THE PHARMACEUTICAL INDUSTRY

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ABSTRACT

Food dyes are largely used in the process of manufacturing pharmaceutical products. The aim of such a procedure is not only to increase the attractiveness of products, but also to help patients distinguish between pharmaceuticals. Various dyes, especially organic colouring agents, may in some cases have a negative impact on the human body. They are incorporated into pharmaceutical products including tablets, hard gelatine capsules or soft gelatine capsules, lozenges, syrups, etc. This article provides an overview of the most widely used colouring agents in pharmaceuticals, their characteristics and the EU legislation which regulates their use.

Key words: analysis; food dye; legislation; pharmaceutical product; potential health risk

INTRODUCTION

In addition to the active ingredients, various additives are used in the manufacture of pharmaceuticals. This group of compounds includes dyes. A colour additive is any dye, pigment, or other substance that imparts colour to food, drink or any non-food applications including pharmaceuticals. Moreover, a colour additive is also any chemical compound that reacts with another substance and causes the formation of a colour [22, 56]. The pharmaceutical industry employs various inorganic and, especially, organic dyes for this purpose. These colour additives are pigments of natural origin or synthetic chemical compounds. However, most of the dyes obtained from natural sources are unstable and can easily undergo degradation during the processing of pharmaceutical products. Therefore, dyes of synthetic origin are widely used, not only because of their stability, but also given the low cost of production when compared to natural dyes [66].

The priority of the dye is to increase the aesthetic appearance of the pharmaceutical products. It can be said that

the dyes are the cosmetics for the pharmaceutical products [2]. Dyes are added to nutritional supplements and pharmaceutical products for commercial, psychological and practical purposes. A variety of coloured tablets, capsules (hard gelatin, soft gelatin), syrups and multivitamin supplements are attracted by children precisely because of their appearance. Colouring of pharmaceuticals improves their easy identification already at the first sight. Various colours of drugs also help distinguish different strengths of the same drug, which can eliminate faults of an over dose or under dose. Synthetic dyes are organic substances that may interact with the human body. These interactions are a potential health risk, especially when they are consumed excessively [51]. There have been various studies focused on neurotoxicity [54], genotoxicity [53] and carcinogenicity [41] of synthetic dyes. The relationship between dyes and the children´s attention deficit disorder (ADD), attention-deficit hyperactivity disorder (ADHD), immune system problems, and certain allergic reactions have also been examined. Due to the fact that the results of various researches have been ambiguous, nowadays, a great deal of attention is paid to study the effects of artificial dyes on human health.

EUROPEAN UNION LEGISLATION ON DYES

Dyes belong to one of the most important classes of pharmaceutical excipients that can affect the health of certain population groups. Therefore, the basic purpose of any laws related to drugs and substances must be the safety of public health. To ensure this task various measures are taken. They protect the health without hindering the development of the pharmaceutical industry and trade with pharmaceuticals. Practical experience so far has shown that in terms of health, there is no reason to disallow the use of foodstuff dyes also in pharmaceutical products.

For this reason, the basic legislation determining the use of dyes in food have in the past, but also currently, overlapped with the legislation on pharmaceutical products and medicines. The first collection of international food standards and guidelines called Codex Alimentarius (CA) was issued in 1962 by the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) [11]. The Codex Committee on Food Additives and Contaminants (CCFAC) operates as part of the CA [35]. The Committee focuses on food additives and contaminants, and implements recommendations for the maximum permitted limits for the use of individual food additives. Codex Alimentarius and WHO/FAO have produced a database in which evidence of the biological activity of food additives are collected, the so called General Standards for Food Additives (GSFA).

Dyes are included as a specific part in the group of additives, because as early as in the Council Directive of 23 October 1962 [17], a list was established of colouring materials authorized for use in foodstuffs intended for human consumption. The basic rule focused on the approximation of the laws of the Member States relating to the colouring materials which may be added to medicinal products under Council Directive 78/25/EEC of 12 December 1977 [18]. In Article 1 of this Directive, it is stated that Member States shall not authorize, for the colouring of medicinal products for human and veterinary use, any colouring materials than those covered by Annex I, Sections I and II, to the Directive of 1962, as subsequently amended. The Directive joined the applicable requirements to food dyes with the requirements applicable to pharmaceutical products. The document, however, did not contain any specific references to earlier legislation that was subsequently cancelled. On the 30th of June 1994, the European Parliament and Council Directive 94/36/EC adopted the use of dyes in food [19], outlining a single list of colouring agents to be used in foodstuffs. The specific purity criteria concerning dyes for the use in foodstuffs were laid down in the Directive 95/45/EC of 26 July 1995 [13]. It can be said that this document essentially replaced the provisions of the Directive of 1962. Directive 2009/35/EC of the European Parliament and of the Council of 23 April 2009 on the colouring materials which may be added to medicinal products [20]. It states that the primary purpose of any laws concerning medicinal products must be to safeguard the public health. In the introduction of the Directive, in point No. 5, it is mentioned that experience has shown that there is no reason, on health grounds, why the colouring materials authorised for use in foodstuffs should not also be authorised for use in medicinal products. Therefore, Annex I to Directive 94/36/EC, as well as the Annex to Commission Directive 95/45/EC of 26 July 1995, should also apply to medicinal products. In Article 1 of that document, it states that Member States shall not authorize, for the colouring of medicinal products for human and veterinary use, any colouring materials, those covered

by Annex I to the Directive 94/36/EC. Legislation relating to the dyes change over time, evolves and adapts.

Based on new research and studies, the appropriateness of the use of certain dyes in pharmaceutical products and their maximum levels are always reviewed. For example, the Scientific Committee on Medicinal Products and Medical Devices adopted an opinion on the suitability and safety of Amaranth, Erythrosine, Canthaxanthin, aluminum and silver as the dyes for medicinal products [61]. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 [21] laid down the general principles and requirements of food law, established the European Food Safety Authority and laid down procedures in matters of food safety. In compliance with this Regulation, additives used in the EU must be first reviewed by the European Food Safety Authority (EFSA), in accordance with the European Commission. EFSA conclusions are based on the recommendations of the Codex Committee on Food Additives and Contaminants (CCFAC). The EFSA publishes, what is officially known as "Scientific Opinion" in the EFSA Journal, on request from the European Commission. The content of the EFSA Scientific Opinion comprises these subject-matters: technical and chemical characteristics of the dye; description of the manufacturing process; analytical methods used for the determination; chemical reactivity with food; summary of the current authorization for use; dose range, toxicokinetic information; toxicological data; carcinogenicity, genotoxicity, developmental and reproductive toxicity; and hypersensitivity to the substance. Approval of the use of these additives depends on the level of scientific knowledge at the time. Therefore, it is necessary to carry out regular reviews and revisions of legislation with respect to new scientific information in evaluating the conditions of a particular use of additives. In 2008, the European Parliament and Council published Regulation (EC) No 1333/2008 on food additives [14]. In this document, it states that food additives which were permitted before 20 January 2009 shall be subject to a new risk assessment carried out by the EFSA. The commission Regulation (EU) No 257/2010 of 25 March 2010 [15] set up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. Considering the above, we can conclude that in practice it is very important to monitor the development of legislation particularly in the field of using the synthetic organic dyes. These rules reflect the new findings on the impact of additive substances on the human body and so help to protect human health.

Given the fact that the European Parliament is well-aware and informed of possible children's health issues linked to the consumption of synthetic dyes, it compiled a bordereau of food dyes allowed to be used in processed food, yet requiring a warning indication in the form of a label on food packaging. Despite this scientific advice, since July 2010, the Annex V of the Regulation (EC) No. 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives, provides a list of food dyes (E110, E104, E122, E129, E102, E124) which must provide labels with the following information for target users: may have an adverse effect on activity and attention in children [32].

The toxicological effects of synthetic dyes are concentration-dependent [44]. Given their toxicity, mostly when consumed in excess, synthetic dyes are strictly governed by applicable legislation, regulations and acceptable daily intake (ADI) values for pharmaceuticals safety. The ADI values and eventual health effects on humans for the most commonly used pharmaceutical dyes are listed in Table 1 [16]. The ADI values are listed in units of mg per kg of body weight.

THE DYES MOST FREQUENTLY USED IN PHARMACEUTICAL INDUSTRY

Every product intended for a medical purpose is an elaborate system of active substances and excipients. In recent time, by virtue of the accelerated growth of the eagerness to make commercially attractive products in the pharmaceutical industry, the human body assembles more and more synthetic molecules. In the composition of medicines to the organisms, arrive excipients such as dyes, among which synthetic dyes are prevalent nowadays. Aspects that affect colour stability include: light, oxidising and reducing agents, heat, pH, microbial contamination, and solubility limits. The synthetic dyes have important technological superiority when being compared to most natural dyes given the fact that: they give vivid colours, they are resistant to light, oxidants and reducing agents impact, to pH changes and they are also less susceptible to various types of impacts that a material is subject to during the technological process.

Table 1. Acceptable daily intake values and potential health effects on humans for the most commonly used pharmaceutical dyes

Pharmaceutical dyes	ADI [mg.kg bw^{-1} day^{-1}]	Potential health effects on humans
E102, Tartrazine	7.5	Tartrazine appears to cause the highest percentage of allergic and intolerance reactions of all the azo dyes, particularly among asthmatics and those with an aspirin intolerance. The use of Tartrazine in susceptible patients has been reported to lead to allergic or adverse reactions, such as urticaria, eczema, asthma and hyperactive behaviour. Reported cases of hypersensitivity, and hyperkinetic activity in children [23, 34, 60].
E104, Quinoline Yellow	0.5	A Quinoline Yellow allergy is an adverse reaction by the body's immune system to a yellow food colouring called Quinoline Yellow which is used in a number of pharmaceuticals. The specific symptoms can vary considerably amongst patients and may range from mild to severe. For this dye, there was some evidence of carcinogenic activity and genotoxicity. E104 causes allergy symptoms in people who are allergic to aspirin. Its consumption after taking aspirin can cause hives, wheezing, sneezing or itching. E104 may result in increased hyperactivity in children [26, 48, 55].
E110, Sunset Yellow	1.0	Gastrointestinal intolerance, with abdominal pain, vomiting, and indigestion, has been associated with sunset yellow. In one case, eosinophilia and hives were present as well. Sunset Yellow may cause allergic or intolerance reactions in certain people. E110 causes side effects such as a hives, allergies, kidney tumours, chromosomal damage, hyperactivity, abdominal pain, nausea and vomiting. E110 may have an adverse effect on activity and attention in children [4, 27].
E120, Cochineal, carminic acid, Carmines	5.0	The natural colourings used, carmine or cochineal extract, can cause allergic reactions. It has been known to cause severe allergic reactions and anaphylactic shock in some people. The hyperactive Children´s Support Group recommends that the dye be eliminated from children´s diets [6, 65].
E122, Azorubine, Carmoisine	4.0	Azorubine has shown no evidence of mutagenic or carcinogenic properties. E122 may have an adverse effect on the activity and attention in children. It may rarely cause skin and respiratory reactions in susceptible individuals, even at the approved dose [28].
E123, Amaranth	0.15	Because Amaranth is an azo dye, it has been proven to provoke asthma, urticaria, rhinitis, eczema and hyperactivity as well as allergic reactions, similar to nettle rash, among asthmatics and individuals who are sensitive to aspirin. Amaranth is considered very dangerous as it increases hyperactivity in affected children [29].
E124, Ponceau 4R	0.7	May increase hyperactivity in affected children. Can adversely affect those that are sensitive to aspirin [38, 50].
E127, Erythrosine	0.1	This substance has been found to have tumorigenic effects in experimental animals, as well as mutagenic effects in both experimental animals and humans. Furthermore, risks include: irritation of eyes, skin, digestive tract, and respiratory tract in its undiluted form. There is a potential risk of negative influences on the thyroid gland due to the fact that E129 has four iodine atoms [3].
E129, Allura Red	7.0	Allura Red may cause allergic reactions (e.g. urticaria, asthma), especially when administered in mixes with other synthetic colour additives [67].
E131, Patent Blue	5.0	Several reports of serious allergic or anaphylactic reaction to Patent Blue have been published [71].
E132, Indigo Carmine	5.0	Indigo Carmine is harmful to the respiratory tract if swallowed. It is also an irritant to the skin and eyes [42].
E133, Brilliant Blue	6.0	It has the capacity for inducing an allergic reaction in individuals with pre-existing moderate asthma [30].
E151, Brilliant Black BN	5.0	It is one of the colourants that the Hyperactive Children's Support Group recommends be eliminated from the diet of children. It appears to cause allergic or intolerance reactions, particularly amongst those with aspirin intolerance. It is a histamine liberator, and may worsen the symptoms of asthma [48].

The European Union has authorised 43 dyes as food additives out of which each one was assigned an E-number. Out of these, 17 are synthetic dyes and 26 are either naturally derived, synthesized to match the naturally occurring counterparts, or are inorganic pigments occurring freely in nature in respective amounts [24].

As a category of the additives in the pharmaceutical industry, dyes are commonly classified as either natural or synthetic. For the most part, synthetic dyes have a higher tinctorial strength than natural dyes. Furthermore, synthetic dyes are predominantly water-soluble, relatively stable and not easily destroyed during the drug processing. These aspects, together with their advantageous price, render synthetic dyes more widely used worldwide these days. However, taking toxicological studies into account, the eventual risks of numerous synthetic azo dyes to human health have been found and are of concern. Scientists interested in this particular field of study have discovered that Amaranth starting at a certain concentration can induce dose-related DNA damage in the mouse colon after oral administration. In addition to the genotoxicity, a part of synthetic dyes can also cause allergic or asthmatic reactions for sensitive people, as well as frequent headaches and children's hyperactivity. To prevent the abuse of synthetic dyes, the permissible type and usage of synthetic dyes are strictly regulated in many countries [51].

There are many different classifications of pharmaceutical coloring agents. One of the most useful ways is to simply divide the colours into water-soluble (dyes) and water-insoluble (pigments) [61].

According to another method of separation, the pharmaceutical colourants are divided into [2]:

1. Organic dyes and their lakes
2. Inorganic or mineral colours
3. Natural colours or vegetable and animal colours

Organic dyes and their lakes

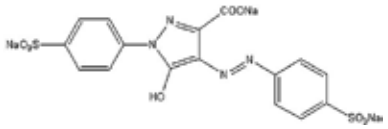
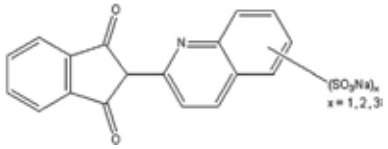
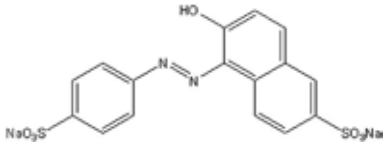
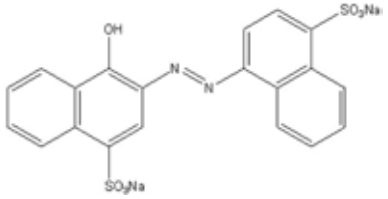
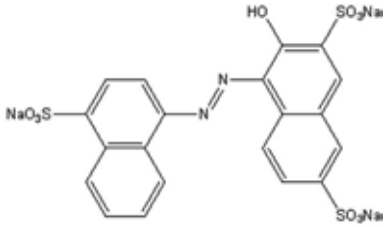
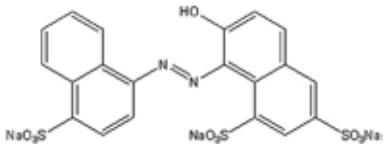
Organic dyes are synthetic chemical compounds that show intense colouring. They have a wide range of shades of greater colour intensity than natural dyes. They are easier to access and cheaper than natural dyes.

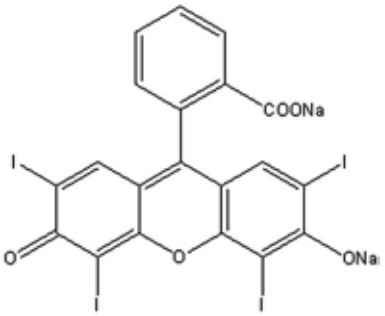
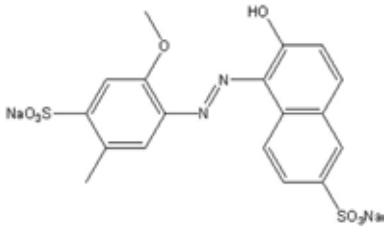
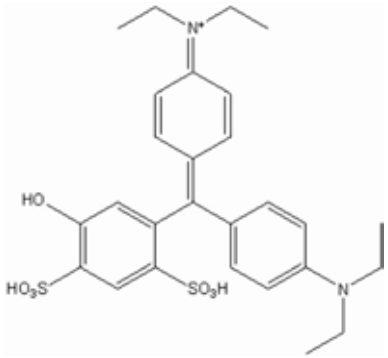
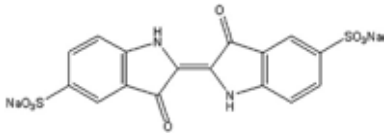
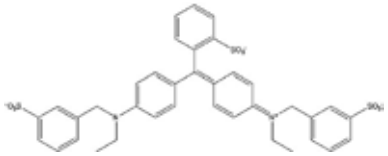
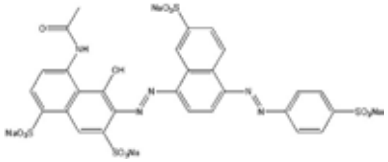
In 1984, FAO/WHO classified synthetic dyes into five major chemical groups: the azo compounds, quinophthalone, triarylmethane group, indigo dyes, and xanthenes. The synthetic dyes are used primarily in their water-soluble form, which is as their sodium, calcium, potassium or ammonium salts. Occasionally, they are used in the form of insoluble lakes, which are produced by precipitating the water-soluble forms onto an alumina or titanium dioxide base. Azo dyes are depicted as having an azo linkage, i.e. a nitrogen-to-nitrogen double bond (–N=N–). Chemically, in their molecules, one may discover benzene and naphthalene rings joined by azo linkages and containing sulfonic acid groups. Dyes in this group are the azo dyes Azorubine, Amaranth, Sunset Yellow, Tartrazine, Ponceau 4R and Allura red. The members of triarylmethane dyes are based on a central triphenylmethane structure and are substituted amine derivatives, with or without sulphuric acid groups. The dyes in this class pharmaceutically used are Brilliant Blue and Patent Blue. The members of xanthene dyes are based chemically on a xanthenes nucleus, for instance Erythrosine. Quinophathalone dyes are based on the naphthalene-quinone structure. From the pharmaceutical perspective, Quinoline Yellow is the constituent of this group. Quinoline Yellow is a mixture of salts, principally the mono- and disulfonate. The group of indigoid dyes stems from indigo which has two isatin molecules joined by a double bond. From the pharmaceutical perspective, the most commonly used dye in this class is Indigo Carmine [31, 59].

Generally, the colouring agents used in commerce have trivial denominations and there are often several synonyms for each substance. The colourants that we mainly resort to for colouring tablets, capsules and lozenges are synthetic water-soluble dyes, pigments (especially the pacifying agent titanium dioxide) and certain dyes of natural origin (cochineal). These dyes are often used as a dye in the manufacturing of pharmaceuticals: E102 Tartrazine, E104 Quinoline Yellow, E110 Sunset Yellow FCF, E120 – Cochineal, carminic acid, Carmines, E122 Azorubine (Carmoisine), E123 Amaranth, E124 Ponceau 4R (Cochineal Red A), E127 Erythrosine, E129 Allura Red, E131 Patent Blue, E132 Indigo Carmine (Indigotine), E133 Brilliant Blue, and E151 Brilliant Black BN. The dyes that can be contained in pharmaceuticals are detailed in a corresponding directive and are listed in Table 2.

Lakes have been defined by the FDA as the "Aluminum salts of FD&C water soluble dyes extended on a substratum of alumina". Lakes, unlike dyes, are insoluble and colour by dispersion. Their colour ability depends on particle size. Smaller particles have greater dyeing ability. They are prepared by reacting synthetic dyes with alumina in an aqueous medium [14].

Table 2. European Union list of the most commonly used pharmaceutical synthetic dyes

Commercial name/ synonym name	E-number	Chemical structure IUPAC name	Chemical formula M.W.
Tartrazine	E102	 Trisodium1-(4-sulfonatophenyl)-4-(4-sulfo-natophenylazo)--5-pyrazolone-3-carboxylate	$C_{16}H_9N_4Na_3O_9S_2$ 534.37
Quinoline Yellow	E104	 Disodium 2-(1,3-dioxoindan-2-yl)quinoline disulfonate	$C_{18}H_9NNa_2O_8S_2$ 477.38
Sunset Yellow FCF	E110	 Disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalene-sulfonate	$C_{16}H_{10}N_2Na_2O_7S_2$ 452.27
Azorubine/	E122	 Disodium4-hydroxy-2-[(E)-(4-sulfonato-1-naphthyl)diazenyl] naphthalene-1-sulfonate	$C_{20}H_{12}N_2Na_2O_7S_2$ 502.44
Amaranth	E123	 Trisodium (4E)-3-oxo-4-[(4-sulfonato-1-naphthyl) hydrazono] naphthalene-2,7-disulfonate	$C_{20}H_{11}N_2Na_3O_{10}S_3$ 604.48
Ponceau 4R/ Cochineal Red A	E124	 Trisodium (8Z)-7-oxo-8-[(4-sulfonatonaphthalen-1-yl) hydrazinylidene] naphthalene-1,3-disulfonate	$C_{20}H_{11}N_2Na_3O_{10}S_3$ 604.48

Commercial name/ synonym name	E-number	Chemical structure IUPAC name	Chemical formula M.W.
Erythrosine	E127	 2-(6-Hydroxy-2,4,5,7-tetraiodo-3-oxo-xanthen-9-yl) benzoic acid	$C_{20}H_6I_4Na_2O_5$ 879.87
Allura Red	E129	 Disodium 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]- -2-naphthalenesulfonate	$C_{18}H_{14}N_2Na_2O_8S_2$ 496.42
Patent Blue	E131	 2-[(4-diethylaminophenyl)(4-diethylimino-2,5-cyclohexadien-1-ylidene) methyl]- -4-hydroxy-1,5-benzene-disulfonate	$C_{27}H_3N_2O_7S_2$ 579.71
Indigo Carmine/ Indigotine	E132	 Disodium salt 3,3'-dioxo-2,2'-bis-indolyden-5,5'-disulfonic acid	$C_{16}H_8N_2Na_2O_8S_2$ 466.36
Brilliant Blue FCF	E133	 Ethyl-[4-[[4-[ethyl-[(3-sulfophenyl)methyl]amino]phenyl]-(2 - sulfophenyl) methylidene]-1-cyclohexa-2,5- dienylidene]-[(3-sulfophenyl)methyl]azanium	$C_{37}H_{34}N_2Na_2O_9S_3$ 792.84
Brilliant Black BN	E151	 Tetrasodium (6Z)-4-acetamido-5-oxo-6-[[7-sulfonato-4-(4-sulfonatophenyl)azo- -1-naphthyl]hydrazono]naphthalene-1,7-disulfonate	$C_{28}H_{17}N_5Na_4O_{14}S_4$ 867.68

Natural colours or vegetable and animal colours

Among natural dyes we include substances with different chemical structures and various physicochemical properties. They are obtained by extraction from different parts of plants, trees or animal products. Some of these colours are the products of chemical synthesis rather than extraction from a natural source, for example, β-carotene of commerce is regularly synthetic in origin. The term *"synthetic in origin"* frequently applied to such materials is "nature identical", which in many ways is more descriptive.

In general, natural dyes are less stable to light, heat and certain pH values than other dye groups. The ability to colour natural products is easier and more readily available than synthetic dyes. They do, however, have an advantage in that they have a wide acceptability [2]. The most commonly used colours in pharmaceutical products are from this group of colours: caramel, cochineal, riboflavin, anthocynins, annato and curcumin. In terms of possible adverse effects on humans, the cohineal is the one that gets the most attention.

Cochineal dye is obtained from the dried bodies of female *Dactylopius coccus* Costa insects (Cochineal) by extraction with water, alcohol or aqueous alcoholic solution. The main component of cochineals is anionic anthraquinone glycoside, carmine acid (7-α-D-glycopyranosyl-9,0-dihydro-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-2-anthracene-carboxylic acid). The molecular structure (Figure 1a) consists of an anthraquinone chromophore, a sugar residue, and a carboxyl group. Carmine is the finished aluminium/calcium lake of carminic acid, and is the commercial dye (E120) used to produce colour to food or pharmaceuticals. The possible structural formula for the aluminium complex of carminic acid is shown in Figure 1b. Both carminic acid and carmine are reasonably stable against light, heat and oxidation. Carminic acid is orange at a pH lower than 5 and bluish-red over 8. A variety of shades from yellow-red to near blue are possible. The solubility of carmine preparations varies depending on the nature of the cations present. Products where the major cation is ammonium (ammonium carminate) are freely soluble in water at pH 3.0 and pH 8.5. Products where the major cation is calcium (calcium carminate) are only slightly soluble in water at pH 3.0, but freely soluble at pH 8.5.

Carmine is used to colour different types of food, beverages, drugs and cosmetics. Increased amounts can cause allergic reactions, childhood hyperactivity and skin tumors in sensitive individuals. Increased hyperactivity has been reported in a few cases. The Hyperactive Children's Support Group recommends that the dye be eliminated from children's diets. As the colourant is of animal origin, it is not acceptable for use in foods or medicinal products for vegans [73].

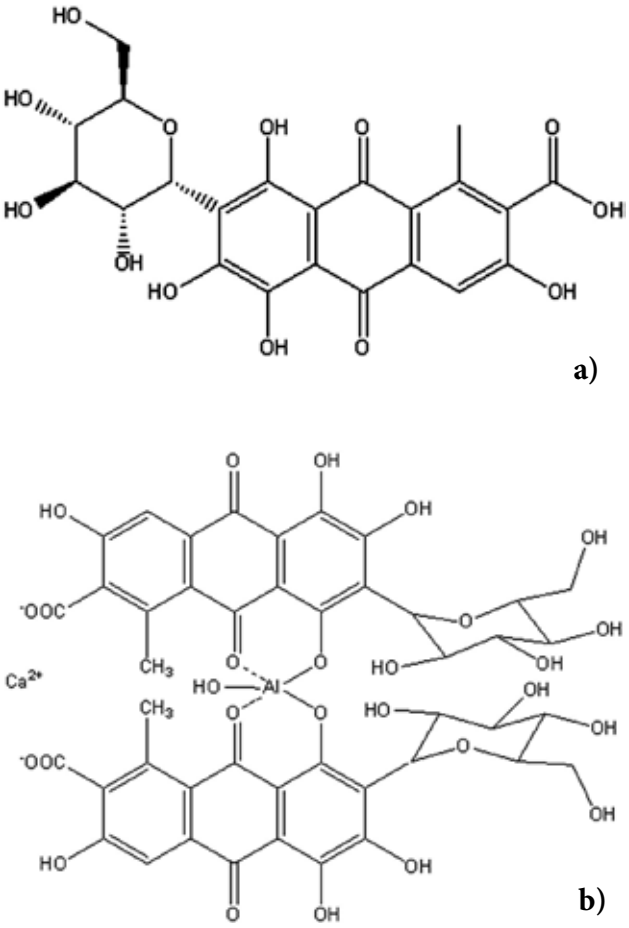


Fig. 1. a) Carminic acid; b) Carmine – possible structural formula for the aluminium complex of carminic acid

Inorganic or mineral colours

Inorganic colours, metal oxides, hydroxides or sulphides are used in particular. These substances are characterized by their stability to light and some are opaque materials. The range of colours we can achieve with these substances is less than that of organic colourings. Today, mainly yellow and red iron oxides (E172) are used to dye medicines. Titanium dioxide (E170) is used to colour and opacify hard gelatin capsules.

POTENTIAL HEALTH EFFECTS OF SYNTHETIC DYES ON HUMANS

An extensive amount of dyes are employed in the area of pharmaceutical manufacturing. The official concession to make use of the synthetic dyes in the pharmaceutical industry is subjected to a wide spectrum of toxicity tests and extremely stringent legislative provisions in all developed states. The crucial chemical structure of artificial dyes consists of azo group and aromatic rings which may be of toxic effects to human health, mainly in cases when they have been over consumed by patients and by pharmaceutical clients in general on a daily basis. In the light of these facts, the producers are obliged to enumerate such ingredients on medicinal packaging to help people avoid potential adverse effects. Most synthetic coloured additives are carcinogenic, teratogenic and are the cause of allergic reactions. Toxicology studies are routinely conducted on an ongoing basis by official institutions — responsible authorities such as the World Health Organisation (WHO), the Food and Drug Administration (FDA), and the European Commission (EC). The outcome of this continuous review is that the various regulatory bodies around the world have compiled lists of permitted colours that are generally viewed as being free from serious adverse, undesired toxicological effects [51].

Some of the groups of dyes have been associated with suspected adverse effects. In 2007, a study scrutinising the use of six dyes (Tartrazine, Quinoline Yellow, Sunset Yellow, Azorubin, Ponceau 4R and Allura Red) was published. The study results connected these dyes to behaviour issues in children. Nonetheless, after reviewing the outcomes of the study, the European Food Standards Agency came to the conclusion that no legislative change or amendment was in fact required [61].

A study by the Food Standards Agency (FSA) in 2007 demonstrated that the consumption of food in which certain dyes were present could potentially negatively influence hyperactive behaviour in children in that they would cause responses that can involve many aspects ranging from excessive movement, to speech and attentiveness. The study focused on three-year-old and eight to nine-year-old children who were given three different types of beverages to drink. Consequently, their behaviour was evaluated by the teachers and their parents. This study, published in 2007 in The Lancet, indicated that the commonly used artificial food colourings may in fact exacerbate hyperactive behaviour in young individuals. On the basis of such investigations, the involved researchers recognised that hyperactive behaviour in children increased with products containing artificial colouring additives. They concluded that the results demonstrated an adverse effect on the behaviour in the sample of individuals after consumption of the food dyes. Recently, it has also been reported that artificial dyes may result in negative impacts on the children's: attention deficit disorder (ADD), immune system problems and certain allergic reactions. Attention Deficit Hyperactivity Disorder (ADHD), represented, for example, by low tolerance to frustration, impulsivity, and inattention, is one of the most widespread behavioral disorders in children. These disorders have symptoms that may begin in childhood and continue into adulthood. ADHD symptoms may be a reason of or directly launch problems at home, school, work and in relationships. Asthmatic symptoms, which include coughing, wheezing and chest tightness, are commonly seen in an asthma attack. Thanks to appropriate treatments for symptoms of asthma, both children and adults are given an opportunity to live a standard, quality of life. Among common causes of allergic symptoms caused by the use of pharmaceuticals, we may enumerate, among others, allergies due to the incorporated dyes [38, 50].

DETERMINATION OF DYES IN PHARMACEUTICALS

Sample preparation constitutes a very important step in order to achieve reliable pharmaceutical analysis, but in general, it is not given sufficient attention. The main pharmaceuticals that are dyed are [61]:

Tablets: either the core itself or the coating.

Hard or soft gelatine capsules: the capsule shell or coated beads.

Oral liquids.

Topical creams and ointments.

For this reason, conventional sample processing techniques are directed to these different dosage forms. Sample preparation can involve a number of steps including: dispersion, particle size reduction (e. g. milling, grinding and homogenization), solubilisation of the target to be analysed, derivatization, sample clean-up (e. g. removing the undesirable interferences), and clarification (e. g. removing insoluble materials). The necessary sample preparation

steps are selected with regard to the particular dosage form and analysis techniques used [10, 58]. The most developed technique is the process usually used for the preparation of solid dosage forms (tablets, capsules). It consists of four basic steps:

1. Grinding
2. Extraction
3. Dilution and
4. Filtration.

When the filtration is non-effective due to the very fine particles of the tablets, centrifugation is recommended. It is a simple procedure for dosage forms that are immediately released. Additional clean-up of a sample is typically not needed which is advantageous in terms of the time of sample preparation [10]. The commercial market offers a large number of automated systems for implementation of various sample preparation actions. Those systems are usually designed in such a way to minimize the possibility of contamination or loss of sample during the preparation steps [52]. The specific procedure for processing the sample of a drug in determining dyes depends on: its form, the number of dyes to be determined, and the analytical method to be used for the determination.

Up to now, a variety of methods for separation, identification and determination of dyes have been suggested. For the purpose of quantification of the dye content in the pharmaceutical products and food, modern analytical methods and techniques are currently used, such as follows: high performance liquid chromatography (HPLC) [39, 40, 43, 46, 47, 51, 64, 70, 74], ion-pair liquid chromatography (IP-LC) [9, 36], thin-layer chromatography (TLC) [5, 49, 63], spectrometry, derivative spectrometry, and spectrophotometric methods [7, 57, 62, 68]. Electroanalytical methods also find its application in this field; mainly adsorptive voltammetry [1] and differential pulse polarography [8, 12].

The behavior of the Carmine (E120) was studied by square-wave adsorptive stripping voltammetry (SW-AdSV) at the hanging mercury drop electrode. It was observed that Carmine gave a sensitive stripping voltammetric peak at –350 mV in pH 3 acetate buffer. The SW-AdSV peak currents depended linearly on the concentration of Carmine from 5×10^{-8} to 1.25×10^{-7} mol.dm^{-3} (r = 0.99). A detection limit of 1.43×10^{-9} mol.dm^{-3} with RSD of 2.2 % and a mean recovery of 97.9 % were obtained.

The differential pulse polarography method was applied for the determination of Carmoisine (E122), Ponceau 4R (E124) and Allura Red (E129) in soft drinks and sweets. They can be easily distinguished from natural dyes, which are not electroactive, using differential pulse polarography. The recovery was in the

96—105 % range and the relative standard deviation was close to 1 % for the three dyes. The limits of quantification in the polarographic cell, estimated from the polarographic data, were 42, 43 and 34 μg.dm^{-3} for Carmoisine, Allura red and Ponceau 4R, respectively [8].

Jaworska et al. [37] reported a comprehensive research in which the detection and separation conditions for the analysis of 15 synthetic dyes (E102, E104, E110, E120, E122, E123, E124, E127, E128, E129, E131, E132, E133, E142 and E151) by micellar electrokinetic capillary chromatography were examined. A large amount of commercially available pharmaceuticals of different dosage forms (coated tablet, film-coated tablet, hard/soft capsule, syrup etc.) were tested during this study. Coated and film-coated tablets were prepared for analysis by elution of a colour layer with 50 % (v/v) methanol (3—5 ml). The obtained solutions were filtered through GD/X Cellulose 0.2 m filters. Gelatin capsules were at first dissolved in 50 % (v/v) methanol (the same volume). The gelatin was then precipitated with acetonitrile (2—4 ml). To prevent the passage of significant amount of gelatin to a solution, it was necessary to make elution of the dyes with 50 % (v/v) methanol. The content of the dyes in the samples prepared by this way was often at the Limit of Detection (LOD) level. For preconcentration of dyes and their separation from the residual matrix, dyes were isolated from eluates by using acidic aluminium oxide. Samples of the liquid pharmaceutical products (syrups, liquids) were analysed without another preparation step, or with only double dilution with water.

Using photoacoustic spectroscopy in the visible range of wavelengths to directly determine Sunset Yellow (E110) content in effervescent multivitamin tablets, the tablets were manually crushed into a fine powder form. Analysis was performed without further filtering or the use of an ultrasonic bath. To determine the dye content by conventional spectrophotometry, a mass of each powdered tablet was weighed and dissolved in 30 ml of deionized water and then diluted to 50 ml. After filtration, the cuvette with filtrate had to be held in an ultrasonic bath for 1 min to eliminate the presence of CO2 bubbles [25].

In one paper [7] authors have described a method for the quantitative determination of Tartrazine in dimetcarb tablets containing a yellow parent compound (dimethpramide). The sample was prepared by adding 25 ml of water to 10 tablets in a 50 ml measuring flask and the mixture was stirred until the disintegration of the tablets. Then, water was added to the mark and the solution was thoroughly stirred and filtered. To 5 ml of the filtrate was added 5 ml of concentrated HCl and the mixture was stirred and characterized by the optical density at 435 nm relative to a reference solution (in 10 mm optical cells).

Derivative spectrophotometric methods were developed for determining Ponceau 4R and Tartrazine in order to resolve their binary mixtures. The method permits the simultaneous determinations of these dyes in various samples [62].

Eight synthetic dyes (Amaranth, Brilliant Blue, Indigo Carmine, New Red, Ponceau 4R, Sunset Yellow, Tartrazine, Allura Red) were determined by high-performance ion chromatography (HPIC) on an anion-exchange analytical column with very low hydrophobicity and visible absorbance detection. Gradient elution with hydrochloric acid–acetonitrile effected both the chromatographic separation of these dyes and the on-line clean-up of the analytical column, which was very advantageous for routine analysis. High-performance ion chromatography may be a solution to the chromatographic analysis for some water-soluble, organic analytes with strong hydrophobicity. The method has been applied to the determination of dyes in drinks and in instant drink powder [9].

From the possibilities of determinations mentioned above, HPLC is one of the most widely used methods nowadays. Generally, HPLC provides a separation of mixtures to component substances whereas a properly chosen detector allows for their identification and for determination of their content. The basis of separation is represented by differences in the affinity of the constituents to two phases (stationary and mobile). Dyes exhibit different adsorption affinity to the stationary phase due to differences in their mass, structural space, and presence of functional groups in a molecule [45].

The most commonly utilized mode in liquid chromatography is represented by reverse phase, where the stationary phase has moderate or no polarity and the mobile phase is strongly polar (e. g. tetrahydrofuran, acetonitrile, methanol, water, etc.) [72]. Liquid chromatography-based techniques are usually coupled with UV-VIS, mass spectrometry (MS) or photodiode array (PAD) detectors. Due to the high polarity of dyes, their elution times are very short. In such cases, the use of C18 ether columns usually increases the retention times, especially of the azo dyes group [33].

Further ion-pair high performance liquid chromatography (IP-HPLC) can also be carried out by means of using ion-pairing (hydrophobic ionic) compounds such as ammonium acetate buffer, 1-hexadecyl trimethylammonium bromide etc., to the mobile phase to improve the separation and elution of the ionic target to be analysed [72]. The used contents of added solutions are usually above 0.1 mol.dm^{-3} [51]. The most significant facts that must be taken into account during the choice of analytic conditions are the hydrophobic properties of dyes and the presence of acidic groups in their structures. As a result of the reaction between the sample and eluent, neutral ionic pairs are formed and are separated chromatographically in the reversed phase system. Another way is the preparation of samples which enables it to conduct the analysis on ionic exchanger or the modification of mobile phase to obtain an ion-exchanger. When the ionic compound is not present, elution times of some dyes (e. g. Tartrazine, Ponceau 4R, Indigo Carmine) are very short. The addition of an ionic agent results in increasing retention time of compounds with acidic functional groups in their structures. It is important for making the separation of dyes simultaneously. The pH value takes a considerable fundamental role in the quantification of dyes using the aforementioned method. In the case of using a column with silica gel, the pH value should be within the range 4—6 because of low hydrolytic gel stability [45].

In most of liquid chromatography methods, various organic solvents are used. Their negative influence on the environment and human health provides a basis for using "green methods" which are in full compliance with the principles of "Green chemistry". One of them is using a surfactant solution Triton X-100 (4-(1,1,3,3-tetramethylbutyl)-phenyl-polyethylene glycol) as a mobile phase. This compound changes the polarity of water and disposition of the C18 column, which becomes more polar, whereas charges of dyes molecules are steadied. Brilliant Blue, Sunset Yellow and Tartrazine had been analysed by applying the above described method. The optimal concentration of Triton X-100 should be in the range of 0.25—1 %. On the one hand, too low a concentration of surfactant results in incorrect preparation of a column; on the other hand, too

high of a concentration causes imprecise separation and quantitative determination of mixture compounds. The pH value should be equal to 7. Below this value, some peaks disappeared. The addition of the phosphate buffer was necessary for a reliable analysis. The charges of buffer constituents balance the charges of dyes molecules. Applying this method, the analysis is completed in 8 min using 15 mg surfactant and 38.8 mg phosphate buffer [69].

High performance ion chromatography (HPIC) can also be used for dye analysis. It involves the use of specific columns filled in resins with functional groups which have constant charge bounded ions. In theory, HPIC is not suitable for organic, multivalent and hydrophobic ions because of strong bonding with the stationary phase. Due to this fact, their elution takes a long time. The use of an anion exchange column that has mild hydrophobicity and acidic eluent is required for this purpose [9, 45].

CONCLUSIONS

In the production of pharmaceuticals different additives that contribute to the formation of the actual drug form or its appearance are used. Various natural or synthetic dyes are used primarily to ensure the appearance and the ability to distinguish the individual drugs. These compounds have different chemical compositions and may interact in different ways with the human body. The safety of dyes used in foodstuffs and pharmaceutical products are assessed by the European Food Safety Authority (EFSA). Most recently in 2008, the safe use of synthetic dyes and ADI values were revised. Based on the available clinical studies and literature, EFSA stated that it is unlikely that these compounds have significant adverse impacts on human health during normal consumption. However, it is known that most studies are focused on the effect of only one dye and the possible combined impact of several dyes from different sources has not been examined. Some investigations point to the increasing consumption of products containing colouring agents. It is particularly evident especially among children. Recently, it has been found that certain dyes have the ability to bind to human serum albumin. This fact has the potential of leading towards the possibility of adverse toxic impacts. The problem is the fact that globally there is no uniformity in the rules relating to the use of dyes in the pharmaceutical field and also in foodstuffs. It often happens that in one country the dye is forbidden and in another it is permitted. Considering this, it is necessary to pay very close attention to the issue of the use of dyes in pharmaceutical products and medicines, and it is necessary to take into account the results of studies focused on pharmacological, neurological and other effects that dyes or their mixtures may have. An essential task is the need for the unification of legislation related to this issue on a global basis.

REFERENCES

1. **Alghamdi, A. H., Alshammery, H. M., Abdalla, M. A., Alghamdi, A. F., 2009:** Determination of Carmine Food Dye (E120) in foodstuffs by stripping voltammetry. *J. AOAC Int.*, 92, 1454—1459.
2. **Allam, K. V., Kumar, G., 2011:** Colorants – the cosmetics for the pharmaceutical dosage forms. *Int. J. Pharm. Pharm. Sci.*, 3, 13—21.
3. **Amchova, P., Kotolova, H., Ruda-Kucerova, J., 2015:** Health safety issues of synthetic food colorants. *Regul. Toxicol. Pharmacol.*, 73, 914—922.
4. **American Academy of Pediatrics, Committee on Drugs, 1997:** Inactive ingredients in pharmaceutical products: Update. *Pediatrics*, 99, 268–278.
5. **Ashkenazi, P., Yarnitzky, Ch., Cais, M., 1991:** Determination of synthetic food colours by means of a novel sample preparations system. *Anal. Chim. Acta*, 248, 289—299.
6. **Attokaran, M., 2011:** *Natural Food Flavours and Colorants*. Wiley-Blackwell, 39.
7. **Bagirova, V. L., Miťkina, L. I., 2003:** Determination of Tartrazine in drugs. *Pharm. Chem. J.*, 37, 558—559.
8. **Chanlon, S., Joly-Pottuz, L., Chatelut, M., Vittori, O., Cretier, J. L., 2005:** Determination of Carmoisine, Allura red and Ponceau 4R in sweets and soft drinks by differential pulse polarography. *J. Food Compos. Anal.*, 18, 503—515.
9. **Chen, Q. Ch., Mou, S. F., Hou, X. P., Riviello, J. M., Ni, Z. M., 1998:** Determination of eight synthetic food dyes in drinks by high-performance ion chromatography. *J. Chromatogr.*, A, 827, 73—81.
10. **Choi, C. K., Dong, M. W., 2005:** Chapter 5 — Sample preparation for HPLC analysis of drug products. In **Ahuja, S., Dong, M. W.:** *Handbook of Pharmaceutical Analysis by HPLC*. Elsevier, United Kingdom, 123—144.
11. **Codex Alimentarius, 1962:** http://www.fao.org/fao-who-codexalimentarius/about-codex/en/ (accessed 05.04.16).

12. **Combeau, S., Chatelut, M., Vittori, O., 2002:** Identification and simultaneous determination of Azorubin, Allura red and Ponceau 4R by differential pulse polarography: application to soft drinks. *Talanta*, 56, 115—122.
13. **Commission Directive 95/45/EC, 1995:** Laying down specific purity criteriaconcerning colours for use in foodstuffs, OJEC L226, 1.
14. **Commission Regulation 1333/2008, 2008:** On food additives, OJEC L354, 16.
15. **Commission Regulation 257/2010, 2010:** Setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No. 1333/2008 of the European Parliament and of the Council on food additives, OJEC L80, 19.
16. **Commission Regulation 232/2012, 2012:** Amending Annex II to Regulation (EC) No. 1333/2008 of the European Parliament and of the Council as regards the conditions of use and the use levels for Quinoline Yellow (E 104), Sunset Yellow FCF/Orange Yellow S (E 110) and Ponceau 4R, Cochineal Red A (E 124), OJEC L78, 1.
17. **Council Directive 2645/62, 1962:** On the approximation of the rules of the Member States concerning the colouring matters authorized for use in foodstuffs intended for human consumption, OJEC 2645/62, 279.
18. **Council Directive 78/25/EEC, 1978:** On the approximation of the laws of the Member States relating to the colouring matters which may be added to medicinal products, OJEC L11, 18.
19. **Council Directive 94/36/EC, 1994:** On colours for use in foodstuffs, OJEC L237, 13.
20. **Council Directive 2009/35/EC, 2009:** On the colouring matters which may be added to medicinal products, OJEC L109, 10.
21. **Council Regulation 178/2002, 2002:** Laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, OJEC L31, 1.
22. **de Boer, L., 2014:** Biotechnological production of colorants. *Adv. Biochem. Eng. Biotechnol.*, 143, 51–89.
23. **Dipalma, J. R., 1990:** Tartrazine sensitivity. *Am. Fam. Physician.*, 42, 1347—1350.
24. **Downham, A., Collins, P., 2000:** Colouring our foods in the last and next millennium. *Int. J. Food Sci. Tech.*, 35, 5—22.
25. **Dóka, O., Bicanic, D., Ajtony, Z., Koehorst, R., 2005:** Determination of sunset yellow in multi-vitamin tablets byphotoacoustic spectroscopy and a comparison with alternative methods. *Food Addit. Contam.*, 22, 503—507.
26. **EFSA, 2009a:** Scientific opinion on the re-evaluation of Quinoline Yellow (E 104) as a food additive. *EFSA J.*, 7, 1329.
27. **EFSA, 2009b:** Panel on food additives and nutrient sources added to food, scientific opinion on the re-evaluation of Sunset Yellow FCF (E 110) as a food additive. *EFSA J.*, 7, 1330.
28. **EFSA, 2009c:** Scientific opinion on the re-evaluation of Azorubine/Carmoisine (E 122) as a food additive. *EFSA J.*, 7, 1332.
29. **EFSA, 2010a:** Scientific opinion on the appropriateness of the food azo-colours Tartrazine (E 102), Sunset Yellow FCF (E 110), Carmoisine (E 122), Amaranth (E 123), Ponceau 4R (E 124), Allura Red AC (E 129), Brilliant Black BN (E 151), Brown FK (E 154), Brown HT (E 155) and Litholrubine BK (E 180) for inclusion in the list of food ingredients set up in Annex IIIa of Directive 2000/13/EC. *EFSA J.*, 8, 1778.
30. **EFSA, 2010b:** Scientific opinion on the reevaluation of Brilliant Blue FCF (E 133) as a food additive. *EFSA J.*, 8, 36.
31. **European Medicines Agency, 2007:** Guideline on excipients in the dossier for application for marketing authorisation of a medicinal product: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003382.pdf (accessed 05.04.16).
32. **Fallico, B., Chiappara, E., Arena, G., Ballistreri, E., 2011:** Assessment of the exposure to Allura red colour from the consumption of red juice-based and red soft drinks in Italy. *Food Addit. Contam.*, 28, 1501—1515.
33. **Gosetti, F., Frascarolo, P., Mazzucco, E., Gianotti, V., Bottaro, M., Gennaro, M.C., 2008:** Photodegradation of E110 and E122 dyes in a commercial aperitif: A high performance liquid chromatography-diode array-tandem mass spectrometry study. *J. Chromatogr.*, A, 1202, 58—63.
34. **Grzelewska-Rzymowska, I., Szmidt, M., Kowalski, M. L., Roźniecki, J., 1986:** Sensitivity and tolerance to tartrazine in aspirin-sensitive asthmatics. *Allergol. Immunopathol.*, 14, 31—36.
35. http://www.codexalimentarius.net/gsfaonline/docs/CXS_192e.pdf (accessed 05.04.16).
36. **Ishikawa, F., Shigeoka. S., Nagashima, M., Takahashi, M., Kamimura, H., 2003:** Simultaneous determination of eleven dyes and their aluminum lakes in drugs. *J. AOAC Int.*, 86, 215—221.
37. **Jaworska, M., Szulinska, Z., Wilk, M., Anuszewska, E., 2005:** Separation of synthetic food colourants in the mixed micellar system application to pharmaceutical analysis. *J. Chromatogr.*, A, 1081, 42—47.
38. **Kanarek, R. B., 2011:** Artificial food dyes and attention deficit hyperactivity disorder. *Nutr. Rev.*, 69, 385—391.

39. **Kaur, A., Gupta, U., 2014:** Identification and determination of binary mixtures of synthetic dyes with Cr (III) complexation in food stuffs and pharmaceutical samples by high performance liquid chromatography. *Indian Journal of Pharmaceutical Science and Research*, 4, 49—52.
40. **Khanavi, M., Hajimahmoodi, M., Ranjbar, A. M., Oveisi, M. R., Ardekani, M. R. S., Mogaddam, G., 2012:** Development of a green chromatographic method for simultaneous determination of food colorants. *Food Analytical Methods*, 5, 408—415.
41. **Khehra, M. S., Saini, H. S., Sharma, D. K., Chadha, B. S., Chimni, S. S., 2006:** Biodegradation of azo dye CI Acid Red 88 by an anoxic–aerobic sequential bioreactor. *Dyes Pigm.*, 70, 1—7.
42. **Kim, S. H., Suk, E. H., Kil, S. H., Hahm, K. D., Hwang, J. H., 2011:** Hypotension in patients administered indigo carmine containing impurities — A case report. *Korean J. Anesthesiol.*, 61, 435—438.
43. **Kirschbaum, J., Krause, C., Pfalzgraf, S., Brückner, H., 2003:** Development and Evaluation of an HPLC-DAD. Method for determination of synthetic food dyes. *Chromatographia*, 57, 115—119.
44. **Kučerová, J., Kotolová, H., 2013:** Toxicity of dyes used in medical preparations (In Czech). *Prakt. Lekáren.*, 9, 83—89.
45. **Kucharska, M., Grabka, J., 2010:** A review of chromatographic methods for determination of synthetic food dyes. *Talanta*, 80, 1045—1051.
46. **Lim, H. S., Choi, J. Ch., Song, S. B., Kim, M., 2014:** Quntitative determination of carmine in foods by high-performance liquid chromatography. *Food Chem.*, 158, 521—526.
47. **Liu, Y. M., Yu, Q., Sun, L., Wang, S., 2006:** Determination of synthetic colorant contents in officinal capsule. *Chin. Pharm. J.*, 41, 303—305.
48. **Macioszek, V. K., Kononowicz, A. K., 2004:** The evaluation of the genotoxicity of two commonly used food colours: Quinoline Yellow (E 104) and Brilliant Black BN (E 151). *Cell Mol. Biol. Lett.*, 9, 107—122.
49. **Materiienko, A. S., Grudko, V. A., Georgiyants, V. A., 2014:** Development of methods for determining Tartrazine and Carmoisine as part of shell of tablets "NITROXOLINE" (In Russian). *Management, Economy and Quality Assurance in the Pharmacy*, 1, 10—14.
50. **McCann, D., Barrett, A., Cooper, A., Crumpler, D., Dalen, L., Grimshaw, K., et al., 2007:** Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *Lancet*, 370, 1560—1567.
51. **Minioti, K. S., Sakellariou, Ch. F. Thomaidis, N. S., 2007:** Determination of 13 synthetic food dyes in water-soluble foods by reversed-phase high-performance liuid chromatography coupled with diode-array detector. *Anal. Chim. Acta*, 583, 103—110.
52. **Moldoveanu, S. C., David, V., 2015:** Chapter 2 — The role of sample preparation. In **Moldoveanu, S. C., David, V.:** *Modern Sample Preparation for Chromatography*. Elsevier, United Kingdom, 33—49.
53. **Mpountoukas, P., Pantazaki, A., Kostareli, E., Christodoulou, P., Kareli, D., Poliliou, S., 2010:** Cytogenetic evaluation and DNA interaction studies of the food colorants amaranth, erythrosine and tartrazine. *Food Chem. Toxicol.*, 48, 2934—2944.
54. **Nagaraja, T. N., Desiraju, T., 1993:** Effects of chronic consumption of metanil yellow by developing and adult rats on brain regional levels of noradrenaline, dopamine and serotonin, on acetylcholine esterase activity and on operant conditioning. *Food Chem. Toxicol.*, 31, 41—44.
55. **National Toxicology Program, U. S., 1997:** Toxicology and Carcinogenesis Studies of D&C Yellow No. 11 (CAS no. 8003-22-3) in F344/N rats (feed studies). *Natl. Toxicol. Program Tech. Rep. Ser.*, 463, 1—190.
56. **Newsome, A. G., Culver, C. A., van Breemen, R. B., 2014:** Nature's palette: the search for natural blue colorants. *J. Agric. Food Chem.*, 62, 6498—6511.
57. **Ni, Y., Gong, X., 1997:** Simultaneous spectrophotometric determination of mixtures of food colorants. *Anal. Chim. Acta*, 354, 163—171.
58. **Nickerson, B., 2011:** Chapter 1—Properties that impact sample preparation and extraction of pharmaceutical dosage forms. In **Nickerson, B.:** *Sample Preparation of Pharmaceutical Dosage Forms (Challenges and Strategies for Sample Preparation and Extraction)*. Springer, USA, 3—20.
59. **Podczeck, F., Jones, B. E., 2004:** *Pharmaceutical Capsules*, 2nd edn., Pharmaceutical Press, 66—67.
60. **Ronald, A., Simon, M. D., 2003:** Adverse reactions to food additives. *Curr. Allergy Asthma Rep.*, 3, 62—66.
61. **Rowe, R. C., Sheskey, P. J., Quinn, M. E., 2009:** Chapter — Coloring agents. In **Rowe, R. C., Sheskey, P. J., Quinn, M. E.:** *Handbook of Pharmaceutical Excipients*, 6th edn., Pharmaceutical Press and American Pharmacists Association, USA, 189—196.
62. **Sayar, S., Őzdemir, Y., 1997:** Determination of Ponceau 4R and Tartrazine in various samples by derivative spectrophotometric methods. *Turk. J. Chem.*, 2, 182—187.

63. Sayar, S., Özdemir, Y., 1998: First-derivative spectrophotometric determination of ponceau 4R, Sunset Yellow and Tartrazine in confectionery products. *Food Chem.*, 61, 367—372.

64. Sindhu, P. R., Bagya, L. J., Surya, P. R., 2011: Estimation of synthetic colorant Tartrazine in food stuff and formulations and effect of colorant on the protein binding of drugs. *Int. J. Pharm. Ind. Res.*, 1, 141–152.

65. Tabar, A. I., Acero, S., Arregui, C., Urdánoz, M., Quirce, S., 2003: Asthma and allergy due to carmine dye. *An. Sist. Sanit. Navar.*, 26, 65—73.

66. Timberlake, C. F., Bridle, P., 1980: In **Walford, J.** (Ed.)**:** *Developments in Food Colours-1*. London, Applied Science Publishers Ltd., 115—149.

67. Tsuda, S., Murakami, M., Matsusaka, N., Kano, K., Taniguchi, K., Sasaki, Y. F., 2001: DNA damage induced by red food dyes orally administered to pregnant and male mice. *Toxicol. Sci.*, 61, 92—99.

68. Üstün Özgür, M., Koyuncu, İ., 2002: The simultaneous determination of Quinoline Yellow (E-104) and Sunset Yellow (E-110) in syrups and tablets by second derivative spectrophotometry. *Turk. J. Chem.*, 26, 501—508.

69. Vidotti, E. C., Costa, W. F., Oliveira, C. C., 2006: Development of a green chromatographic method for determination of colorants in food samples. *Talanta*, 68, 516—521.

70. Vlase, L., Muntean, D., Cobzac, S. C., Filip, L., 2014: Development and validation of an HPLC-UV method for determination of synthetic food dyes. *Re. Roum. Chim.*, 59, 719—725.

71. Wu, T. T., Chang, Y. Ch., Lee, J. M., Hung, M. H., 2015: Anaphylactic reaction to patent blue V used in preoperative computed tomography-guided dye localization of small lung nodules. *JFMA*, 1—2.

72. Yamjala, K., Nainar, M. S., Ramisetti, N. R., 2016: Methods for the analysis of azo dyes employed in food industry — A review. *Food Chem.*, 192, 813—824.

73. Yilmaz, U. T., Ergun, F., Yilmaz, H., 2014: Determination of the food dye carmine in milk and candy products by differential pulse polarography. *J. Food Drug Anal.*, 22, 329—335.

74. Yoshioka, N., Ichihashi, K., 2008: Determination of 40 synthetic food colours in drinks and candies by high-performance liquid chromatography using a short column with photodiode array detection. *Talanta*, 74, 1408—1413.

Received March 21, 2017
Accepted July 13, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0026

FOLIA VETERINARIA, 61, 3: 47—51, 2017

TESTING THE POTENTIAL CLASTOGENIC/ CYTOTOXIC EFFECTS OF PESTICIDE CALYPSO 480 SC

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ABSTRACT

The detection of chromosomal damage serves as a tool for the verification of the genotoxic effects of chemical substances *in vitro*. We used conventional cytogenetic analysis in order to test for the potential genotoxic action of the insecticide thiacloprid (the active ingredient in commercial preparation CALYPSO 480 SC). The test cultures of bovine lymphocytes obtained from the peripheral blood were incubated with the insecticide in concentrations of: 30, 120, 240 and 480 μg.ml^{-1} for 24 and 48 hours. After 24 hours of incubation, we observed that the increasing concentrations resulted in a significant ($P < 0.05$; $P < 0.01$) increase in the frequency of DNA damage. Our experiments showed the presence of aberrations of a non-stable type (chromatid and chromosome breakage). The conventional chromosome analysis was supplemented with fluorescence *in situ* hybridization for the detection of numeric and stable structural aberrations. Whole chromosome probes for bovine chromosomes 1, 5 and 7 (BTA 1, BTA 5 and BTA 7) were used in the experiments.

Key words: conventional cytogenetics; chromosomal aberrations; fluorescence *in situ* hybridization

INTRODUCTION

Pesticides are a heterogeneous group of chemical substances designed specifically for the protection against pests. The intensive application of these substances in agriculture results in heavy contamination and degradation of the environment, which is reflected in the health of humans and other animals, and the presence of various residues in the food of animal origin [14].

Genotoxicity and its impact on the body are considered the most serious side effects of agricultural chemicals [2]. Their negative influence is amplified by other factors, such as environmental pollution, presence of heavy metals, nitrates and many other contaminants in our food and water.

There is available, a range of methods and models that can be used in a multistage system of evaluation of genetic risk related to the action of mutagens. One of the most important biological parameters reflecting exposure to geno-

toxic substances are chromosomal aberrations (CA) detected in lymphocytes of the peripheral blood [12]. They include changes in the structure or number of chromosomes in the cell genome that considerably contribute to the development of tumorous diseases, reproduction disorders (reduced fertility, increased embryonal mortality, abortions, perinatal mortality) and inherent malformations. In addition to the evaluation of chromosomal aberrations, the genotoxic effects of chemicals can be confirmed by other cytogenetic markers, such as sister chromatid exchanges (SCE) and micronucleus frequences (MN).

Neonicotinoids are one of the most important groups of insecticides used in agriculture. Due to their high selectivity, they are suitable also for controlling skin parasites in dogs and cats [15]. They are broad-spectrum systemic insecticides acting as stomach and contact poisons. They persist little in the outer environment and do not accumulate in the tissue of mammals, so their toxicity to mammals is lower than that of the older classes of insecticides [3].

Their mechanism of action consists in blockage of the post-synaptic nicotinic acetylcholine receptors, which results in the disturbance of the transfer of impulses and subsequent paralysis and death. In insects, these receptors are localised exclusively in the CNS and thus they are much more sensitive to these insecticides than mammals [11].

The aim of our study was to test the potential clastogenic/cytotoxic effects of commercial insecticide CALYPSO 480 SC, with the effective ingredient of thiacloprid, in bovine lymphocytes. Fluorescence *in situ* hybridization was used for the detection of stable and numeric aberrations.

MATERIALS AND METHODS

The experiments were carried out using the peripheral blood from two clinically healthy young bulls of the Slovak spotted breed. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 $\mu g.ml^{-1}$) and ethylmethane sulphonate (EMS, Sigma, St. Louis, MO, USA, 250 $\mu g.ml^{-1}$) served as a positive control. Thiacloprid (CALYPSO 480 SC; thiacloprid 480 $g.l^{-1}$) was dissolved in water (negative control) and added before the final 24 and 48 hours of cultivation at concentrations of: 30, 120, 240 and 480 $\mu g.ml^{-1}$. Colchicine (Merck, Darmstadt, Germany) with a final concentration of 5 $\mu g.ml^{-1}$ was added 90 min before the termination of the cultivation.

The preparations with chromosomes in metaphase were prepared by a standard cytogenetic method. Structural aberrations were evaluated in 100 metaphases for each donor and concentration. The value of the mitotic index was determined by the calculation from the number of metapahases per total number of 1000 cells. The statistical significance of the effects of the insecticide tested on the induction of chromosomal aberrations and reduction of mitotic index in lymphocytes of bovine peripheral blood was determined by parametric $\chi 2$ test. The standard deviations (SD) were calculated using variation analysis.

The preparations intended for fluorescence *in situ* hybridization were subjected to the process following a 3-day storage. Whole chromosome fluorescence-labelled probes BTA 1, BTA 5 and BTA7 were used in the experiments. The probes were applied to lymphocyte cultures after a 48-hour exposure to the pesticide in a concentration of 30 $\mu g.ml^{-1}$. The results of hybridization were evaluated and recorded under a fluorescence microscope Nikon (Labophot 2A/2) by means of dual filters.

RESULTS

The hybridization experiments failed to detect chromosomal aberrations of a stable type. Of the numeric aberrations of the type of aneuploidia and polyploidia, we observed one polyploidy (Fig. 1).

The frequency of the induced chromosomal aberrations following the exposure to thiacloprid for the last 24 and 48 h is presented in Figures 2 a) b). A significant increase in aberrations was detected after the exposure to the two highest concentrations of 240 and 480 $\mu g.ml^{-1}$ ($P < 0.05$ and $P < 0.01$). In both donors, we detected a moderate reduction in the mitotic index (MI) which was insignificant with the exception of the concentration of 480 $\mu g.ml^{-1}$ in Donor 1 ($P < 0.05$) (Fig. 3a). Chromatid and chromosomal breakages were the most frequently detected types of chromosomal aberrations in both donors. We also evaluated gaps (achromatic lesions) which were not subjected to statistical analysis. After the prolonged action of the insecticide (48 h), we observed a moderate insignificant increase in the frequency of breakages in comparison with the control cultures. The decrease in the mitotic activity was dose-dependent and the changes were significant after exposure to the highest concentration of 480 $\mu g.ml^{-1}$ ($P < 0.001$) in

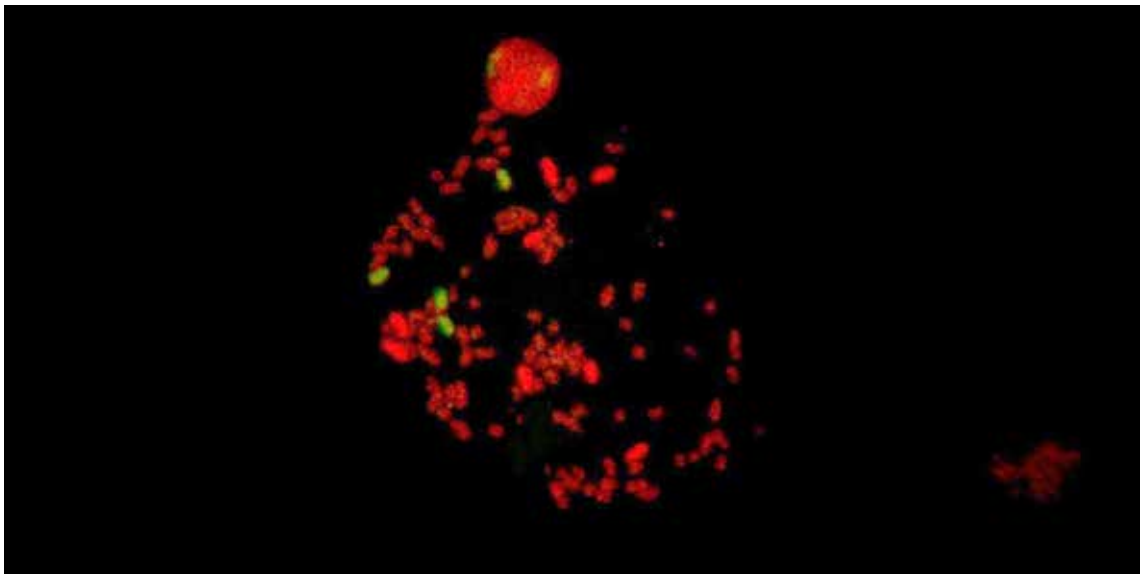

Fig. 1. Metaphase HD, 60 (XY), BTA 1 and BTA7-red, BTA5-green

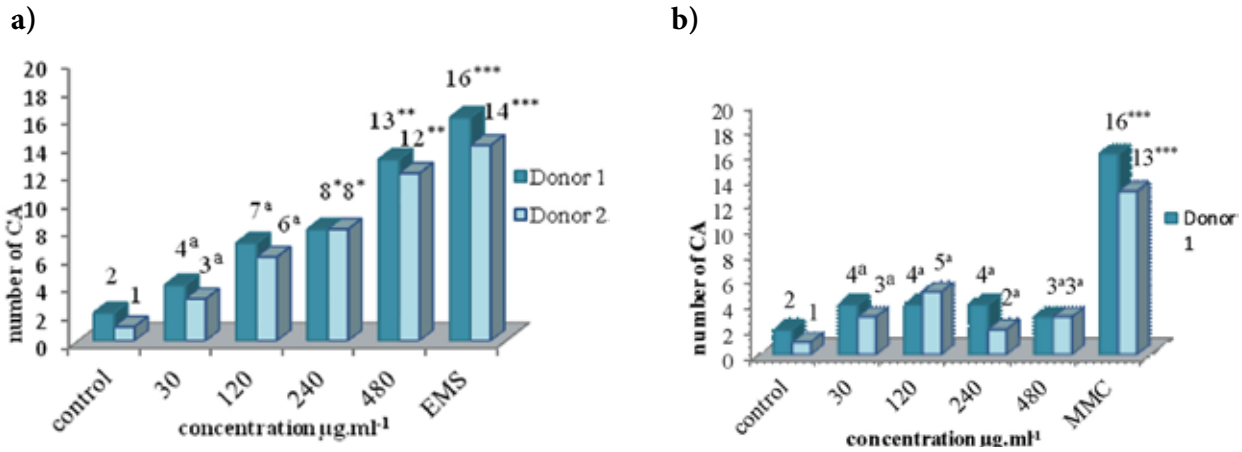

Fig. 2. Frequency of chromosomal aberrations after 24h (a) and 48 h(b) exposure to thiacloprid

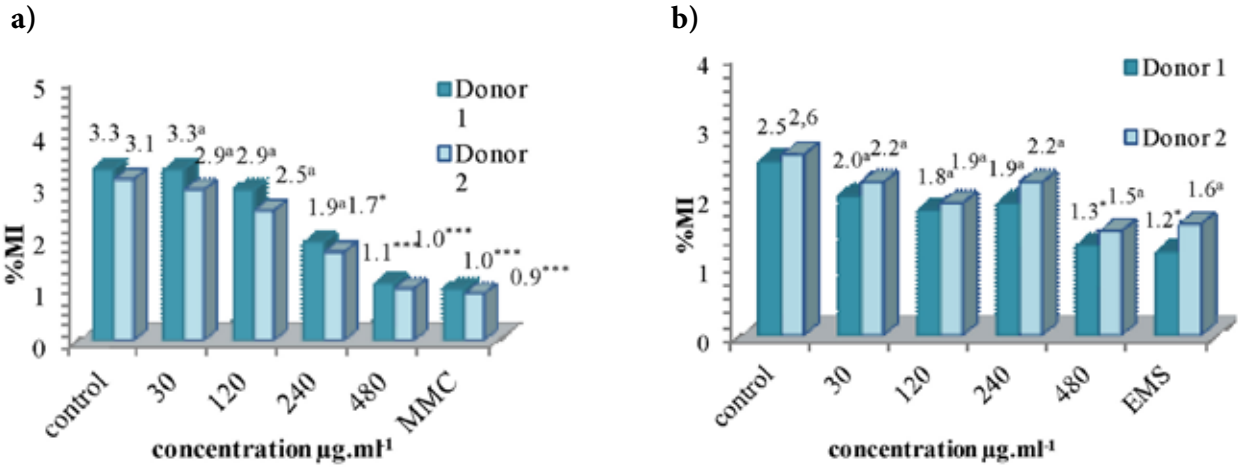

Fig. 3: Reduction in mitotic index after 24 h (a) and 48 h (b) exposure to thiacloprid

Donor 1 and to the two highest concentrations ($P < 0.05$ and $P < 0.001$) in Donor 2 (Fig. 3b).

DISCUSSION

The progressive development of industry and agriculture results in the increasing introduction of complex chemical substances of varying character and origin into the environment, such as agrochemicals, heavy metals, medicines and many various industrial wastes. We refer to them by the common name of xenobiotics. One of the most serious classes of xenobiotics are pesticides that are intensively applied in the agroindustry sector to control pests and weeds in order to ensure adequate protection of crop and increase agricultural yields. Pesticides belong among the most used chemicals around the world [4] and their negative impact on the environment is increasingly noticeable.

Farm animals exposed to the influence of pesticides may present risk to human health as they are an important part of the human food chain. Genotoxicity and its impact on the health of individuals is considered the most serious side-effect of pesticides [2]. Studies were conducted reporting the effects of the commercial fungicide Raxil with the active ingredient of tebuconasole [6], tolylfluanid [10], herbicide bifenox [14], insecticide bendiocarb [8] and others.

By the Decision of the European Comission from 2013, the use of three types of neonicotinic insecticides (imidacloprid, thiamethoxam and clothianidin) is currently limited due to their negative effect on honey bees. The fact that bees favour crops containing these substances raises a suspicion that they act as drugs and can gradually induce addiction in bees. At the same time, neonicotinic pesticides disturb their immune system, making them more susceptible to viral infections to which they are normally resistant [5].

The basis of confirmation of the genotoxic effects of a chemical *in vitro* is the detection of chromosomal damage linearly dependent on concentrations of the active ingredient, while the lowest dose should not cause any significant changes. The highest frequency of the induced chromosomal aberrations is observed during the first cellular cycle and is decreased to about half in subsequent divisions [1].

In our experiments, after a 24 h exposure, we observed a significant increase in aberrations after the application of the two highest concentrations of 240 and 480 $\mu g.ml^{-1}$ ($P < 0.05$ and $P < 0.01$). After a longer exposure to the insecticides (48 h), no clastogenic effect of thiacloprid was observed.

Chromosomal aberrations are recognised as a valuable biological marker of genotoxic effects and probably the only internationally standardized and validated cytogenetic biomarker [1]. Despite extensive use of thiacloprid there have not been published many studies involved in testing of its genotoxic effect. In V79 cells of Chinese hamsters no statistically or biologically important increase in chromosomal aberrations (96.8—97.2 %) was observed after their exposure to pure thiacloprid [13]. Kocaman et al. [9] described, in their study, genotoxic and cytotoxic/cytostatic effects of thiacloprid *in vitro* in human lymphocytes, where they had observed an increased frequency of chromosome aberrations, sister chromatid exchanges and micronucleus after application of all tested concentrations. Galdíková et al. [7] carried out complex evaluations of potential genotoxic effects of commercial preparation based on thiacloprid (CALYPSO 480 SC) *in vitro* in lymphocytes of the peripheral blood of cattle. In agreement with our results, they observed a significant increase in breakages starting from concentration of 120 $\mu g.ml^{-1}$, after 24 h of exposure to thiacloprid.

In the hybridization experiment we failed to record stable aberrations and observed one polyploidy included among numeric aberrations. The inability to detect any translocations in our experiments can be ascribed to the fact that we had at our disposal only three whole-chromosome probes and thus could investigate only a small percentage of the genome.

CONCLUSION

Our results obtained by conventional cytogenetic analysis allowed us to report the genotoxic effects of the insecticide thiacloprid on bovine peripheral blood lymphocyte chromosomes.

ACKNOWLEDGEMENT

This study was supported by projects VEGA 1/0043/15 and 1/0176/16.

REFERENCES

1. **Albertini, R. J., Anderson, D., Douglas, G. R., Hagmar, K., Hemmiki, F., Merlo, A. T., et al., 2000:** IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutat. Res.*, 463, 111—172.
2. **Anwar, W. A., 1997:** Biomarkers of human exposure to pesticides. *Environ. Health Perspect.*, 105, 801—806.
3. **Calderón-Segura, M. E., Gómez-Arroyo, S., Villalobos-Pietrini, R., Martínez-Valenzuela, C., Carbajal-López, Y., Calderón-Ezquerro, M. C., et al., 2012:** Evaluation of genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed *in vitro* to neonicotinoid insecticides news. *J. Toxicol.*, 2012, 1—11.
4. **Costa, C., Teixeira, J. P., Silva, S., Roma-Torres, J., Coelho, P., Gaspar, J., et al., 2006:** Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. *Mutagenesis*, 21, 343—350.
5. **Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., et al., 2013:** Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States*, 110, 18466–18471.
6. **Fisun, K., Rasgelle, P. G., 2009:** Genotoxic effects of Raxil on root tips and anthers of *Allium cepa* L. *Caryologia*, 62, 1—9.
7. **Galdíková, M., Šiviková, K., Holečková, B., Dianovský, J., Drážovská, M., Schwarzbacherová, V., 2015:** The effect of thiacloprid formulation on DNA/chromosome damage and changes in GST activity in bovine peripheral lymphocytes. *J. Environ. Sci. Health B*, 50, 698—707.
8. **Holečková, B., Šiviková, K. Dianovský, J., 2009:** Effect of N-metylcarbamate pesticide bendiocarb on cattle lymphocytes after *in vitro* exposure. *Acta Biol. Hung.*, 60, 167—175.
9. **Kocaman, A. Y., Regencűzoğullari, E., Topaktasş M., 2014:** *In vitro* investigation of the genotoxic and cytotoxic effects of thiacloprid in cultured human peripheral blood lymphocytes. *Environ. Toxicol.*, 29, 631—641.
10. **Lakatošová, M., Holečková, B., 2007:** Chromosomal aberrations in lymphocyte of cattle exposed to fungicide Euparen Multi (tolylfluanid) (In Slovak). In *Proceedings from Seminar of PhD. Students Devoted to the Memory of Academician Boďa*, UVLF, Košice, 59—62.
11. **Marrs, T. C.** (Ed.)**, 2012:** *Mammalian Toxicology of Insecticides*. Royal Society of Chemistry, Cambridge, 506 pp.
12. **Natarajan, A. T., 2001:** Fluorescence *in situ* hybridization (FISH) in genetic toxicology. *J. Environ. Pathol. Toxicol. Oncol.*, 20, 293—298.
13. **Pesticide Residues in Food, 2006:** *Evaluations 2006*, Part II Toxicological. Joint FAO/WHO Meeting on Pesticide Residues, Rome, Italy, 595 pp.
14. **Šivikova, K., Dianovský, J., Piešová, E., 2002:** Genotoxic effects of pesticides *in vitro* (In Slovak). *Ekotoxikologické biotesty*, 1, 156—161.
15. **Tomizawa, M., Casida, J. E., 2003:** Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.*, 48, 339—364.

Received May 29, 2017
Accepted July 15, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0027

FOLIA VETERINARIA, 61, 3: 52—56, 2017

THE EFFECT OF INORGANIC OR ORGANIC ZINC ON THE MORPHOLOGY OF THE INTESTINE IN BROILER CHICKENS

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ABSTRACT

This study compared the effect of dietary supplementation with an inorganic or organic zinc source on the gut morphology in the jejunum of broilers. One-day-old chickens were fed a basal diet (Control group: BD — 32 mg Zn.kg^{-1} DM), or the same BD supplemented with 30 mg or 70 mg of Zn per kg of DM in the form of $ZnSO_4$. H_2O (Group 1: 30 mg $ZnSO_4$; Group 2: 70 mg $ZnSO_4$), and 30 mg or 70 mg of Zn per kg of DM in the form of zinc chelate of glycine hydrate (Group 3: 30 mg Zn-Gly; Group 4: 70 mg Zn-Gly) for 40 days. The villus height was increased in the groups which received 30 mg $ZnSO_4$ and 70 mg $ZnSO_4$ and or 70 mg $ZnSO_4$, as compared to the BD and 30 mg Zn-Gly. The villus surface was higher in all groups receiving the Zn supplements in comparison to the BD.

Key words: chickens; intestine; morphometry; zinc

INTRODUCTION

The nutritional importance of zinc has been known for a long time, but in the past decades its importance in immune modulation has gained increased recognition. Zn can come from organic or inorganic sources. The organic forms of zinc include: amino acid chelates, bioplexes, proteins, as well as lactates and acetates. Zinc from amino acid complexes has been reported to be more bioavailable than Zn from the inorganic sources [1, 15]. Zinc-glycin (Zn-Gly) complex has a slightly higher stability constant for Zn than methionine which could be important for better availability for absorption. Zn-Gly can directly or indirectly influence the function of intestinal mucosa and improve the utilization of dietary energy. The immune response of chickens may be modified by the level of zinc in the diet. Supplementation of Zn in diets also improves intestinal morphology by increasing the villus height and reducing the crypt depth in animals [4, 10].

The National Research Council [9] recommended 40 ppm for broiler chickens, which appeared to be based on the results that considered growth performance as the only

criterion [5, 12]. However, there are several reports that demonstrate that higher Zn levels [60—180 ppm] produce better immune growth performance and intestinal function of broiler chickens [13, 14].

These discrepancies prompted us to evaluate the morphology in the caudal part of jejunum after feed supplementation with different levels of organic and inorganic zinc.

MATERIALS AND METHODS

Animals

A total of 210 one-day-old ROSS 308 hybrid broilers (MACH Hydina Budmerice Ltd., Slovakia) of both sexes were randomly assigned into 5 treatment groups consisting of 6 replicate pens with 7 chickens in each pen. Dietary treatments included the unsupplemented basal diet (BD, Control) and the same BD supplemented with 30 or 70 mg.kg^{-1} added Zn from $ZnSO_4.H_2O$ (Sigma-Aldrich, USA) or zinc chelate of glycine hydrate (Glycinoplex-Zn 26, Phytobiotics, Germany). Commercial broiler starter (1—19 days) and grower (20—39 days) diets were formulated as a basal diet (BD) with no supplemental zinc for the control treatment (Table 1). The mean analysed values of the Zn content in the starter and grower basal diets were 31.6 and 31.9 mg.kg^{-1}, respectively. During the 40-day feeding trial, all birds were offered the BD supplemented with two levels of the inorganic Zn source ($ZnSO_4$ 30 mg.kg^{-1}, $ZnSO_4$ 70 mg.kg^{-1}) or organic Zn chelate (Gly-Zn 30 mg.kg^{-1}, Gly-Zn 70 mg.kg^{-1}).

The birds were housed in large pens on wood shavings. The environmental temperature was kept at about 35 °C during the first week and then was gradually reduced to reach a final temperature of about 24 °C. The broilers were exposed to 23 h constant light/1 h darkness light schedule and had free access to experimental diets and tap water throughout the experiment.

All procedures were in accordance with European Community guidelines (Directive 2010/63/EU) for animal experiments and the experimental protocol was approved by the Ethics Committee of the Institute of Animal Physiology of the Slovak Academy of Sciences and by the State Veterinary and Food Administration (Ro-4160/13-221).

Table 1. Composition of the basal diets (BD)

Ingredient [%]	Starter diet (Days 1 to 19)	Grower diet (Days 20 to 39)
Wheat, ground	30.96	26.56
Maize, ground	35.00	45.00
Soybean meal, extracted	28.20	25.00
Fish meal	2.50	–
Monocalcium phosphate	0.90	0.95
Limestone	1.70	1.70
Feed salt	0.35	0.36
Coccidiostat	0.05	0.05
Lysine	0.10	0.10
Methionine	0.16	0.20
Vitamin premix a	0.04	0.04
Mineral premix b	0.04	0.04
Nutrient composition		
Dry matter [g.kg^{-1}]	883.02	881.55
Crude protein [g.kg^{-1}]	210.75	182.19
Crude fat [g.kg^{-1}]	28.27	27.44
Crude fibre [g.kg^{-1}]	30.85	29.97
Lysine [g.kg^{-1}]	12.10	9.90
Methionine [g.kg^{-1}]	5.09	4.89
Zinc [mg.kg^{-1}]	31.64	31.86
Manganese [mg.kg^{-1}]	90.99	88.48
Copper [mg.kg^{-1}]	14.23	13.58
ME [MJ.kg^{-1}]	12.08	12.24

[a]The vitamin premix provided per kg of diet: vitamin A 12.000 IU; vitamin D3 4000.0 IU; vitamin K 3.0 mg; vitamin E 45.5 mg; vitamin B1 2.0 mg; vitamin B2 6.0 mg; vitamin B6 4.0 mg; vitamin B12 0.02 mg; niacin 40.0 mg; pantothenic acid 12.0 mg; biotin 0.2 mg; folic acid 1.5 mg.
[b]The mineral premix provided per kg of diet: I 0.64 mg; Mn 64.0 mg; Cu 6.4 mg; Se 0.1 mg; Fe 48.0 mg.

Sample collection

After the 40-day feeding period, two birds from each replicate (12 birds/group) were slaughtered for sample collection. Tissue samples from the terminal section of the jejunum were collected for the determination of villus height and surface area.

Intestinal histomorphology

Jejunum samples were fixed in 10 % neutral buffered formalin and prepared using paraffin embedding techniques. Three consecutive sections (5 µm) from each jejunum were stained using haematoxylin and eosin and observed for histomorphology. The villus height and its area (from the tip of the villus to the crypt opening) were measured from 70 to 100 randomly selected villi with one section per chicken at 100× magnification (Fig. 1). The morphometry was evaluated using the NIS-Elements Advanced Research 3.0 Programme (commercial purchased programme).

Statistical analysis

Statistical analysis of the data was done by one-way analysis of variance (ANOVA) with the post hoc Tukey multiple comparison test using GraphPad Software (USA). The differences between the mean values for the different treatment groups were considered statistically significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The values are expressed as means ± standard deviation (SD).

RESULTS

Intestinal morphometry

The dietary Zn supplementation increased the villus height of the jejunum in both groups fed the diets enriched with inorganic Zn source ($ZnSO_4$ 30 mg, $ZnSO_4$ 70 mg.kg^{-1}) and also in Gly-Zn 70 mg.kg^{-1} group (ab $P < 0.001$) compared to the BD and Gly-Zn 30 mg.kg^{-1} groups (Table 2).

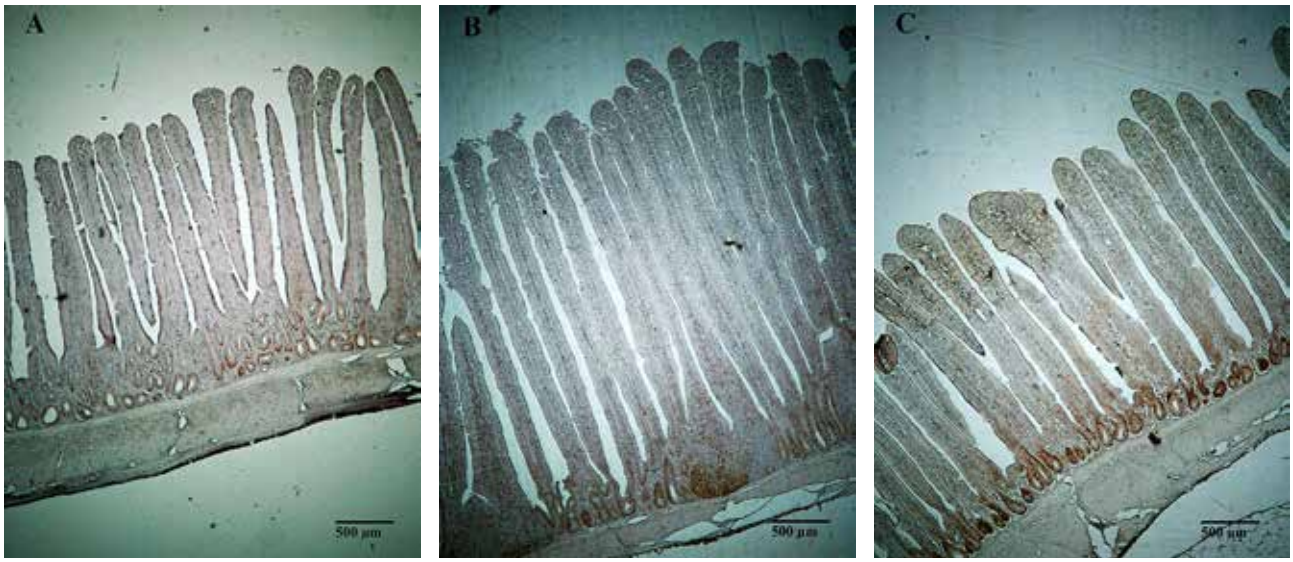

Fig. 1. Histological sections of the jejunum of broilers supplemented with different amount and Zn source (A) — BD; (B) — $ZnSO_4$ 30 mg.kg^{-1}; (C) — Gly-Zn 70 mg.kg^{-1}

Table 2. Effect of Zn supplementation on the histomorphology of the jejunum in broilers

Group	BD	$ZnSO_4$.30 mg^{-1}	$ZnSO_4$.70 mg^{-1}	Gly-Zn.30mg^{-1}	Gly-Zn.70 mg^{-1}
Villus height [µm]	1284 ± 180^{bd}	1462 ± 360^{a}	1492 ± 330^{a}	1204 ± 240^{b}	1376 ± 310^{c}
Villus surface [µm^2]	$152^3 \pm 680^{f}$	$192^3 \pm 1370^{ae}$	$184^3 \pm 910^{a}$	$160^3 \pm 580^{bd}$	$184^3 \pm 640^{c}$

All values are expressed as means ± SD. Means with different superscripts within a row differ significantly (^{ab} — $P < 0.001$; ^{cd} — $P < 0.01$; ^{ef} — $P < 0.05$)

The villus height was higher in the group Gly-Zn 70 mg.kg^{-1} (cd $P < 0.01$) compared to the jejunal villi of broilers fed the BD only.

Low-dose supplementation with inorganic Zn source ($ZnSO_4$ 30 mg.kg^{-1}) led to the increase of the villus surface area in compare to the BD (ef $P < 0.05$) and Gly-Zn 70 mg.kg^{-1} groups (ab $P < 0.001$). Similarly, the higher villus surface area was measured in the $ZnSO_4$ 70 mg.kg^{-1} group (ab $P < 0.001$) compared to the Gly-Zn 30 mg.kg^{-1} as well as in the group Gly-Zn 70 mg.kg^{-1} (cd $P < 0.01$) compared to Gly-Zn 30 mg.kg^{-1}.

DISCUSSION AND CONCLUSIONS

Zinc is known to influence the intestinal morphology and improve absorptive capacity, and enhance growth performance [5, 6]. Moreover, zinc is essential for cell proliferation and differentiation, especially for the regulation of DNA synthesis and mitosis [2). Southon et al. [11] demonstrated that Zn deficiency in rats is accompanied with a reduction of the jejunal villus height, while a short period of zinc supplementation returned the morphology into its normal condition in experimental animals. On the other hand, 42-day-old chickens fed the diet supplemented with 90 mg.kg^{-1} Zn-Gly increased the villus height and decreased the crypt depth of the jejunum [5]. In our experiments, the intake of both diets supplemented with the inorganic Zn source and also with the higher-dose organic chelate of zinc (Gly-Zn 70 mg.kg^{-1}) increased the height of the jejunal villi. Similarly, the surface villus area followed the same pattern as the height of the villi. It should be stressed that the villus height and surface villus area of broilers fed the diet with the addition of 70 mg.kg^{-1} of Zn chelate reached the similar value of both morphometric parameters as the broilers supplemented with the low-dose inorganic source of zinc.

The height of villi and their area can influence the source of supplemented zinc in diets [8]. It is known, that organic zinc chelate used in our trial improves zinc absorption comparing to the inorganic form of zinc. The absorption difference of zinc between the organic and inorganic forms can influence the growth of the intestinal villi. Our results suggest that organic zinc chelate after part absorption, supported better growth of the villi after supplementation of the diet with higher doses of organic zinc chelate. On the other hand, recently published immunological parameters from that experiment [3, 7] as quantification of the expression of MUC-2, IgA gene, and evaluation of secretory IgA in the lumen of the intestine, resulted in better affects found in birds fed diet supplemented with low doses of organic source of zinc.

In conclusion, our results demonstrated that an inorganic zinc source increased the height of villi and surface area of villi already after supplementation of feed with a low dose of zinc. On the other hand, the positive effect on the growth of villi was seen only after administration of a high dose of organic zinc in the feed. The villus surface was higher in all groups receiving the Zn supplements in comparison to just the basal diet.

ACKNOWLEDGEMENTS

This study was supported by the Slovak Research and Development Agency under the contracts No. APVV-15-0165, VEGA 1/0575/16, VEGA 1/0562/16, and Centrum of excellences for infectious disease and zoonosis (Operational program of research and development) financed by EU funds for the regional improvement.

REFERENCES

1. **Bao, Y. M., Choct, M., Iji, P. A., Bruerton, K., 2010:** The digestibility of organic trace minerals along the small intestine in broiler chickens. *Asian-Australasian J. Anim. Sci.*, 23, 90—97.
2. **Beyersmann, D., Haase, H., 2001:** Functions of zinc in signalling, proliferation and differentiation of mammalian cells. *BioMetals*, 14, 331—341.
3. **Bobíková, K., Levkut, M., Jr., Husáková, E., Levkutová, M., Kissová, V., Ivanišinová, O., et al., 2016:** Effect of glycin-zinc complex on mucin and IgA expression, secretory IgA concentration and lengths of intestinal villi in chickens. *J. Comp. Pathol.*, 154, 81.
4. **Burrell, A. L., Dozier, W. A., Davis, A. J., Compton, M. M., Feeman, M. E., et al., 2004:** Responses of broilers to dietary zinc concentrations and sources in relation to environmental implications. *British Poult. Sci.*, 45, 225—263.
5. **Feng, J., Ma, W. Q., Niu, H. H., Wu, X. M., Wang, Y., Feng, J., 2010:** Effects of zinc glycine chelate on growth, haematologi-

cal, and immunological characteristics in broilers. *Biol. Trace Elem. Res.*, 133, 2003—2011.

6. **Katouli, M., Melin, L., Jensen-Waern, M., Wallgren, P., Möllby, R., 1999:** The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. *J. Appl. Microbiol.*, 87, 564—573.
7. **Levkut, M., Jr., Husáková, E., Bobíková, K., Karaffová, V., Levkutová, M., Ivanišinová, O., et al., 2017:** Inorganic or organic zinc and MUC-2, IgA, IL-17, TGF-β4 gene expression and sIgA secretion in broiler chickens. *Food Agricult. Immunol.*, 28, 801—811.
8. **Lönnerdal, B., 2000:** Dietary factors influencing zinc absorption. *J. Nutr.*, 130, 1378S–1383S.
9. **National Research Council, 1994:** *Nutrient Requirements of Poultry*. 9th edn., Washington, DC, National Academic Press, 157 pp.
10. **Payne, R. L., Bidner, T. D., Fakler, T. M., Southern, L. L., 2006:** Growth and intestinal morphology of pigs from sows fed two zinc sources during gestation and lactation. *J. Anim. Sci.*, 84, 2141—2149.
11. **Southon, S., Gee, J. M., Bayliss, C. E., Wyatt. G. M., Horn, N., Johnson, I. T., 1986:** Intestinal microflora, morphology and enzyme activity in zinc-deficient and Zn-supplemented rats. *Br. J. Nutr.*, 55, 603—611.
12. **Sunder, G. S., Panda, A. K., Gopinath, N. C. S., Rama Rao, S. V., Raju, M. V. L. N., Reddy, M. R., et al., 2008:** Effects of higher levels of zinc supplementation on performance, mineral availability, and immune competence in broiler chickens. *J. Appl. Poult. Res.*, 17, 79—86.
13. **Tang, Z., Wen, C., Li, P., Wang, T., Zhou, Y, 2014:** Effect of zinc-bearing zeolite clinoptololite on growth performance, nutrient retention, digestive enzyme activities, and intestinal function of broiler chickens. *Biol. Trace Elem. Res.*, 158, 51—57.
14. **Wenqiang, M. A., Niu, H., Feng, J., Wang, Y., Feng, J., 2011:** Effect of zinc glycine chelate on oxidative stress, contents of trace elements, and intestinal morphology in broilers. *Biol. Trace Elem. Res.*, 142, 546—556.
15. **Yu, Y., Lu, L., Wang, R. L., Xi, L., Luo, X. G., Liu, B., 2010:** Effects of source and phytate on zinc absorption by in situ ligated intestinal loops of broilers. *Poult. Sci.*, 89, 1146—1155.

Received May 17, 2017
Accepted August 17, 2017

DE GRUYTER
OPEN

DOI: 10.1515/fv-2017-0028

FOLIA VETERINARIA, 61, 3: 57—64, 2017

DETERMINATION OF ANTIBIOTIC RESIDUES IN MILK BY MICROBIAL INHIBITORY TESTS

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ABSTRACT

Undesirable substances enter the organism of animals mostly via feed, water or veterinary medicines and their residues pass subsequently into the products of animal origin. In dairy cows, sheep and goats these residues are eliminated particularly in milk. Milk intended for human consumption must comply with safety criteria also with respect to residues of antibiotics. The aim of this study was to determine the presence or absence of antibiotic residues in the milk using the tests Milchtest and Premi®Test. While the Milchtest was developed for the determination of antibiotic residues in cow, sheep and goat milk, the Premi®Test is intended for the determination of antibiotic residues in meat juice, liver, kidneys, fish, eggs and in the urine of animals treated with antibiotics. As examined matrices, we used 45 samples of raw cow's milk collected at 3 agricultural farms and 10 samples of milk offered to consumers at grocery stores. When using the Milchtest, 8 samples tested positive and 10 provided dubious results while testing with the Premi®Test showed that only 6 samples were positive for antibiotics. Comparison of the results confirmed a higher detection sensitivity of Milchtest reflected in higher numbers of positive samples and the detection of dubious results in samples of raw cow's milk. However, it should be noted that even the Premi®Test, although not intended preferably for the determination of antibiotics in milk, can be used, if needed, for the preliminary screening of antibiotic residues in such a matrix.

Key words: antibiotics; milk; residues; screening

INTRODUCTION

Milk is considered one of the most valuable foods and plays an important role in the life of animals and especially in humans. It is the first source of nutrition for infant mammals before they are able to digest other types of food. Therefore it is important to ensure the highest possible nutritional and hygiene standard of milk [5].

Milk is a valuable source of important substances such as milk fat, proteins, lactose (milk sugar), vitamins and

minerals. These substances are unique for the human body providing a considerable proportion of energy and nutrients. Milk fat is easily absorbable and digestible. Milk proteins are a source of a number of essential amino acids essential for the organisms. Lactose is a valuable and easily digestible source of energy, which also supports the regulation of gut function.

According to the Regulation (EC) No. 853/2004 of the European Parliament and the Council [8], laying down specific hygiene rules for food of animal origin, raw milk must originate from animals that show no signs of infectious diseases transmissible to humans via milk. The animals must be in good health, show no clinical signs of diseases that may contaminate milk, they should not suffer from detectable udder inflammation, nor udder trauma that can affect the milk. The animals should not be administered any unpermitted substances or medications and if they were administered permitted substances or medications, the withdrawal periods prescribed for these substances or medicines must be observed [8].

The use of antibiotics for the treatment of animals kept for the production of food of animal origin can result in the presence of residues of these substances in the products, including the milk, pass through the food chain directly to the consumer and seriously affect human health. In the interest of protecting consumer's health and ensuring food safety, European legislation specifies a maximum residue limits (MRL) for all pharmacologically effective substances administered to animals kept for the production of food of animal origin. MRL is defined as the maximum concentration of residue of pharmacologically active substance accepted in a food product as a guarantee of its safety [1, 9].

Antibiotics belong among pharmacologically active substances with potent health risks. As their finding in food of animal origin is not rare, the development of reliable screening methods that can detect their presence in food appears essential. Currently, there are available many screening methods differing in: the type of the test strain used; susceptibility of test strains to individual types or groups of antibiotics; and the determination procedures and temporal or financial demands [7].

Milchtest (Packhaus Rockmann, Germany) is one of the most recent microbial inhibitory tests developed for the determination of antibiotic residues in raw, thermally treated and dried cow, sheep and goat milk. It is simple, provides accurate results and exhibits adequate sensitivity in the determination of residues of: beta-lactams, tetracyclines, macrolides, aminoglycosides, sulphonamides and trimetoprim at the level of MRL. Version Milchtest MT 25 FARM is recommended for small milk producers for the purpose of self-control and Milchtest MT 50 was developed for large producers and industrial milk-processing units. Flexible microplates MT 96 FP and MT 288 FP are recommended for the examination of a greater number of milk samples and official controls. Just as the Premi®Test, the Milchtest also combines the principle of agar diffusion tests with indicator colour changes due to the active metabolism of the test strain Bacillus stearothremophillus var. calidolactis at inhibitor absence. The blue-violet colour of the agar nutrient medium indicates the presence of inhibitory substances (antibiotics) in the tested sample [3].

The Premi®Test (R-Biopharm AG, Germany) is a broad-spectrum microbiological screening test intended for the determination of antibiotic residues in food and raw materials of animal origin. It is usable also for the determination of antibiotic residues in feed and body fluids (urine). The Premi®test allows one to determine the presence of the most important groups of antibiotics (β-lactams, cephalosporins, macrolides, tetracyclines, aminoglycosides) at or below their MRL. Premi®Test also combines the principle of agar diffusion tests with changes in an indicator colour due to the active metabolism of the test strain in the absence of an inhibitor. The tested sample is dosed to vials containing the test strain Bacillus stearothremophillus var. calidolactis. Normal growth of the test strain during incubation results in a colour change of a pH indicator from blue-violet to yellow. If the sample contains substances inhibiting the growth of the test strain (antibiotics), the blue-violet colour of the indicator remains unchanged. The Premi®Test is available in two versions, Premi®Test 25 and Premi®Test 4x25 [4, 10].

With regard to the fact that both tests are based on the inhibition of growth of the test strain Bacillus stearothremophillus var. calidolactis and are performed using the same principle, conditions of application and incubation of samples, and evaluation of results with the exception of the tested food matrices, we decided to use these two microbial inhibitory tests for determination of the presence or absence of antibiotics in milk intended for human consumption and compare their performances.

MATERIALS AND METHODS

During 2016 and 2017, we tested 45 samples of raw cow's milk collected at 3 agricultural farms registered in Slovakia and 10 samples of milk produced in Slovakia and Czechia and offered to consumers at grocery stores. The samples were stored in a freezer at 1—8 °C. Immediately before analysis, the samples were defrosted and mixed thoroughly. They were examined for the presence of residues using the procedures specified by the manufacturers of Premi®test and Milchtest.

Milchtest: Using disposable tips, 50 µl aliquots of the samples were transferred to vials supplied with the test kit. The vials were marked, sealed with foils supplied by the manufacturer as a part of the test kit and transferred to a thermoblock (Dry Bath EMK 20, Euroclone S. p. A, Pero, Italy) where they were incubated for about 3 hours at a temperature of 65 ± 0.5 °C. The test was terminated when the colouring of the agar medium of the negative control changed from violet to yellow. The results were evaluated using a colour scale supplied by the manufacturer. Yellow colour indicated the absence of antibiotics in the sample, while violet colour indicated the presence of antibiotic residues. Yellow-green or yellow-violet colouring of the agar medium indicated the presence of antibiotics at a level equal to the test detectability level.

Premi®Test: By means of a micropipette with a defined volume, 100 µl aliquots of a milk sample were transferred into the provided vials. The vials were marked, sealed with foils supplied by the manufacturer and transferred to a thermoblock (Acublock Digital Dry Bath D 1200, Labnet, Edison, USA) where they were incubated for approximately 3.5 hours at a temperature of 64 ± 0.5 °C. The test was terminated when the colouring of the agar medium of the negative control changed from violet to yellow. After incubation, we examined the vials for a positive or negative reaction. A yellow colour indicated the absence of antibiotics in the sample, while a violet colour indicated the presence of antibiotic residues. Yellow-violet colouring of the agar medium indicated the presence of antibiotics at a level equal to the test detectability level.

RESULTS AND DISCUSSION

Samples of raw cow's milk and milk obtained from grocery stores were tested for the presence of antibiotic residues. Also, the suitability of both methods for the rapid screening of antibiotic residues in milk were compared.

When using the Milchtest, a positive result was obtained in 8 samples of milk, while using the Premi®Test only 6 samples were positive. The positive reaction indicating the presence of residues exceeding the level of test detectability was manifested by the violet colour of the agar medium, as the colour of the Ph indicator remained unchanged due to the inhibition of growth of the test strain (Bacillus stearothermophilus var. calidolactis) by the presence of the antibiotics.

Negative results were indicated by the yellow colour of the agar medium resulting from the metabolic activity of the test strain and relevant change in the indicator colouring from blue-violet to yellow. When using the Milchtest, 37 samples were negative, while the Premi®Test indicated negativity in 49 of the samples.

Other colour shades from yellow, yellow-green to yellow-violet, which did not allow us to decide unambiguously whether the samples were positive or negative, resulted from the inhibition of the growth of the test strain by the presence of antibiotic at the level of test detectability or immediately below it. Such samples were considered dubious and must be subjected to confirmation analysis for confirmation or negation of the positive result. When using the Milchtest, we obtained 10 dubious results, while there were no dubious results with the Premi®Test.

The results obtained in this study are presented in Tables 1—4 and Figures 1—8.

The basic aim of food legislation is to prevent any health risks associated with food. The Regulation (EC) No. 853/2004 sets safety criteria for raw milk. These criteria involve the so-called trigger values — if any of them is exceeded, the operators of the food processing establishments are obliged to take corrective measures and report this to the relevant authority. Animals treated with antibiotics that could result in excretion of their residues in milk must be identified so that the milk produced by them before the end of withdrawal period is not used for human consumption. This means that raw milk containing residues of antibiotics in amounts exceeding MRL permitted according to Commission Regulation (EU) No. 37/2010 or milk containing combination of residues exceeding in total any maximum acceptable value must not be placed on the market [1, 8].

In our study we used two microbial inhibitory tests to determine presence/absence of antibiotics in raw cow's

Table 1. Results of the examination of raw cow's milk for the presence/absence of antibiotic residues using the Milchtest and Premi®Test — Farm 1

Test	Sample														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Milchtest	+	+	–	+	±	–	–	–	–	–	+	–	–	–	–
Premi®Test	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–

+ — positive; ± — dubious; – — negative

Table 2. Results of the examination of raw cow's milk for the presence/absence of antibiotic residues using the Milchtest and Premi®Test — Farm 2

Test	Sample														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Milchtest	±	–	±	–	–	–	–	±	–	+	–	+	–	–	±
Premi®Test	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–

+ — positive; ± — dubious; – — negative

Table 3. Results of the examination of raw cow's milk for the presence/absence of antibiotic residues using the Milchtest and Premi®Test — Farm 3

Test	Sample														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Milchtest	±	–	–	–	–	±	–	–	–	±	+	+	–	+	±
Premi®Test	–	–	–	–	–	–	–	–	–	+	–	+	–	+	–

+ — positive; ± — dubious; – — negative

Table 4. Results of the examination of cow's milk for the presence/absence of antibiotic residues using the Milchtest and Premi®Test — grocery store

Test	Sample									
	1	2	3	4	5	6	7	8	9	10
Milchtest	–	–	–	–	–	–	–	–	–	–
Premi®Test	–	–	–	–	–	–	–	–	–	–

– — negative

Fig. 1. Examination of raw cow's milk for the presence/absence of antibiotic residues using the Milchtest — Farm 1

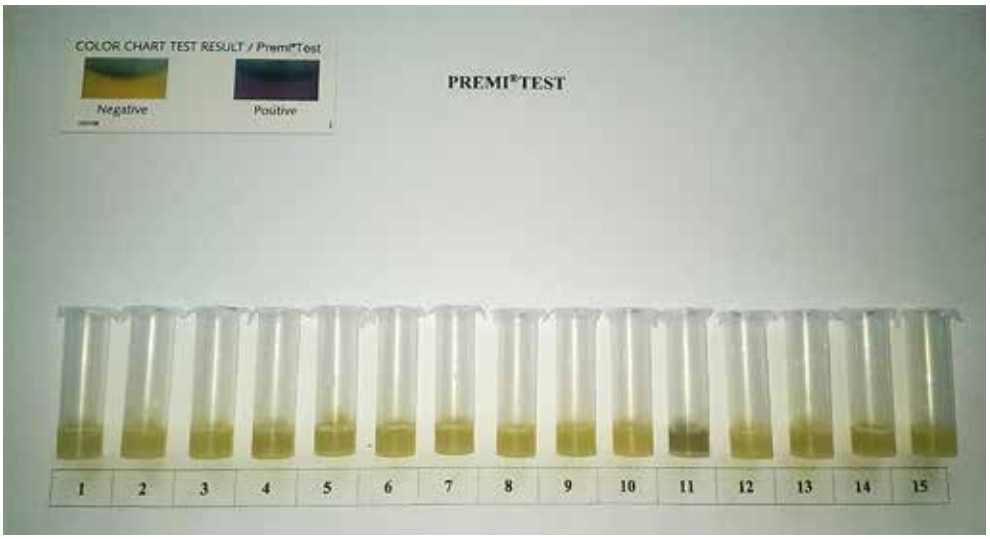

Fig. 2. Examination of raw cow milk for the presence/absence of antibiotic residues using the Premi®Test — Farm 1

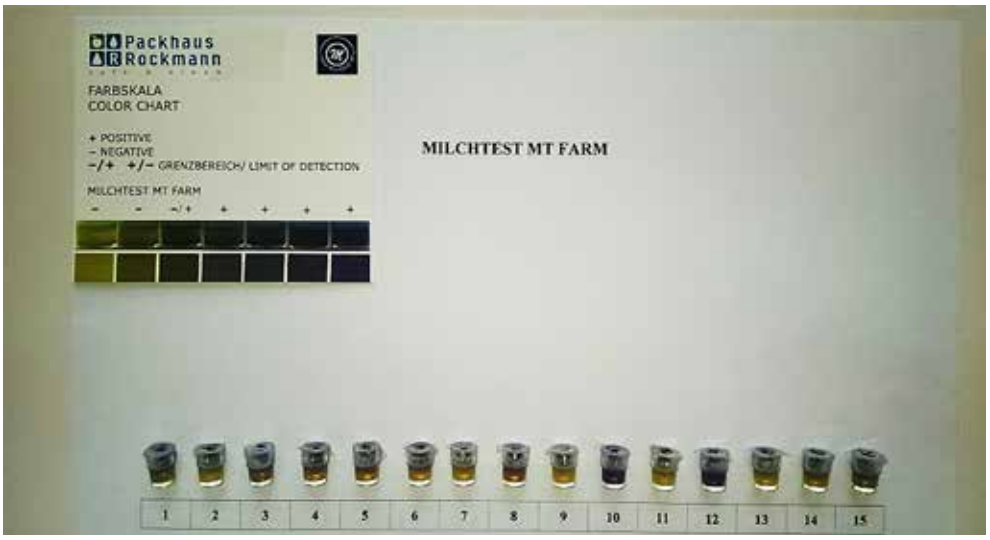

Fig. 3. Examination of raw cow's milk for the presence/absence of antibiotic residues using the Milchtest — Farm 2

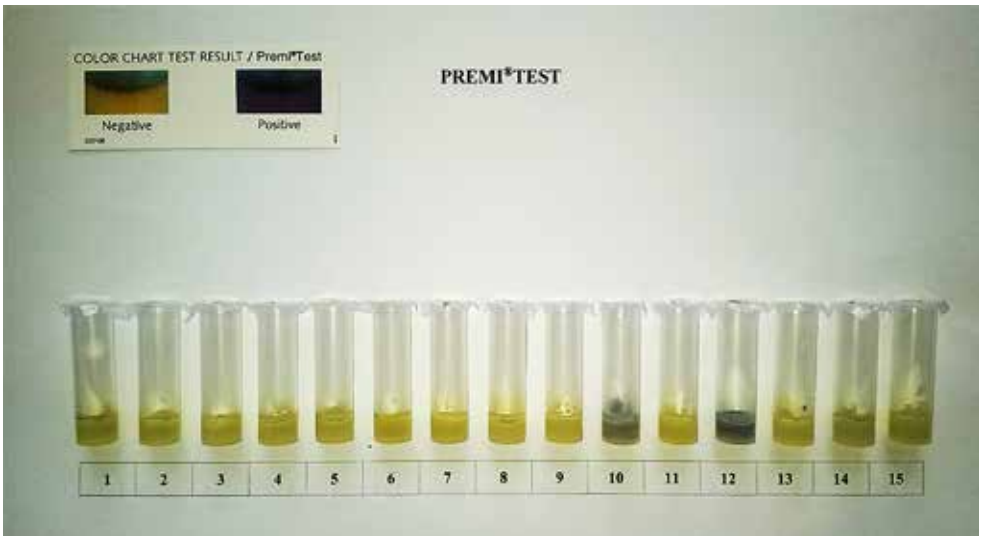

Fig. 4. Examination of raw cow's milk for the presence/absence of antibiotic residues using the Premi®Test — Farm 2

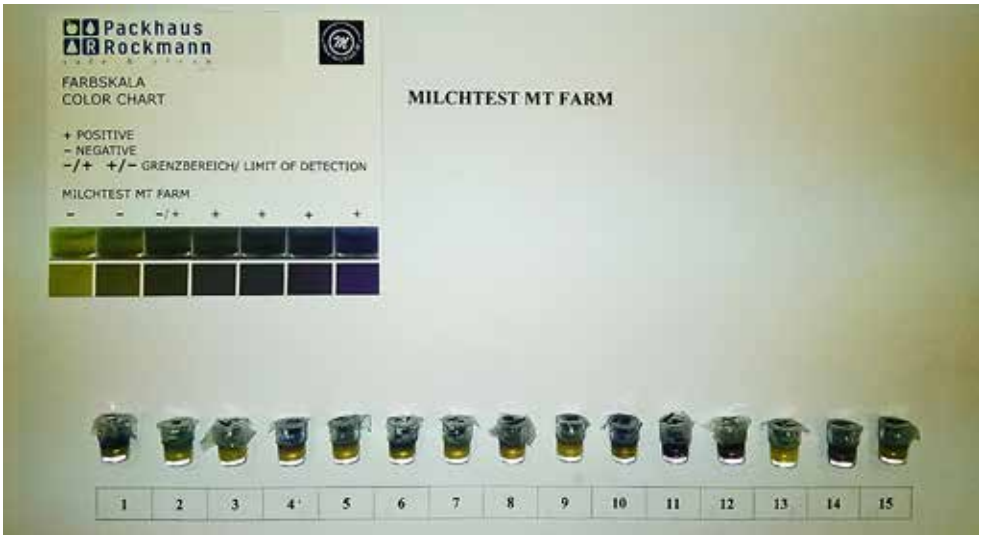

Fig. 5. Examination of raw cow's milk for the presence/absence of antibiotic residues using the Milchtest — Farm 3

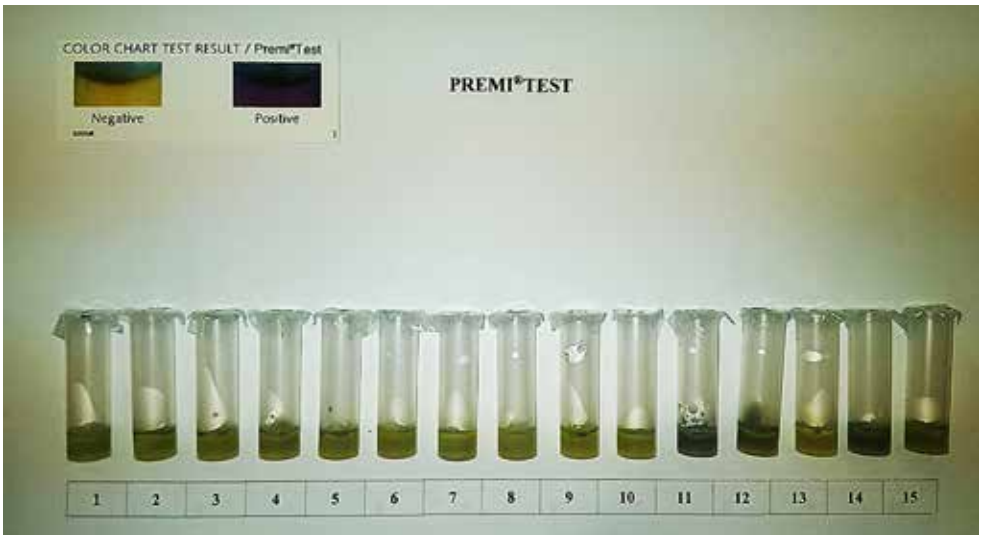

Fig. 6. Examination of raw cow's milk for the presence/absence of antibiotic residues using the Premi®Test — Farm 3

Fig. 7. Examination of cow's milk for the presence/absence of antibiotic residues using the Milchtest — grocery store

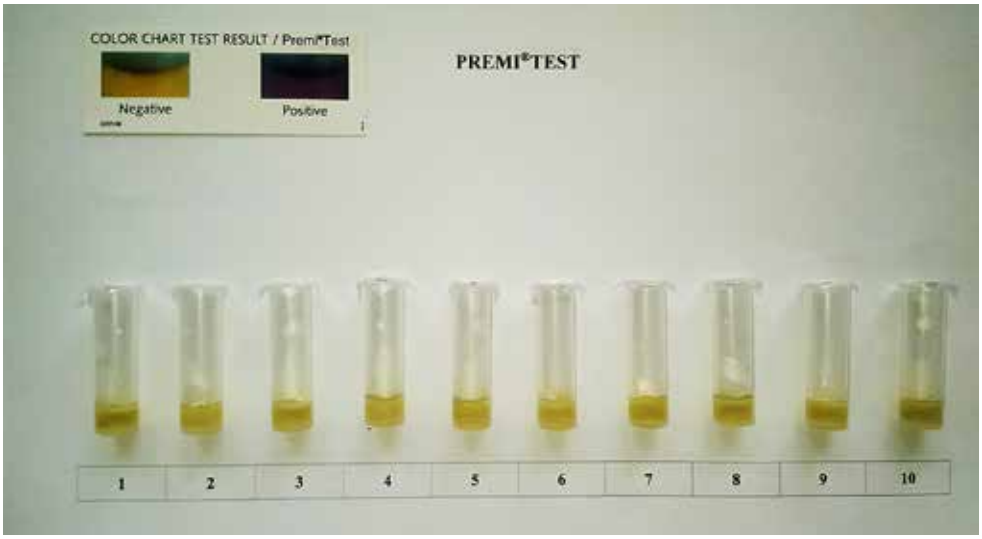

Fig. 8. Examination of cow's milk for the presence/absence of antibiotic residues using the Premi®Test — grocery store

milk and milk offered for sale. The test strain, Bacillus stearothermophilus var. calidolactis, used in these tests is generally considered the most susceptible microbial strain for broad-spectrum analysis of antibiotic residues. It showed the highest susceptibility particularly to those groups of antibiotics, for example beta-lactam antibiotics, that are most frequently used in veterinary practice to treat animals producing milk for human consumption [2, 6].

The rapid microbial inhibitory tests are a practical and valuable tool for the primary screening of antibiotic residues in milk, not only from the point of view of eliminating potential health risk, but also prevention of economic losses. They allow one within a short time to obtain results confirming or negating the presence of residue/residues of antibiotics in the milk samples. Although manufacturers of these rapid screening tests supply with them a relevant colour change scales declaring positive, dubious and negative results that support the objectivity of evaluation and the drawing of relevant conclusion, each positive or dubious result must be validated by confirmation analysis and result in adequate correction measures.

CONCLUSIONS

Our study compared the results of two commercial mi-

crobial inhibitory tests, the Milchtest and Premi®Test, used for the detection of presence/absence of antibiotic residues in raw cow's milk and milk placed on the market. Both tests showed the presence of residues only in raw cow's milk obtained directly from agricultural farms. With regard to the higher number of positive and dubious results obtained with the Milchtest, this tests appeared more sensitive, however, if immediate screening and preliminary conclusions are needed, it is possible to use also the Premi®Test for the detection of the presence of antibiotics in milk intended for human consumption.

ACKNOWLEDGEMENT

The study was supported by the projects VEGA MŠVVaŠ SR and SAV No. 1/0576/17.

REFERENCES

1. **Commission Regulation (EU) No. 37/2010 of 22 December 2009**, on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union*, L15, 2010, 1—72.
2. **Gaudin, V., Maris, P., Fuselier, R., Ribouchon, J. L., Cadieu, N., Rault, A., 2004:** Validation of microbiological method: the STAR protocol, a five-plate test, for the screening of antibiotic residues in milk. *Food Addit. Contam.*, 21, 422—433.
3. http://www.milchtest.de/product/.
4. http://www.r-biopharm.com/products/food-feed-analysis/residues/antibiotics/premitest/item/premitest-25.
5. **Kardošová, A., 2010:** Inspection of farm milk production hygiene (In Slovak). In *Proceedings of lectures and posters of the international scientific conference Hygiena Alimentorum XXXI*, May 25–27, University of Veterinary Medicine and Pharmacy, Košice, Slovakia, 275—277.
6. **Pikkemaat, M. G., 2009:** Microbial screening methods for detection of antibiotic residues in slaughter animals. *Anal. Bioanal. Chem.*, 395, 893—905.
7. **Poláková, Z., Kožárová, I., 2014:** History of the development of screening methods (In Slovak). In *Proceedings of lectures and posters of the XLIVth conference on food hygiene and technology "Lenfeldovy a Höklovy dny"*, October 15—16. Veterinary and Pharmaceutical University in Brno, Czechia, 150—154.
8. **Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004** laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union*, L139, 2004, 14—74.
9. **Regulation (EC) No. 470/2009 of the European Parliament and of the Council of 6 May 2009** laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No. 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No. 726/2004 of the European Parliament and of the Council. *Official Journal of the European Union*, L152, 2009, 1—12.
10. **R-26:** Determination of residues of inhibitory substances in meat by Premi®Test method (In Slovak). http://www.svssr.sk/dokumenty/zakladne_info/Diagnostika/R%2026%20PREMi%20test.pdf.

Received May 22, 2017
Accepted August 19, 2017

DE GRUYTER
OPEN

DOI: 10.1515/fv-2017-0029

FOLIA VETERINARIA, 61, 3: 65—70, 2017

THE INCIDENCE OF HEPATITIS E VIRUS IN DOMESTIC PIGS

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of hepatitis E — an emerging zoonotic disease distributed worldwide. The aim of this study was to determine the prevalence of HEV in Slovakian domestic pigs, as this has not been studied yet. Clinical samples (n = 269) from fourteen randomly selected domestic pig farms for three different age categories of pigs were analysed and the subsequently detected isolates were genetically characterized. The reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that 32 pigs (11.9 %) of all age categories were HEV RNA positive. The highest occurrence was detected in fattening pigs (14.8 %) and the lowest in weaning pigs (12.5 %). The HEV RNA was not observed at all in the youngest category (the suckling piglets). The phylogenetic analysis revealed that all Slovak HEV isolates clustered into two genetic groups of the genotype HEV-3.

Key words: domestic pig; hepatitis E virus (HEV); rectal swab

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a small non-enveloped virus with a virion size of approximately 27—34 nm in diameter containing a positive-sense single-stranded RNA with a length of 6.6—7.3 kb. The genome is organized into three open reading frames (ORF1-3). ORF1 and 3 encoded non-structural proteins and ORF2 encodes the viral capsid protein [13]. HEV belongs to the *Hepeviridae* family, which has recently been split into two genera, *Orthohepevirus* and *Piscihepevirus*. Genus *Orthohepevirus* is classified into four species designated *Orthohepeviruses* A–D. *Orthohepevirus* A is formed by seven genotypes, that infect humans (HEV-1, -2, -3, -4 and -7), pigs (HEV-3 and -4), rabbit (HEV-3), wild boar (HEV-3, -4, -5 and -6), mongoose (HEV-3), deer (HEV-3), yak (HEV-4) and camel (HEV-7) [23].

After the first characterization of HEV in domestic pigs by Meng et al. [13] in the USA, the virus has been detected in the same animal species in many other countries including Canada [18], Netherlands [25], United Kingdom [1], Italy [3], France [21] and Spain [11]. At the same time

swine HEV has been detected and characterized in such neighbouring countries as Hungary and Czechia [8, 4].

Domestic pigs are believed to be an important source of zoonotic transmission of HEV-3 and HEV-4 and they are responsible for sporadic human hepatitis E cases worldwide [17]. It is critical to know the prevalence of HEV in domestic pig farms so as to be able to assess the health situation of the pig population and thus estimate the risk to public health.

The objective of this study was: to observe HEV in domestic pigs in Slovakia, which has not been determined as yet: to characterize the genetically detected isolates; and to gain an insight into the incidence of HEV in the Slovak domestic pig population.

MATERIALS AND METHODS

Samples

Clinical samples (rectal swabs, n = 269) were obtained from the pigs of fourteen randomly selected Slovakian domestic pig farms. Three different age categories were distinguished: suckling piglets (< 28 days, n = 35), weaners (28—70 days, n = 112), and fattening pigs (> 70 days, n = 122). No clinical sign of any disease was observed. The health status of each pig was evaluated by a qualified veterinarian on each farm.

Isolation of RNA and synthesis of cDNA

The rectal swabs were processed in the laboratory by elution into 1 ml of 0.01 mol.l^{-1} PBS (Merck Millipore Corp., USA) for 30 min. The eluted solution was vortexed at 2000 rev. min^{-1} for 3 min and then centrifuged at 17 500 × g for 5 min. The total RNA was isolated using TRIzol Reagent (Life Technologies, USA) from 200 µl of a sample according to the manufacturer's instructions and dissolved in 20 µl of molecular biology grade water (Merck, GmbH, Germany). Samples with aliquots of the isolated RNA were stored at –80 °C. The cDNA was synthesized in a 20 µl reaction mixture comprising 5 µl of isolated RNA, 5 µM of random hexamers (Invitrogen, USA), 50 µl dNTPs (Thermo Fisher Scientific, Inc., USA), 200 U RevertAid Premium reverse transcriptase with 1xRT buffer (Thermo Fisher Scientific, Inc., USA), 20 U RNase inhibitor (Takara Bio, Inc., Japan), and molecular biology grade water (Merck, GmbH, Germany). The mixture was incubated at 65 °C for 5 min and then chilled on ice to destroy the RNA secondary structure. Subsequently, the mixture was incubated at 25 °C for 10 min, then at 50 °C for 30 min to synthesise cDNA, and at 85 °C for 5 min to terminate the reaction.

Detection of HEV by nested reverse transcriptase polymerase chain reaction (RT-PCR)

The detection of the partial HEV genome was based on the amplification of a 242 bp fragment of ORF1 gene using outer and inner primers published by Erker et al. [5]. The PCR reaction mixture (25 µl) was composed of 1x ThermoPol reaction buffer (New England Biolabs, Inc., USA), 200 µM dNTPs (Thermo Fisher Scientific, Inc., USA), 0.3 µM of outer primers, 0.5 U Taq DNA polymerase (New England Biolabs, Inc., USA), 2 µl cDNA, and molecular grade water (Merck, GmbH, Germany). The first PCR was run with the following thermal profile: 1 cycle at 95 °C for 1 min, and 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 68 °C for 1 min, and a final extension at 68 °C for 5 min. In the second PCR inner primers, a similar thermal profile was used. The size of the PCR products was checked by electrophoresis in 2 % agarose gel after staining with GelRedTM (Biotium, Inc., USA) and visualization by Gel Doc EZ imager (Bio-Rad Laboratories, Inc., USA).

Sequencing of DNA and phylogenetic analysis of HEV

Purified PCR products were sequenced using an automatic sequencer ABI PRISM (Microsynth Austria, GmbH, Austria). The sequences from both strands of the PCR products were determined with the same primers as those used for the nested PCR amplification. Partial ORF1 sequences (242 nt) were edited and aligned by the computer programme SeqMan, EditSeq and MegAlign (Lasergene, DNASTAR, Inc. USA). The phylogenetic tree was constructed by the neighbour-joining method using MEGA 4.0 [24].

RESULTS

The results of the prevalence of hepatitis E virus in the domestic pigs are summarized in Table 1. The RT-PCR analysis of 269 enteric samples revealed that 32 pigs (11.9 %) of all age categories were HEV RNA positive. When looking across age categories, HEV RNA was most often detected

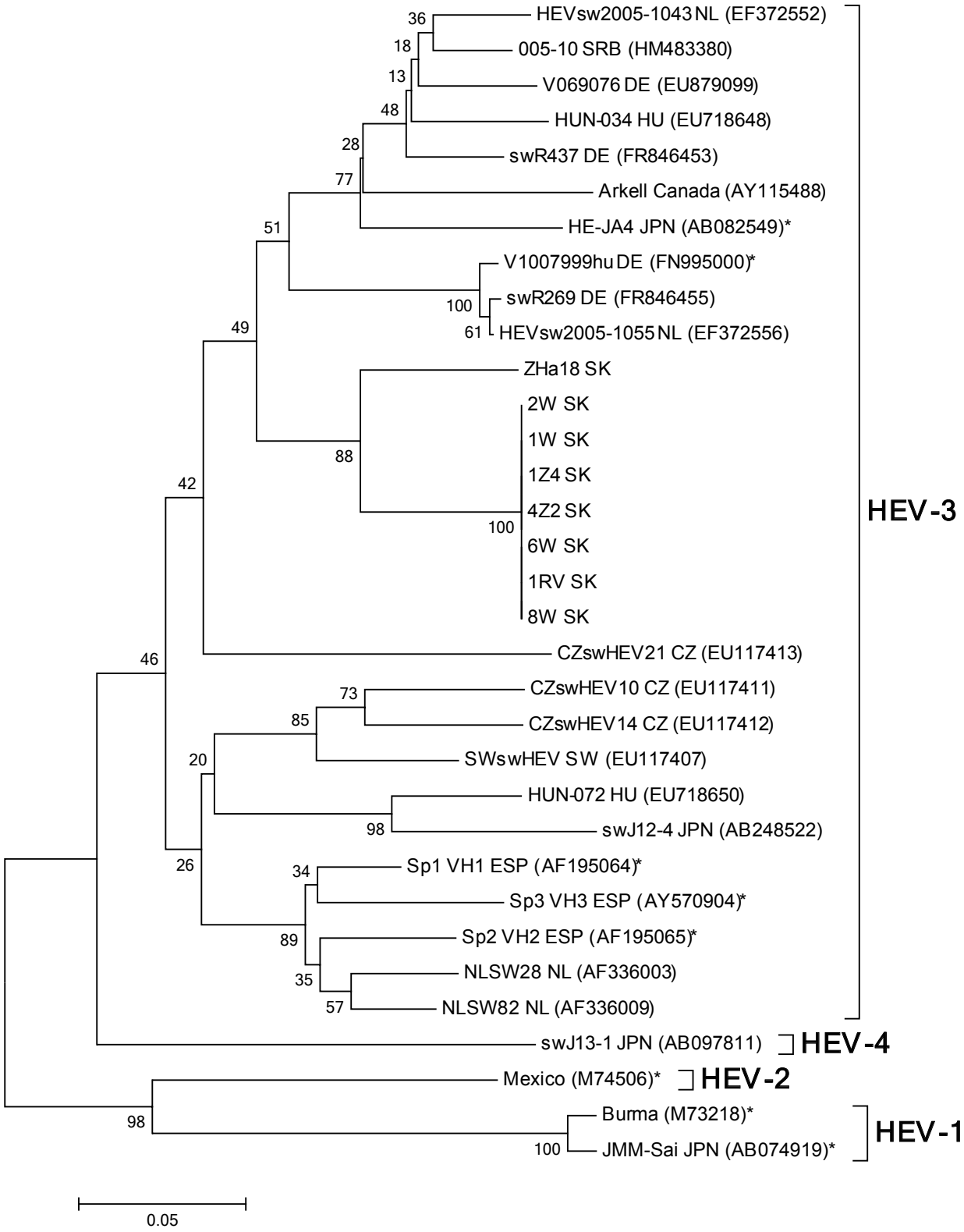

Fig. 1. The phylogenetic tree based on the partial ORF1 region of HEV-1, -2, -3, -4 genotypes
All HEV isolates from GenBank — in parentheses with GenBank Acc. No.
Human HEV isolates from GenBank — in parentheses with GenBank Acc. No., in addition labelled *
Slovak swine HEV isolates without GenBank Acc. No. in parentheses

Table 1. The incidence of HEV in different age categories of domestic pigs

Age category [days]	Number of samples [n]	Number of HEV RNA positive [n]	Percentage of HEV RNA positive [%]
Suckling piglets < 28 days	35	0	0
Weaners 28–70 days	112	14	12.5
Fattening > 70 days	122	18	14.8
Total	**269**	**32**	**11.9**

in fattening pigs (14.8 %), while a lower prevalence was detected in weaned piglets (12.5 %). HEV RNA was not observed in suckling piglets. Positive HEV RNA samples were detected only on two pig farms from all of the 14 analysed farms.

Phylogenetic analysis revealed that Slovak HEV isolates clustered into two genetic groups (Fig. 1). All Slovak HEV isolates were typed as HEV-3. The comparison of nucleotide sequences of Slovak HEV isolates to each other showed 81.0—100 % identity. The identity of the nucleotide sequences of Slovak HEV isolates with HEV isolates from neighbouring countries, Czechia and Hungary, was observed 81.8—86.8 % and 80.686.8 %, respectively.

DISCUSSION

After the first characterization of swine HEV in USA by Meng et al. [13], the virus has been detected in the same species in many countries including Spain, Netherlands, Canada, United Kingdom [19, 25, 18, 1] and recently has been described in Africa [16].

Our study investigated the occurrence of HEV in clinically healthy pigs on Slovak domestic pig farms indicating a relatively high presence of HEV (11.9 %). Very low occurrence of RNA HEV (3 %) was observed in faeces samples collected in a Czech slaughterhouse, similarly to clinically healthy pigs [4]. Besides Czechia, swine HEV was detected and characterized in neighbouring Hungary. HEV infection among domestic swine appears to be at quite a high level (39 %) on the Hungarian swine farms [20].

This study detected HEV in clinically healthy pigs. Most of the published studies are based on the detection in naturally or experimentally infected pigs [2, 9, 19].

Up to now only a few studies have been aimed at HEV detection in relation to the age of pigs. Although two published studies pointed out that 2—4 months old pigs usually showed HEV infection [13, 14], our study demonstrated that the virus can be detected in pigs younger than 2 months. In our study, we have observed the occurrence of HEV in 12.5 % of weaned piglets (28—70 days old). The highest percentage of HEV (14.8 %) was observed in the fattening category (> 70 days old). Similarly, the highest percentage of pigs with RNA HEV (36 %) was observed by Forgách et al. [8] in a comparable age group (11—16 weeks old) of pigs.

Contrary to the results indicating a low HEV prevalence (9 %) in 1—4 week old piglets detected by Forgách et al. [8], we failed to detect HEV in the youngest age category, the suckling piglets (< 28 days old). The protective role of passive immunity is probably the reason for the absence of HEV in suckling piglets in our study. We assume that the influence of maternal antibodies in this age category of pigs has a high protective effect.

By RT-PCR detection in the Netherlands, 22 % and 55 % of the pig farms were found HEV-positive in 1999 and 2005, respectively [22]. In the USA, 54 % and 40 % of the pig farms were found HEV-positive in 2002 and 2005, respectively [10, 12]. Surprisingly, HEV RNA was detected in 100 % of the 6 pig farms located in northern Italy [3], in 73 % of the pig farms investigated in Shanghai [15]. In Spain the prevalence of HEV among pig farms increased from 38 % in 2006 to 76 % in 2007 [6, 7].

The nucleotide sequences of ten HEV isolates from Slovakia were aligned and compared to each other and to the selected strains deposited in GenBank. The comparison of the nucleotide sequences identity of Slovak HEV isolates to each other showed high identity in nearly 100 %. By comparison of nucleotide sequences from neighbouring countries, Czechia and Hungary, an identity within 80.6—86.8 % was observed. By phylogenetic analysis of partial ORF1, we found that Slovak isolates clustered into two genetic groups (Fig. 1). All Slovak HEV isolates were typed as genotype HEV-3, which is a common genotype of animal, including human isolates. This is considered an important characteristic of the potential source of human infections.

CONCLUSIONS

In our study we investigated the prevalence of HEV on domestic pig farms in Slovakia which has not been addressed before. The analysis of three different age categories of domestic pigs showed some differences. The highest occurrence was observed in the fattening category and lowest in weaned piglets. HEV RNA was not observed in the youngest category (the suckling piglets). The phylogenetic analysis revealed that the Slovak HEV isolates clustered into two genetic groups. All Slovak HEV isolates were typed as HEV-3, which are capable of infecting humans.

In the near future, we would like to extend our investigations to other domestic pig farms in Slovakia and to characterize more HEV isolates.

ACKNOWLEDGEMENT

This study was supported by the project INFEKTZOON — Center of excellence for infectious disease and zoonosis ITMS 26220120002 (operational programme of research and development) financed by European Union funds for regional improvement.

REFERENCES

1. **Banks, M., Heath, G. S., Grierson, S. S., King, D. P., Gresham, A., Girones, R. et al., 2004:** Evidence for the presence of hepatitis E virus in pigs in the United Kingdom. *Vet. Rec.*, 154, 223—227.
2. **Cooper, K., Huang, F. F., Batista, L., Rayo, C. D., Bezanilla, J. C., Toth, T. E. et al., 2005:** Identification of genotype 3 hepatitis E virus (HEV) in serum and faecal samples from pigs in Thailand and Mexico, where genotype 1 and 2 HEV strains are prevalent in the respective human populations. *J. Clin. Microbiol.*, 43, 1684—1688.
3. **Di Bartolo, I., Martelli, F., Inglese, N., Pourshaban, M., Caprioli, A., Ostanello, F. et al., 2008:** Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy. *Vet. Microbiol.*, 132, 47—55.
4. **Di Bartolo, I., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G. et al., 2012:** Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. *Emerg. Infect. Dis.*, 18, 1282—1289.
5. **Erker, J. C., Desai, S. M., Mushahwar, I. K., 1999:** Rapid detection of Hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. *J. Virol. Methods*, 81, 109—113.
6. **Gracia, M. T., 2006:** Detection of hepatitis E virus shedding in faeces of pigs at different stages of production using reverse transcription polymerase chain reaction. *J. Vet. Diagn. Invest.*, 18, 462—465.
7. **Fernandez-Barredo, S., Galiana, C., Garcia, A., Gomez-Munoz, M. T., Vega, S., Rodriguez-Iglesias, M. A. et al., 2007:** Prevalence and genetic characterization of hepatitis E virus in paired samples of faeces and serum from naturally infected pigs. *Can. J. Vet. Res.*, 71, 236—240.
8. **Forgách, P., Nowotny, N., Erdélyi, K., Boncz, A., Zentai, J., Szucs, G. et al., 2010:** Detection of hepatitis E virus in samples of animal origin collected in Hungary. *Vet. Microbiol.*, 143, 106—116.
9. **Halbur, P. G., Kasorndorkbua, C., Gilbert, C., Guenette, D., Potters, M. B., Purcell, R. H. et al., 2001:** Comparative pathogenesis of infection of pigs with Hepatitis E viruses recovered from a pig and a human. *J. Clin. Microbiol.*, 39, 918—923.
10. **Huang, F. F., Haqshenas, G., Guenette, D. K., Halbur, P. G., Schommer, S. K., Pierson, F. W. et al., 2002:** Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J. Clin. Microbiol.,* 40, 1326—1332.
11. **Jiménez de Oya, N., de Blas, I., Blázquez, A. B., Martin-Acebes, M. A., Halaihel, N., Gironés, O. et al., 2011:** Widespread distribution of hepatitis E virus in Spanish pig herds. *BMC Res. Notes*, 4, 412—419.
12. **Kasorndorkbua, C., Opriessnig, T., Huang, F. F., Guenette, D. K., Thomas, P. J., Meng, X. J. et al., 2005:** Infectious swine hepatitis E virus is present in pig manure storage facilities on United States farms, but evidence of water contamination is lacking. *Appl. Environ. Microbiol.*, 71, 7831—7837.
13. **Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S. et al., 1997:** A novel virus in swine is closely related to the human hepatitis E virus. *Proc. Natl. Acad. Sci. USA*, 94, 9860—9865.
14. **Meng, X. J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., 1998:** Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J. *Virol.*, 72, 9714—9721.

15. **Ning, H., Yu, S., Zhu, Y., Dong, S., Yu, R., Shen, S. et al., 2008:** Genotype 3 hepatitis E has been widespread in pig farms of Shanghai suburbs. *Vet. Microbiol.*, 126, 257—263.
16. **Owolodun, O. A., Gerber, P. F., Gimenez-Lirola, L. G., Kwaga, J. K., Opriessnig, T., 2014:** First report of hepatitis E virus circulation in domestic pigs in Nigeria. *Am. J. Trop. Med. Hyg.*, 91, 699—704.
17. **Pavio, N., Meng, X. J., Renou, C., 2010:** Zoonotic hepatitis E: Animal reservoirs and emerging risks. *Vet. Res.*, 41, 46.
18. **Pei, Y., Yoo, D., 2002:** Genetic characterization and sequence heterogeneity of a Canadian isolate of swine hepatitis E virus. *J. Clin. Microbiol.*, 40, 4021—4029.
19. **Pina, S., Buti, M., Cotrina, M., Piella, J., Girones, R., 2000:** HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J. Hepatol.*, 33, 826—833.
20. **Reuter, G., Fodor, D., Forgách, P., Kátai, A., Szucs, G., 2009:** Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. *J. Clin. Virol.*, 44, 277—281.
21. **Rose, N., Lunazzi, A., Dorenlor, V., Merbah, T., Eono, F., Eloit, M. et al., 2011:** High prevalence of hepatitis E virus in French domestic pigs. *Comp. Immunol. Microbiol. Infect. Dis.*, 34, 419—427.
22. **Rutjes, S. A., Lodder, W., Bouwknegt, M., de Roda Husman, A M., 2007:** Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55 % by using appropriate internal quality controls for RT-PCR. *J. Virol. Methods*, 143, 112—116.
23. **Smith, D. B., Simmonds, P., Jameel, S., Emerson, S. U., Harrison, T. J., Meng, X. J. et al., 2014:** Consensus proposals for classification of the family *Hepeviridae. J. Gen. Virol.*, 96, 1191—1192.
24. **Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007:** MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 24, 1596—1599.
25. **Van der Poel, W. H. M., Verschoor, F., van der Heide, R., Herrera, M. I., Vivo, A., Kooreman, M. et al., 2001:** Hepatitis E virus sequences in swine related to sequences in humans, the Netherlands. *Emerg. Infect. Dis.*, 7, 970—976.

Received July 24, 2017
Accepted September 4, 2017

DE GRUYTER OPEN

DOI: 10.1515/fv-2017-0030

FOLIA VETERINARIA, 61, 3: 71—77, 2017

IN VITRO ANTIBACTERIAL ACTIVITY OF MENTHA ESSENTIAL OILS AGAINST *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Plant extracts and essential oils (EOs) are characterized by their antibacterial properties against various bacterial pathogens, including staphylococci. Some strains of these bacteria are resistant against the adverse effects of the environment including antibiotics, e. g. methicillin-resistant *Staphylococcus aureus* (MRSA). EOs alone cannot substitute for antibiotics but their treatment may be useful to intensify and strengthen the effects of antibiotics on pathogenic staphylococci. In this work, we tested the antibacterial effects of the essential oils of *Mentha* species with menthol as one of the effective substances against different strains of *S. aureus*. Two *in vitro* methods were used, the qualitative disc diffusion assay and the quantitative minimal inhibitory concentration (MIC) of selected essential oils. Peppermint oil from *Mentha piperita*, spearmint oil from *Mentha spicata* var. *crispa* and cornmint oil from *Mentha arvensis* were tested in this study against the various strains of *Staphylococcus aureus*, including methicillin resistant *Staphylococcus aureus* (MRSA). The oils were dissolved in DMSO (dimethyl sulfoxide) and diluted at the following ratios: 1 : 1, 1 : 2, 1 : 5, and 1 : 10. Based on the results determined by the agar disc diffusion test, the highest antibacterial properties were observed in spearmint oil against *S. aureus* CCM 4223 at 1 : 2 ratio where the inhibition zone varied at a range of 35.67 ± 6.81 mm. We determined also the MIC of all the oils where concentrations of the oils were as follows: 1 %; 0.5 %; 0.25 %; 0.125 % and 0.0625 %. The lowest concentrations of essential oils that possessed inhibitory effects on the growth of *S. aureus* varied between 0.125 % and 0.25 %.

Key words: antibacterial activity; essential oils; *in vitro* methods; *Staphylococcus aureus*

INTRODUCTION

The essential oils (EOs) are a group of various natural chemicals which are characterized by their volatility and aroma. The term "essential oil" comes from the times of the Middle Ages. At this time, alchemists considered every

liquid floating on the water as an oil. After the invention of the distillation procedure, they thought that EOs are "the essence of life".

The plants produce EOs for their protection against herbivores. EOs can concentrate in a specific plant organ or they pervade the whole plant (conifers). Naturally they are colourless. At room temperature the majority of EOs occurs in liquid form; some are solids (camphora). Mostly they have lower density than water, with the except of cinnamon and clove oils. They dissolve in alcohol and fats. The main chemical compounds in EOs are terpenes-monoterpenes (ocimene, limonene, linalool, α-pinene) and sesquiterpenes (β-caryophyllene). More complex terpenes have higher molecular weights and because of that, they cannot be distilled. Other chemical groups are alcohols (geraniol, menthol), aldehydes (cinnamaldehyde, vanillin), ketones (carvone), and phenols (eugenol, thymol, carvacrol) [12].

EOs have a wide range of applications. They can be used as antiflogistics, stomachics, carminatives, diuretics, sedatives, antimycotics, antivirotics, disinfectants, etc. One of the main benefit is also their antibacterial effects. EOs act by various mechanism on different bacterial structures. The structure of gram-positive bacteria facilitates the penetration of hydrophobic molecules into the cell and act on the bacterial wall, cytoplasmic membrane or cytoplasm. At low concentrations, they can react with enzymes responsible for producing energy, at higher concentration they can denaturate proteins. Because of the reduced proton gradient by influencing the transfer of H^+, essential oils reduce the synthesis of adenosine triphosphate (ATP) and thus the intracellular store of ATP. They can cause the degradation of bacterial cell walls, damage of cytoplasmic membranes and coagulation of the cytoplasm. By damaging the membrane proteins, they increase the permeability of the membrane and cause leakage of the cell contents [4]. In general, gram-negative (G^-) bacteria are more resistant against EOs in comparison to gram-positive (G^+) because of the different composition of the bacterial cell walls [15]. G^- bacteria have a thin layer of peptidoglycan and lipopolysaccharide layer (LPS) on their outer membrane that is lacking in gram-positive bacteria. Small hydrophilic molecules can penetrate through the porin proteins of G^- bacteria. The porins are relatively resistant to hydrophobic molecules, but not completely. Some EOs, e. g. from basil, sage or oregano, act on *E. coli, S. aureus, B. cereus* and *Salmonella* spp. However, they are less effective against *Pseudomonas* spp. because it increases its resistency by producing exopolysaccharides and by creating biofilms [7].

Bacteria of the species *Staphylococcus aureus* are Gram-positive, facultative anaerobic and non-motile cocci. They cause many diseases in humans and animals that can be local or even systemic. Staphylococci can be found on the skin, skin wounds or abrasions. The infection can be spread by hands or by secretion from the nose or mouth. Some *S. aureus* strains produce at least 11 enterotoxins designated SEA to SEJ. Toxin types A and D are most frequently implicated in the outbreaks of food poisoning. These toxins are resistant to a temperature of 100 °C within 20 minutes. Bacteria are cold resistant, while heating over 60 °C will killing them [1]. An intoxication called staphyloccocal enterotoxicosis results from contaminated food where the bacteria are spreading and producing the toxins. Pathogenic staphylococcal strains can cause also pneumonia, post-operative infections and nosocomial bacteremia [13]. Methicillin resistant *Staphylococcus aureus* (MRSA) strains are dangerous, because infections caused by these strains cannot be treated with many antibiotics (ATB), as they are multiresistant. The resistance against ATB is caused by the overuse of ATB or inappropriate dosage. Furthermore, many MRSA strains produce biofilms. The biofilm is a product of the microbial population attached on the substrate or on to another. It is a polymeric substance that arises due to a changed bacterial fenotype related to its growth, gene expression and synthesis of proteins. The production of biofilm is strictly regulated by genetic factors. Immune response against the biofilm infections is uneffective and leads to chronic diseases [2].

Some of the more popular methods used for testing antimicrobial activity of EOs is the disc diffusion method, the determination of minimum inhibitory concentration (MIC) and the vapour phase method. Additional methods are bioautography TLC method for testing the antimicrobial activity of individual components of EOs [3].

The purpose of this study was to investigate the antibacterial properties of three various species of the mint essential oils and menthol against *Staphylococcus aureus* strains using two *in vitro* methods.

MATERIALS AND METHODS

Bacterial strains

All together 4 strains of *Staphylococcus aureus* were

used in this study: *S. aureus* (clinical isolate from a dog wound), *S. aureus* CCM 4750 (MRSA) and 2 strains of *S. aureus* with the ability to produce biofilms (CCM 3953 and CCM 4223). The strains with CCM numbers were obtained from the Czech Collection of Microorganisms in Brno (Czechia).

Essential oils

Three different species of the mint essential oils and menthol were tested in this study. *Mentha x piperita* L. — peppermint oil (Calendula a.s., Nová Ľubovňa) with its mayor components L-menthol (40.7 %) and menthone (23.4 %). *Mentha spicata* L. var. *crispa* — spearmint oil (Hanus s. r. o., Nitra) with its mayor components L-carvone (70 %) and limonene (10 %). *Mentha arvensis* L. — cornmint (Hanus s. r. o., Nitra) with its mayor components L-menthol (28.8—34.7 %), menthone (16.3—1.1 %) and isomenthone (6.8—12.1 %). And finally, we used also a pure component, L-menthol (Galvex, s. r. o.).

Growth conditions

Growth media: PYG broth (Pepton Yeast Extract Glucose Broth) was used for overnight cultivation of the bacterial strains. MHA (Mueller Hinton Agar, HiMedia, India) was used for the disc diffusion method and also for the MIC determinations.

The preparation of the overnight culture: 3 ml of PYG broth was inoculated with the various staphylococcal strains and incubated on a shaker at 37 °C for 18 h.

Determination of CFU (colony forming units): After incubation, the counts of bacteria in overnight culture were determined. The results for each strain are shown in Table 1.

Disc diffusion method

Table 1. Number of CFU.ml^{-1} after 18 h incubation at 37 °C

Bacterial strain	CFU.ml^{-1}
Staphylococcus aureus — clinical dog isolate	3.32×10^{11}
Staphylococcus aureus (MRSA) CCM 4750	3.16×10^{12}
Staphylococcus aureus CCM 3953	3.31×10^{12}
Staphylococcus aureus CCM 4223	9.4×10^{10}

This method was used as qualitative method to determine the antimicrobial activity of selected essential oils. 0.1 ml of overnight culture was spread onto the surface of MHA using a sterile bacteriological spreader. After the absorption of inoculum, we transferred the paper discs (6 mm, Becton, Dickinson and Company, USA) with sterile needles onto the surface of the inoculated MHA agar and applied with a micropipette 10 µl of each EO in several dillutions (1 : 1, 1 : 2, 1 : 5, 1 : 10) onto the paper discs. Pure concentrated DMSO (dimethyl sulfoxide) was used as a negative control and specific ATBs (oxacillin 5 µg/disc and methicillin 5 µg/disc) were used as positive controls. The Petri dishes were incubated for 24 h at 37 °C. After the incubation time, the diameter of the inhibition zones (in mm) around the paper discs were measured. This *in vitro* test was repeated in triplicate to get an average number with the standard deviation as a result.

Determination of MIC

(Minimal Inhibitory Concentration)

Two-fold dilutions of EOs with concentrations of 1 %, 0.5 %, 0.25 %, 0.125 % and 0.0625 % in MHA with 0.5 % Tween 20 (Sigma Aldrich, Germany) were prepared before starting the test. Two µl of overnight culture were inoculated with a micropipette drop by drop onto the surface of MHA [11]. Medium without EOs was used as a negative control. The Petri dishes were incubated for 24 h at 37 °C. The experiments were carried out in triplicate. We determined the MIC as a minimal concentration of the EOs that suppressed the growth of bacteria on the agar dishes.

Statistics

The results obtained by the disc diffusion method are reported as an arithmetical average ± standard deviation in mm. We considered $d > 6$ mm as a positive result. The statistic software GraphPad Prism version 3 was used and the results were evaluated by one-way ANOVA and Tukey's test with $^{***}P < 0.001$ as a significance level. The results obtained by the MIC method were evaluated only as presence/absence of growth of bacteria on the surface of the agar plate.

RESULTS AND DISCUSSION

Disc diffusion method

The evaluation of antibacterial activity of mint essential oils showed that they effectively inhibited the staphylococcal strains, hovewer, with different sensitivities.

Table 2. Clinical isolate *Staphylococcus aureus*, disc diffusion method (n = 3), inhibition zones [mm]

Dilution	*M. piperita*	*M. arvensis*	*M. spicata*	L-menthol	Oxacillin 5 µg/disc
1 : 10	2.33 ± 4.04	7 ± 0	0 ± 0***	7.5 ± 0	22
1 : 5	9 ± 0***	9.33 ± 0.58***	9.33 ± 0.58***	15.67 ± 4.04	24
1 : 2	18.17 ± 1.26	15.33 ± 2.52	22.33 ± 2.31	20.5 ± 0.87	23
1 : 1	15.5 ± 0.87	16.67 ± 1.16	15 ± 2	15.17 ± 0.29	21

*** — significantly different from Oxacillin (P < 0.001)

Table 3. Methicillin resistant *S. aureus* (MRSA) CCM 4750, disc diffusion method (n = 3), inhibition zones [mm]

Dilution	*M. piperita*	*M. arvensis*	*M. spicata*	L-menthol	Oxacillin 5 µg/disc
1 : 10	0 ± 0	0 ± 0	0 ± 0	0 ± 0	22
1 : 5	5 ± 4.33	4.67 ± 4.04	0 ± 0	8 ± 0	24
1 : 2	10.67 ± 1.53	10.67 ± 2.02	11.33 ± 9.87	12 ± 1	23
1 : 1	11.5 ± 0.5	13.83 ± 3.62	9.33 ± 0.29	10.67 ± 0.29	21

Table 4. *Staphylococcus aureus* CCM 3953, disc diffusion method (n = 3), inhibition zones [mm]

Dilution	*M. piperita*	*M. arvensis*	*M. spicata*	L-menthol	Oxacillin 5 µg/disc
1 : 10	**0 ± 0*****	**0 ± 0*****	**0 ± 0*****	**2.5 ± 4.33*****	**15**
1 : 5	**7.67 ± 0.29*****	**7.5 ± 0.5*****	**6.5 ± 0*****	**9.5 ± 1.32*****	**18**
1 : 2	**17 ± 1**	**15.83 ± 2.02**	**15.33 ± 0.58**	**14.67 ± 1.61**	**19**
1 : 1	**12.33 ± 1.53**	**12.33 ± 1.44**	**10.83 ± 0.76**	**12.5 ± 0.5**	**14**

*** — significantly different from Oxacillin (P < 0.001)

Table 5. *Staphylococcus aureus* CCM 4223, disc diffusion method (n = 3), inhibition zones [mm]

Dilution	*M. piperita*	*M. arvensis*	*M. spicata*	L-menthol	Methicillin 5 µg/disc
1 : 10	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a, b]	7.83 ± 0.29 [b]	20
1 : 5	18 ± 2	10.67 ± 1.26	12.67 ± 1.53	14.33 ± 2.08	15
1 : 2	22.83 ± 0.76	17.67 ± 0.58 [c]	35.67 ± 6.81 [a, b]	18 ± 1	18
1 : 1	14.17 ± 0.76	15.5 ± 0.5 [a]	16.33 ± 0.58 [a]	12.33 ± 0.29	14

[a] — significantly different from L-menthol; [b] — significantly different from Methicillin
[c] — significantly different from *Mentha spicata* (P < 0.001)

The results of individual dilutions 1 : 1, 1 : 2, 1 : 5, 1 : 10 are shown in Tables 2—5. The strongest effect was obtained with *Mentha spicata* var. *crispa* and the highest resistance was shown by the MRSA strain. It was interesting that almost in every experiment the concentration 1 : 1 exhibited lower efficiency in comparison to the concentration of 1 : 2. We used L-menthol which exhibited the major efficiency. L-menthol was used at the same volume as EOs. The best reflection of the effects of L-menthol would be the same volume as in the essential oils. But in some EOs, menthol did not represent the major part.

Significant differences were detected between *Mentha spicata* var. *crispa* and menthol and the positive control (***$P < 0.001$).

Determination of MIC

The concentrations which inhibited the growth of staphylococci varied between 0.125 % and 0.25 %. The strongest effects against the MRSA was observed for *M. spicata* at the concentration of 0.125 %. *M. arvensis* was effective at the same concentration against both biofilm producing staphylococcal strains (CCM 3953 and CCM 4223) (Table 6).

Table 6. Minimal inhibitory concentration (MIC) [mm]

	S. aureus	MRSA CCM 4750	*S. aureus* CCM 3953	*S. aureus* CCM 4223
M. piperita	0.25 %	0.25 %	0.25 %	0.25 %
M. arvensis	0.25 %	0.25 %	0.125 %	0.125 %
M. spicata	0.25 %	0.125 %	0.25 %	0.25 %

*** — $P < 0.001$

The antibacterial activity of the essential oils is a topic for many investigations. We observed antibacterial activity of EOs for 3 different species of *Mentha* sp. gender and L-menthol against 4 different strains of *Staphylococcus aureus*.

Using the disc diffusion method, we observed that the highest antibacterial effect was achieved mostly at the concentration of 1 : 2, not 1 : 1 as expected. EO from *Mentha spicata* var. *crispa* showed the strongest activity against the clinical isolate of *S. aureus* at a concentration of 1 : 2. On the other hand, the concentration of 1 : 1 was the strongest in the case of EO from *M. arvensis*. The biggest diameter of inhibition zones against MRSA were measured with EO from *M. spicata* at the concentration of 1 : 2 and at the concentration of 1 : 1 with EO from *M. arvensis*. We tested also 2 species of staphylococcal strains producing biofilms. *S. aureus* CCM 3953 was inhibited by EO from *M. piperita* at the dilution of 1 : 2, *S. aureus* CCM 4223 by EO from *M. spicata* var. *crispa* at the same dilution.

For MIC determination we exposed all 4 strains of *S. aureus* to 5 different concentrations of EOs: 1 %, 0.5 %, 0.25 %, 0.125 % and 0.0625 %. The difference in the sensitivity within staphylococci strains was not so noticeable in comparison to the disc diffusion method. The results varied between 0.125 % and 0.25 %. The biofilm producing staphylococci CCM 3953 and CCM 4223 were the most sensitive to EO from *Mentha arvensis*. These results were affected by the emulgation of the EOs in agar. For better EOs homogenization, Tween 20 at 0.5 % concentration was used. It appears desirable to use different concentrations of an emulgator and to compare the results.

In another study it was found that EO from *M. spicata* var. *crispa* also affected *S. aureus*. The sizes of the inhibition zones in the disc diffusion method were 10 mm at *S. aureus* ATCC 6538 and 8 mm at *S. aureus* ATCC 25923 despite the application of double dose (20 µl) of 100 % EO in comparison to a dose 10 µl used in this study [10]. The reason for the different results may be caused by the different composition of EOs. *S. aureus* ATCC 6538 was inhibited at 0.1 % concentration compared to our 0.25 %. In another study, it was observed that *Mentha piperita* also had inhibitory effects on *S. aureus* (the strain was not specified) [9]. Using the disc diffusion method, the measured inhibition zone was 7.6 ± 0.57 mm at 1 : 10 concentration. No inhibition zone was observed in our study.

Imai et al. [6] confirmed the effectivity of all three species of mint against a MRSA strain.

The results of the antimicrobial activity of essential oils can be influenced also by various extraction methods and extracts used for the determination. Para et al. [8] confirmed that among ethanol, methanol, ethyl acetate, chloroform, hexane and petroleum ether, the ethyl acetate leaf extract of *Mentha piperita* caused more pronounced inhibition of *Staphylococcus aureus*, *Bacillus subtilis* and *Proteus vulgaris* than chloroform, petroleum ether or hexane extracts.

The antibacterial activity of *M. spicata* was confirmed also in the study by Golestan et al. [5]. They observed that *M. spicata* EO had the highest inhibition activity against *S. aureus* and *Clostridium perfringens*.

Zaidi and Dahiya [14] used the agar well difusion method for the determination of the antimicrobial activity

of EOs from *Mentha spicata* and *Mentha piperita* against 11 bacterial and 4 fungal clinical isolates. They reported that both of the EOs showed the maximum activity against *S. aureus*, producing a maximum zone of inhibition of 21 ± 0.09 mm with *Mentha spicata* and 19.2 ± 0.07 mm with *Mentha piperita*.

CONCLUSIONS

This study tested the antibacterial activity of EOs from *Mentha* sp. and L-menthol by means of the disc diffusion method and the determination of MIC. All three EOs had various contents of menthol and exhibited different effects. In spearmint oil, the main ingredient was L-carvone and not menthol.

The disc diffusion method revealed the strongest effect of EO from *Mentha spicata* var. *crispa* and the highest resistance of the MRSA strain. The EO from *M. spicata* var. *crispa* had the strongest effect against *S. aureus* CCM 4223. We noticed that almost in every experiment the 1 : 1 dilution exhibited lower efficiency than the 1 : 2 dilution. At the 1 : 2 dilution, EO from *Mentha spicata* var. *crispa* exhibited stronger or similar effect as the oxacillin/methicillin that were used as positive controls. The effect of EO from *Mentha piperita* was stronger against *S. aureus* CCM 3953.

The values of MIC varied between 0.125 % and 0.25 %. The strongest effect against MRSA showed EO from *M. spicata* var. *crispa* that inhibited the growth of bacteria even at 0.125 % concentration. *M. arvensis* had the strongest effect on the biofilm forming staphylococci also at this low concentration (0.125 %).

The results from our study indicated that the mint essential oils can be used as a potential source of natural antimicrobial compounds against staphylococcal infections.

ACKNOWLEDGEMENT

This study was supported by the project APVV-15-0377 and INFEKTZOON — Center of excellence for infectious disease and zoonosis ITMS code 26220120002 (operational program of research and development) financed by European Union funds for regional improvement.

REFERENCES

1. **Adams, M. R., Moss, M., 2008:** *Food Microbiology*. The Royal Society of Chemistry, 463 pp.
2. **Archer, N. K., Mazaitis, M. J., Costerton, J. W., Leid, J. G., Powers, M. E., Shirtliff, M. E., 2011:** *Staphylococcus aureus* biofilms. Properties, regulation and roles in human disease. *Virulence*, 2, 445—459.
3. **BaşEr, K. H. C., Buchbauer, G., 2010:** *Handbook of Essential Oils: Science. Technology, and Applications*. Boca Raton. CRC Press/Taylor, 975 pp.
4. **Burt, S., 2004:** Essential oils: their antibacterial properties and potential applications in foods — a review. *International Journal of Food Microbiology*, 94, 223—253.
5. **Golestan, L., Seyedyousefi, L., Kaboosi, H., Safari, H., 2016:** Effect of *Mentha spicata* L. and *Mentha aquatica* L. essential oils on the microbiological properties of fermented dairy product, kashk. *Int. J. Food Sci. Technol.*, 51, 581—587.
6. **Imai, H., Osawa, K., Yasuda, H., Hamashima, H., Arai, T., Sasatsu, M., 2001:** Inhibition by the essential oils of peppermint and spearmint of the growth of pathogenic bacteria. *Microbios*, 106 Suppl. 1, 31—39.
7. **Nazzaro, F., Fratianni, F., Martino, L., Coppola, R., De Feo, V., 2013:** Effect of Essential Oils on Pathogenic Bacteria. *Pharmaceuticals*, 6, 1451—1474.
8. **Para, S., Thulasi, M. S., Penchalaneni, J., Challagundla, V. N., 2013:** Antibacterial activity and phytochemical analysis of *Mentha piperita* L. (Peppermint) — An important multipurpose medicinal plant. *American Journal of Plant Sciences*, 4, 77—83.
9. **Ramos, S., Rodrigues, R. A. B. L, Farias, A. L. F. et al., 2017:** Chemical composition and *in vitro* antioxidant, cytotoxic, antimicrobial, and larvicidal activities of the essential oil of *Mentha piperita* L. (*Lamiaceae*). *The Scientific World Journal*, 2017, 1—8.
10. **Shahbazi, Y., 2015:** Chemical composition and *in vitro* antibacterial activity of *Mentha spicata* essential oil against common food-borne pathogenic bacteria. *Journal of Pathogens*, 2015, 1—5.
11. **Silva, F., Ferreira, S., Queiroz, J. O., Domingues, F. C, 2011:** Coriander (*Coriandrum sativum* L.) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. *Journal of Medical Microbiology*, 60, 1479—1486.
12. **Tomko, J., 1999:** *Farmacognozy* (In Slovak). Textbook for pharmaceutical faculties. 2nd edn., Martin, Osveta, 424 pp.

13. **Tong, S. Y. C, Davis, J. S, Eichenberger, E., Holland, T. L., Fowler, V. G., 2015:** *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.*, 28, 603—661.
14. **Zaidi, S., Dahiya, P., 2015:** *In vitro* antimicrobial activity, phytochemical analysis and total phenolic content of essential oil from *Mentha spicata* and *Mentha piperita*. *International Food Research Journal*, 22, 2440—2445.
15. **Zaika, L. L., 1988:** Spices and herbs: their antibacterial activity and its determination. *J. Food Saf.*, 23, 97—118.

Received July 7, 2017
Accepted September 8, 2017