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MEDICINAL MUSHROOMS *OPHIOCORDYCEPS SINENSIS* AND *PAECILOMYCES HEPIALI*

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ABSTRACT

The fungi of the genus *Ophiocordyceps sinensis* (Berk.) belong to entomopathogenic fungi. *Ophiocordyceps sinensis* has been used as a tonic and medicinal product in China for more than 2,000 years. A number of scientific papers have described the clinical use of this parasitic fungus with subsequent biological and pharmacological effects. The various chemical compounds identified in these fungi are responsible for a wide range of biological activity: cordycepin, cordycepic acid, D-mannitol, polysaccharides, nucleotides, proteins, amino acids, and unsaturated fatty acids. Our research focused on the determinations of the biologically active chemical compounds in extracts from cultivated *Ophiocordyceps* fungi using UV/VIS (Ultraviolet/Visible) spectrophotometry and NMR (Nuclear Magnetic Resonance) spectroscopy. The testing of antimicrobial activity of extracts against the collection strains of *Escherichia coli* and *Staphylococcus aureus* demonstrated that the percentage of RIZD (relative inhibition zone diameter) ranged from 83 % to 166 %. The increased antimicrobial activity against *E. coli* was observed in comparison with that against *S. aureus*.

Key words: cultivation; *Ophiocordyceps sinensis*; *Paecilomyces hepialid*; UV/VIS and NMR spectroscopy

INTRODUCTION

Cordyceps sinensis (Fr.) Link (CS), after the new *Ophiocordyceps sinensis* (OS) (Fig. 1.) from the family *Ophiocordycipitaceae* [11], is one of the enthomopathogenic fungi, i.e. fungi parasitizing insects. It occurs naturally in Asia at altitudes above 3500 meters in the Tibetan Plateau, especially in the provinces of Yunnan, Qinghai, Sichuan and Gansu, as well as in the north of Nepal, Bhutan, and India [10].

The life cycle of OS consists of a sexual (teleomorph) and asexual stage (anamorph). Host larvae are infected between July and late August [14]. In the later autumn period, chemicals from the surface of the larvae interact with the spores of the fungus released from the head of the

Fig. 1. ***Ophiocordyceps sinensis*** **[13]**

stroma. A fungal mycelium is formed, which then infects a larva surviving the Tibetan winter buried underground. The larva, under the influence of the fungus begins to travel closer to the earth's surface. The OS gradually begins to grow at the expense of the inner tissue of the larva, initially only killing non-vital tissue. By spring, the contents of the larva are completely replaced by fungal tissue, and the mycelium have formed by the fibrous hyphae. The larval exoskeleton remains intact [13]. At the beginning of summer, it is possible to observe a fruiting body (stroma) growing from the head of the larva. This, then produces spores that are released into the environment, which can infect other larvae, and the cycle is repeated [15]. The natural form of OS is harvested from April to August. It is also relatively rare, which, along with its medical and culinary status, has made Chinese consumers willing to pay astronomical prices for it. The harvest of this mushroom is the main source of income for the Tibetan countryside [13]. The demand for OS has increased significantly since about 1950, and the scientific community has therefore attempted to cultivate it artificially, or to cultivate other species of the genus *Cordyceps* with a similar positive effect on the human body [11]. One of the first isolated strains of OS used for cultivation was a strain called CS-4, *Paecilomyces hepialid* (HP). The fermented mycelium of this strain contains pharmacologically active substances similar to those of native OS. In total, more than 20 bioactive substances have been detected in OS [4]. The two most important active substances are cordycepin and D-mannitol (cordycepic acid) [3, 13]. In addition, others chemical compounds have been discovered, i. e., nucleosides, cyclic peptides, sterols, polyketides, alkaloids, and polysaccharides [15]. These chemical compounds are responsible for a wide variety of biological activities, such as, hyposexuality, hyperlipidemia, and weakening of the immune system, respiratory problems, kidney, liver, and heart problems [5]. It is also used as a complement to modern cancer treatments (chemotherapy, irradiation and surgery).

The aim of our study was to determine the biologically active chemical compounds in cultivated *Ophiocordyceps sinensis* using UV/VIS spectrophotometry and NMR spectroscopy, and to examine the isolated extracts for their antioxidant and antibacterial activities.

MATERIALS AND METHODS

Samples of OS and PH were provided by the Technical University in Zvolen, Slovakia. The mushroom samples were cultured and prepared into a final powder form [1]. Six samples were tested: four samples of OS and two samples of PH. Mushroom samples were cultivated on two different rice substrates, *Oryza sativa* var. *japonica* and *Oryza sativa* var. *indica*: Sample 1—OS, MFTCCB026/0216, *Oryza sativa* var. *japonica*; Sample 2—OS, MFTCCB026/0216, *Oryza sativa* var. *indica*; Sample 3—OS, MFTCCB025/0216, *Oryza sativa* var. *indica*; Sample 4—OS, MFTCCB025/0216, *Oryza sativa* var. *japonica*; Sample 5—PH, MFTCCB023/0216, *Oryza sativa* var. *japonica*; Sample 6—PH, MFTCCB023/0216, *Oryza sativa* var. *indica*.

The extracts were prepared by two methods, reflux and ultrasound (Bandelin, Sonorex Digitec, Germany), in order to develop the most effective extraction method. All extractions were performed in a 1 : 1 water-methanol (methanol, Sigma Aldrich, USA) solvent. The solvent was evaporated to dryness by a digital vacuum rotary evaporator (IKA RV 10, Germany).

A Libra S12 UV/VIS spectrophotometer (Biochrom Ltd., USA) was used to test the antioxidant activity employing the DPPH method (2,2-diphenyl-1-picrylhydrazyl radical, Merck Millipore, USA). A Varian VNMRS 600 MHz spectrometer (Agilent Technologies, Palo Alto, USA) was used to analyse the content of the sample extracts.

NMR spectra of the samples were measured in deuterated water (D_2O, d_2, Organic Acros, USA) and deuterated dimethyl sulfoxide (DMSO, d_6, Merck Millipore, Germany). These spectra were measured at the University of Pavol Jozef Šafarik in Košice, Slovakia.

The antimicrobial activities of the extracts were tested by the agar diffusion method according to Rojas et al. [8]. The test bacteria *Staphylococcus aureus* and *Escherichia coli* were obtained from the Czech Collection of Microorganisms, Brno, CR (*S. aureus* CCM 4223, ATCC 29213, *E. coli* CCM 3988, ATCC 10536). The bacteria were cultured in BHI broth (Brain Heart Infusion broth, Oxoid) at 37 °C for 20 hours. Gentamicin sulphate (50 $\mu g.ml^{-1}$) was used as a positive and dimethyl sulfoxide (Sigma Aldrich, USA) as a negative control.

After culturing the bacteria, they were diluted in phosphate saline to a concentration of 0.5—1.0 McFarland turbidity. This scale indicated the number of colony forming units (CFU) per 1 ml. At a concentration of 0.5 to 1.0, there were $1.5—3 \times 10^8$ bacteria in 1 ml of suspension. One ml of this suspension was inoculated into 100 ml of tempered liquid agar (Standard plate count agar, Oxoid). Twenty (20) ml of this agar were transferred to Petri dishes with a diameter of 9 cm. After solidification, wells with a diameter of 0.5 cm were cut into the agar and 50 µL of a sample were applied to the wells. An aqueous solution of 50 $\mu g.ml^{-1}$ gentamicin sulphate was used as a positive control and DMSO as a negative control. The plates were incubated at 37 °C for 24 hours.

RESULTS

The series of samples of *Ophiocordyceps* fungi consisted of six samples (species *Ophiocordyceps sinensis* and *Paecilomyces hepiali*) that were cultivated on two types of rice: *Oryza sativa* var. *indica* and *Oryza sativa* var. *japonica*. Of the two extraction methods used the most effective was the extraction by reflux (the highest yield was 9.86 % in the extract of sample 4, OS, *Oryza sativa* var. *japonica*). The extracts obtained by refluxing had the lowest value of the half-maximal inhibitory concentration $IC_{50} = 3.03 \pm 0.05\ mg.ml^{-1}$ (sample 5, PH, *Oryza sativa* var. *japonica*). Ultrasound extraction was the least effective method. The antioxidant activity of the extracts prepared by this method was 2.5-times lower (the lowest value of IC_{50} was $6.67 \pm 0.01\ mg.ml^{-1}$, sample 3, OS, *Oryza sativa* var. *indica*) in the uptake of DPPH radical compared to the extracts obtained by reflux.

To determine the content of individual chemicals in the extracts, 1D, 2D NMR was used. By comparing the signals in the measured proton NMR spectra with the NMR database of chemical compounds [9] and the available literature [6], we were able to identify the chemical structure of the respective compounds. The results showed that the ex-

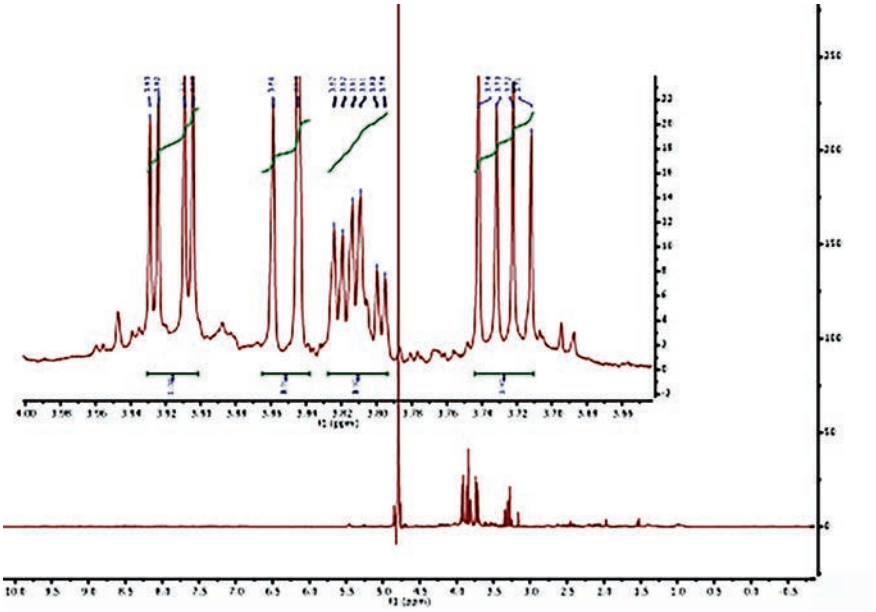

Fig. 2. 1H NMR spectrum of the chemical structure of D-mannitol in sample 5, PH extract

Tab. 1. Antimicrobial activity of extracts 1—4 and 6 tested on *S. aureus* and *E. coli* strains

Sample	Strain, substrate	Extraction method	IZD (mm)		RIZD (%)	
			S. aureus	*E. coli*	*S. aureus*	*E. coli*
1	***O. sinensis*** **MFTCCB026/0216** ***Oryza sativa* var. *japonica***	reflux	10	9	166	150
		ultrasound	5	5	83	83
2	***O. sinensis*** **MFTCCB026/0216** ***Oryza sativa* var. *indica***	reflux	9	9	150	150
		ultrasound	0	8	0	133
3	***O. sinensis*** **MFTCCB025/0216** ***Oryza sativa* var. *indica***	reflux	5	10	83	166
		ultrasound	7	10	116	166
4	***O. siensis*** **MFTCCB025/0216** ***Oryza sativa* var. *japonica***	ultrasound	7	8	116	133
6	***P. hepiali*** **MFTCCB023/0216** ***Oryza sativa* var. *indica***	reflux	6	10	100	166
		ultrasound	0	5	0	83

IZD—inhibition zone diameter [mm]; RIZD—relative inhibition zone diameter
concentration of gentamicin = 50 μg.ml^{-1}; IZDDMSO = 0 mm; IZDGENTAMICIN = 6 mm

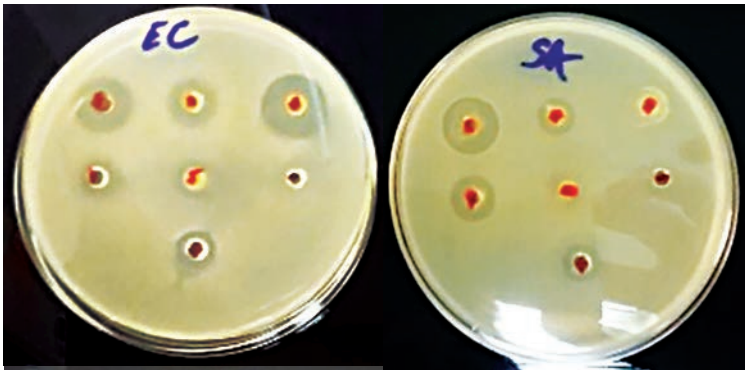

Fig. 3. Antimicrobial activity of the most active extracts of OS against *E. coli* (EC, left) and *S. aureus* (SA, right) collection strains

traction into 1:1 water-methanol gives: oleic acid, isomers of linoleic acid, acetic acid, 1,4-butanedioic acid, malonic acid; polysaccharides were present in some samples; D-mannitol, tyrosine, uracil and some amino acids.

Figure 2 shows the 1H NMR spectrum of the chemical structure of D-mannitol present in sample 5, PH, MFTCCB023/0216, *Oryza sativa* var. *japonica*.

The antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* strains, expressed as a percentage of relative inhibition zone diameter RIZD, varied from 83 % to 166 % for the active samples. The following formula was used to calculate the % of RIZD:

$$\%\,\mathrm{RIZD} = (\mathrm{IZD}_{\mathrm{SAMPLE}} - \mathrm{IZD}_{\mathrm{NEGATIVE\,CONTROL}})/\mathrm{IZD}_{\mathrm{GENTAMICIN}} \times 100$$

where IZD is the inhibition zone diameter (mm).

The values of the antimicrobial activity of extracts are presented in Table 1.

Table 1 shows an average increase in antimicrobial activity of extracts against *E. coli* in comparison with *S. aurus*. It is interesting that the extracts prepared from sample 5, PH strain MFTCCB023/0216, *Oryza sativa* var. *japonica*, did not show any antimicrobial activity.

Figure 3 illustrates the results of the agar diffusion test of the antimicrobial activity of extracts against *E. coli* and *S. aureus* (IZD).

DISCUSSION

The aim of this study was to isolate the substances contained in fungi of the species *Cordyceps*. The prepared extracts were examined for antioxidant and antimicrobial activities and the content of chemical compounds in the extracts were determined.

Extracts of substances were prepared by two different extraction methods, reflux and ultrasound extraction. Reflux is one of the most widely used methods for isolating substances from fungi. According to our previous experience, water-methanol mixture was used as a suitable solvent. The yield of the extracts ranged from 1.93 to 9.86 %. The values of the given yields were comparable with the yields reported in the literature [12]. Yields ranging from 1.7 % to 10 % have been reported.

The antioxidant activities of the prepared solutions were determined by the DPPH method. It is one of the most frequently used methods for determining the antioxidant activity *in vitro*. From the prepared extracts, the highest antioxidant activity (IC_{50} = 3.03 ± 0.05 mg.ml^{-1}) was shown by the extract from sample number 5, which represented the PH strain MFTCCB023/0216 cultured on *Oryza Sativa* var. *japonica* substrate. In the case of ultrasonic extraction, the highest IC_{50} value was shown by sample 3 (IC_{50} was 6.67 ± 0.01 mg.ml^{-1}, OS, *Oryza sativa* var. *indica*).

D o n g et al. [2] compared the antioxidant activities of natural and artificially cultured mycelia by the DPPH method. They found that the aqueous extracts from natural mycelium had an IC_{50} of 0.93 mg.ml^{-1} and for artificially cultured mycelium 1.03 mg.ml^{-1}. The difference in these values may be due to the different *Cordyceps* strain used, the different culture conditions and the substrate, or different procedure. An interesting observation was that the naturally growing *Cordyceps* showed better antioxidant activity in the uptake of DPPH radical than the artificially cultured one [10]. However, the common result was that *Cordyceps* fungi are a functional antioxidant.

Comparison of the signals in the measured proton NMR spectra with the NMR database of chemical compounds and the available literature [6] showed that the extracts contained oleic acid, isomers of linoleic acid, acetic acid, 1,4-butanedioic acid and malonic acid. Some samples contained also polysaccharides D-mannitol, tyrosine, uracil and some amino acids.

D-mannitol belongs to the category of compounds that show a significant therapeutic potential [7]. When evaluating the NMR spectra, D-mannitol was captured only in sample 5. In the other spectra, the above compounds were detected as the majority. We assume that the bands of minor compounds, including D-mannitol, overlap with the bands belonging to the major compounds.

Testing of the antimicrobial activity on *Escherichia coli* and *Staphylococcus aureus* strains showed that the % RIZD varied from 83 % to 166 % for active samples. Compounds belonging to the group of peptides, glycoproteins and, to a large extent, cordycepin itself are responsible for this antimicrobial activity.

CONCLUSIONS

Our research focused on the determination of antioxidant and antimicrobial activities of OS and PH methanol extracts. The extract of sample 5, PH, *Oryza sativa* var. *japonica* (sample 5) had the best ability to scavenge the DPPH radical (IC_{50} = 3.03 ± 0.05 mg.ml^{-1}). The analysis of NMR spectra confirmed the presence of the following compounds in extracts 1—6 in various ratios: oleic acid, isomers of linoleic acid, acetic acid, 1,4-butanedioic acid, malonic acid, polysaccharides, D-mannitol, tyrosine, uracil, and some amino acids. The testing of extracts for their antimicrobial activities against collection bacterial strains *Escherichia coli* and *Staphylococcus aureus* showed that the % of RIZD ranged from 83 % to 166 %. An increased antimicrobial activity against *E. coli* was observed in comparison with that against *S. aureus*.

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ECTOPIC PREGNANCY DIAGNOSED POST CAESAREAN SURGERY IN A THREE YEAR OLD BOERBOEL BITCH

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ABSTRACT

A three year old nulliparous Boerboel bitch presented with complaints of fever and inappetence six weeks after an elective caesarean section was diagnosed with an extrauterine foetus. A per-cutaneous abdominal ultrasound revealed a foetal sac showing a well-developed skeletal structure and the absence of foetal movement or heartbeat. During laparotomy, a foetal sac containing a dead foetus was located between the spleen and the stomach. The foetal sac was excised following ligation of its mesenteric attachment to the spleen. The previously operated uterus was observed to have involuted but revealed a small bud observed on the middle portion of the left uterine horn. The histological findings of the foetal sac revealed fibro-adipose tissue with numerous congested vessels. It was concluded that the Boerboel bitch had a secondary abdominal ectopic pregnancy and recommended that owing to the difficulty of diagnosing the condition before or during routine elective caesarean surgery, post-operative abdominal ultrasound would have been instructive.

Key words: Boerboel; dog; ectopic; extrauterine; foetus; pregnancy

INTRODUCTION

Extrauterine (ectopic) pregnancy is an abnormal pregnancy state in which a conceptus develops and attaches in an abnormal location outside the uterus [7]. It is a relatively common condition in humans but rare in animals [3]. Ectopic pregnancy has been reported in rabbits [4], cats [3] and dogs [9]. Four main types of extrauterine pregnancy based on location have been reported in human and other animals with various subtypes. These include: (1) tubal, (2) abdominal, (3) cervical and (4) ovarian ectopic pregnancies. In humans, the development of a gestational sac on an ovary [10] and implantation of a foetus on a cervical canal [2] have been reported but never in animals. Tubal extrauterine pregnancy occurs due to an implantation of the foetus within the oviduct [2]. It is the most common type of ectopic pregnancy in humans, but is rarely found in domestic animals because of the different physiology of the

uterus between humans and animals. Abdominal ectopic pregnancy is the most common type of ectopic pregnancy in animals, including bitches [4]. An abdominal ectopic pregnancy occurs when the foetus is implanted or existed in the abdominal cavity [2]. All reported cases of ectopic pregnancies in bitches were abdominal ectopic pregnancies [1].

An abdominal ectopic pregnancy can also be described as primary or secondary. Primary abdominal ectopic pregnancies occur as a result of entering and the implantation of a fertilized egg forming a placental relationship on the peritoneal or omental surface through the oviductal fimbria [4]. The placental attachment in the primary form may also be on the mesentery, liver, spleen and the large and small intestines [2]. The secondary abdominal ectopic pregnancy is considered to have occurred as a consequence of the rupture of the wall of the upper reproductive tract, thereby creating a communication between the uterus or oviduct and the peritoneal cavity with subsequent propulsion of the already gestating foetus into the abdominal cavity [3]. The majority of reported cases of abdominal ectopic pregnancies in bitches are considered to be secondary [7, 8].

Canine ectopic pregnancies are uncommon and the aetiology is not clearly understood, but it has been reported to be caused by uterine rupture due to road accidents, infection, uterine torsion and inappropriate obstetrical technique during the peri-parturient period [7]. Until now, most cases of canine ectopic pregnancy have been diagnosed in bitches that have whelped normally. There has been no report of canine ectopic pregnancy after caesarean section in bitches. This report presents the diagnosis and management of an unusual case of ectopic pregnancy in a three year old Boerboel bitch, six weeks after an elective caesarean surgery was performed.

Case Presentation

A three year old nulliparous Boerboel bitch was presented with fever and anorexia. The bitch had an elective caesarean operation six weeks earlier in another veterinary hospital during which five healthy puppies were removed. According to the owner, the bitch had been doing fine until about two days prior to presentation when the dog was observed with reduced appetite. At presentation, the dog was alert with pink mucous membrane. Rectal temperature (40.3 °C) was elevated, while the heart rate (88 beats.min^{-1}) and respiratory rate (24 breaths.min^{-1}) were within the normal range. The dog's abdomen was moderately enlarged and palpation revealed a mid-abdominal mass. Other parameters were within normal limits. An abdominal ultrasound performed with a portable ultrasound machine (Kaixin KX 2000R, Xuzhou, China), revealed a foetal sac with a well-developed skeletal structure and the absence of a foetal heart beat (Fig. 1). Thus, a diagnosis of a retained foetus was made and an exploratory laparotomy was scheduled to determine the location and removal of the retained foetus. Blood was obtained from the cephalic vein for the determination of selected haemato-biochemical parameters. The results showed severe lymphocytic (absolute lymphocyte count: 21.5 × 103.l^{-1}) leukocytosis (total leukocyte count: 42.9 × 103.l^{-1}), while other parameters were normal.

ETHICAL CONSIDERATIONS

Informed owner's consent and ethical approval (FUNAAB/COLVET/CREC/2020/09/02) were obtained.

Case Management

The dog's ventral abdomen was prepared aseptically. Premedication was done with 3 mg.kg^{-1} intramuscular injection of 5 % tramadol (Tramadol®, Gland Pharma, India) and one hour later with a combination of intramuscular

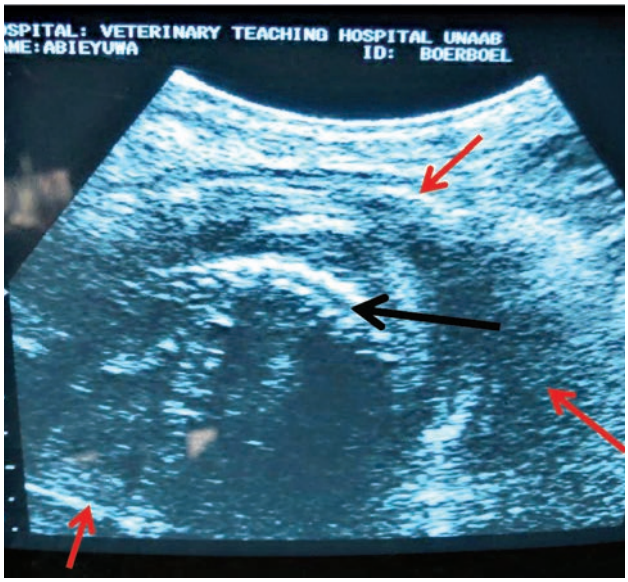

Fig. 1. Transcutaneous abdominal ultrasound of a three year old Boerboel dog with ectopic pregnancy showing a foetal sac (red arrows) and foetal skeletal structure (black arrow)

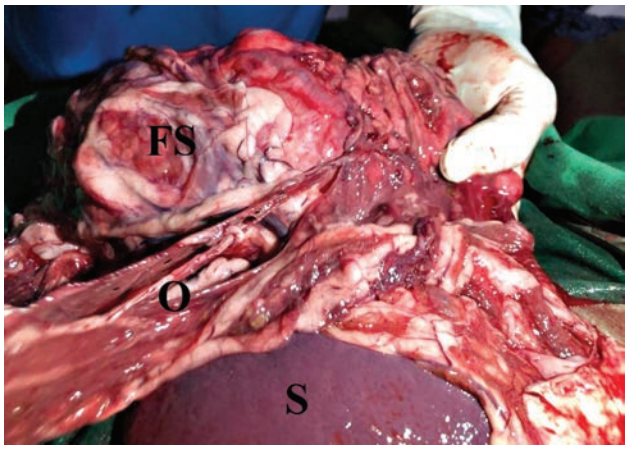

Fig. 2. Intraoperative picture of a three year old Boerboel dog with ectopic pregnancy showing a foetal sac (FS), omentum (O) and spleen (S)

Fig. 3. Intraoperative picture of a three year old Boerboel dog showing the uterine horn with a bud at the left horn (black arrow)

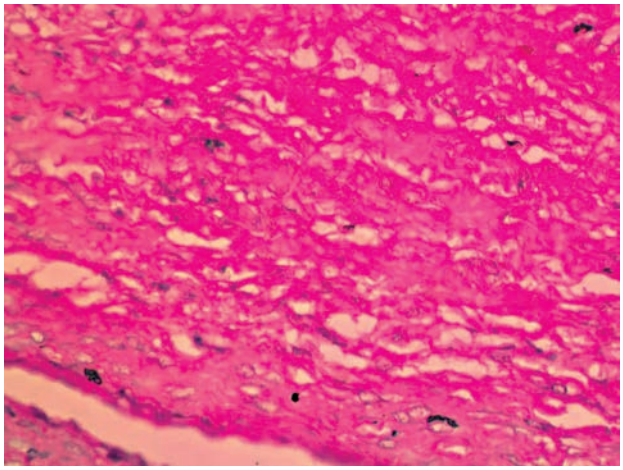

Fig. 4. Histology of the ectopic foetal sac from a three year old Boerboel dog showing fibroadipose tissue with numerous congested blood vessels. H and E, Magn. × 100

injections of 0.04 mg.kg^{-1} of atropine sulphate (Atocan®, Sishui Xierkang Pharma, China) and 0.5 mg.kg^{-1} of Xylazine Hydrochloride (Xylased, Bioveta, Ivanovice, Czech Republic). Venous access was secured at the cephalic vein and circulating blood volume maintained with Lactated Ringers solution at the rate of 5 ml.min^{-1}. Anaesthesia was induced with a loading dose of 4 mg.kg^{-1} intravenous injection of propofol (Hyprovan 200®, Celon Laboratories PVT LTD, Telangana, India) and maintained with constant infusion of propofol at the rate of 0.12 mg.kg-1.min^{-1}. After anaesthesia induction, the dog was positioned in dorsal recumbency and a standard ventral midline incision was made to gain access into the abdominal cavity. The foetal sac was located between the spleen and the stomach (Fig. 2) and a stab incision was made on the sac, and the incision was extended to expose a mummified foetus, after which the foetus was removed. The foetal sac was excised following ligation of its omental attachment to the spleen. Haemorrhage was controlled and the abdominal cavity was explored. The uterus was observed to have involuted and the hysterotomy incision around the inter-cornual junction had healed with no evidence of dehiscence. However, a small bud measuring about 0.5 cm was observed around the middle portion of the left uterine horn (Fig. 3). The abdominal incision was then closed routinely.

Postoperatively, tramadol injection at 4 mg.kg^{-1} and Amoxicillin injection (Amoxinject LA R, Bremer Pharma, Warburg, Germany) at 15 mg.kg^{-1} were administered intramuscularly for one week. The resected foetal sac was fixed in 10 % formalin for histopathology. Histologic findings (Fig. 4) revealed mesenteric fat, loose connective tissue, smooth muscles and numerous congested vessels. On the basis of the abdominal ultrasound, operative findings and histopathological examination, a final diagnosis of secondary abdominal ectopic pregnancy was made.

DISCUSSION

Most reported cases of extrauterine pregnancy were usually an incidental finding [7]. They are often associated with previous normal parturition and have been reported several months after normal parturition [11]. In this case, the ectopic pregnancy was diagnosed six weeks after an

elective caesarean section was performed in the bitch. This case is possibly the first report of ectopic pregnancy after a caesarean section. The cranial location of the foetal sac between the spleen and the stomach might have probably been responsible for its non-recognition preventing subsequent removal during the elective caesarean surgery. It might also have been difficult to recognize the ectopic pregnancy as different from the uterine pregnancy in this dog during routine pregnancy ultrasonographic or radiographic examination because of the multiple number of conceptuses. Although, radiographic examination is occasionally done to predict the expected number of puppies to be removed during caesarean surgery, this information might not be available to the surgeon at the time of the elective caesarean surgery. Owing to this unusual experience, we suggest that abdominal cavity be explored properly before routine closure of abdominal incision or an immediate postoperative abdominal ultrasound should be done after an elective caesarean surgery is performed to detect the presence of any extrauterine foetuses.

Abdominal ectopic pregnancy is described to be secondary when a foetus enters the abdominal cavity because of rupture of the uterine wall, usually as a result of trauma or injury, creating a communication between the uterus or oviduct and the peritoneal cavity with subsequent propulsion of already gestating foetus into the abdominal cavity [11]. The bud on the left uterine horn might be the possible point of uterine rupture that led to the detachment of the foetus and possible relocation between the spleen and the stomach. The exact cause of uterine bud is unknown but might be associated with a tear prior to the elective caesarean section. Histopathological examination was not performed on the uterus because it appeared normal and intact grossly. However, it has been reported that the myometrium is regenerative with little or no scar tissue [7].

It is often difficult to diagnose extrauterine pregnancy in the bitch because the majority of dogs seems healthy and may not show any clinical signs [1]. However, ectopic pregnancy can be diagnosed using imaging techniques such as radiography, ultrasonography, diagnostic laparoscopy, endometrial biopsy and computed tomography [7]. Although ultrasonography is the most preferred diagnostic imaging modality, it may be difficult to separate an extrauterine foetus from those in the uterine location, making its diagnosis prior to elective caesarean surgery difficult. Thus, advanced imaging modalities such as computed tomography or magnetic resonance imaging (MRI) can play important roles [5]. A differential diagnosis of ectopic abdominal pregnancy should be made for any intact bitch with a mid-abdominal mass in a recently whelped bitch or bitches with recently performed caesarean section.

Several management methods have been reported for human ectopic pregnancies such as surgical treatment, and medical treatment [6]. Medical treatment involves the administration of methotrexate and is indicated only in patients in which the foetal sac is intact. Surgical resection of the foetal sac following laparotomy is indicated in patient with a ruptured extrauterine foetal sac and is the only reported treatment in the literature for ectopic pregnancies in animals [11]. In this bitch, the ectopic pregnancy was surgically resected. This allowed for exploration of the abdominal cavity.

In conclusion, canine ectopic pregnancies associated with caesarean surgery are rare in dogs. The patient described in this case had a secondary abdominal ectopic pregnancy with no scar tissue grossly visible in the uterus but a presence of a bud on the left uterine horn. An ectopic pregnancy should be included in the differential diagnosis of a dog presenting with an abdominal mass. Surgical resection of the foetus is recommended whether clinical signs are present or not. We also recommend that post-operative abdominal ultrasound should be done following caesarean surgery owing to the inability to detect this case during the initial caesarean section.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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FASCINATING DENDRITIC CELLS— SENTINEL CELLS OF THE IMMUNE SYSTEM A REVIEW

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ABSTRACT

Dendritic cells (DC) are specialized antigen presenting cells which have the unique ability to activate naive T-lymphocytes. Their role in the immune system is much more sophisticated than it seems, as they do not kill the pathogens directly, but provide a long-lasting antigen specific immune response thanks to that sufficiently bridging the innate and the adaptive immunity. In recent years, there has been a growing interest in studies of their role in immune regulation, autoimmune reactions, as well as in immune responses against pathogens and tumours. Processing and presentation capabilities of a highly specific and unique tumour antigen makes them an interesting tool for stimulating effective anti-tumour immunity. *In vitro* generations of DC represent a preferred model for more detailed studies of DC biology in other fields. The aim of this review was to discuss the main role of dendritic cells in the body as well as their current use as experimental models for further scientific studies.

Key words: anti-tumour immunity; dendritic cells; immune response; T-lymphocytes

INTRODUCTION

The immune system (IS) can be thought of as a set of interactive cellular and molecular networks where the presence of foreign invaders trigger changes in cellular activities and generates an expanding set of cellular and molecular responses. These reactions eventually result in the elimination of the invaders and increased resistance to infection in the body [66]. The IS represents all the mechanisms that allow a living organism to distinguish between "self" and "non-self" [34]. It can be activated by different molecular patterns—antigens, which the body does not recognize as its own. These antigens attach to special receptors on the immune cells triggers a whole series of processes in the body [54]. Immunity is divided into two parts based on the speed and specificity of the reactions called innate and adaptive immunity [22]. The main purpose of the innate immune response is to immediately prevent the

spread and movement of foreign pathogens in the organism. The adaptive IS provides a defense system that can recognize and destroy specific invaders and then learn from the process that if they attack a second time, they could be destroyed even more effectively [10]. Adaptive immunity in order to be triggered first needs to capture, process, and correctly present a given sample of foreign material to cells that can recognize it. The responsible cells for this antigen processing process are so-called professional APCs (antigen-presenting cells), which include dendritic cells, macrophages, and B-lymphocytes [57, 66].

Dendritic cells biology

The theory of the cell base, which links antigens presented in tissues with immune responses, was unexplained until the late 1980s [1]. Dendritic cells (DC) were discovered in 1868 by Paul Langerhans, who claimed in his work that the epidermal layer of the skin consists of a network formed by cells that are probably part of the nervous system. He described them as "tree-like" cells, so he called them dendritic cells, from the Greek word dendreon which means tree [43].

It took up to hundred years for Ralph Steinman and Zanvil Cohn to elucidate and publish an article in 1973 stating that dendritic cells with their specific properties belong to the group of white blood cells [62]. Subsequently, during many experimental studies of DCs, it has been clearly shown that these professional APC have unique immunostimulatory and immunomodulatory properties and are among the most important cells of the immune system [60].

Steinman proposed in his studies a classical model of how DC work and what is their function [1]. These cells are able to absorb surrounding antigens by the process of endocytosis and phagocytosis in tissues, then they migrate through lymph nodes, where they present processed antigens to naive T-lymphocytes [61].

DCs are professional antigen-presenting cells that are the only ones in the body able to activate naive T-lymphocytes and initiate a primary cellular response. After absorbing and processing foreign antigens in peripheral tissues, they migrate to secondary lymphoid organs to present these processed antigens to T-lymphocytes, providing long-lasting antigen-specific responses sufficiently bridging innate and adaptive immunity [13].

DCs have the unique ability to activate naive T cells and direct the immune response toward the production of other T cell subpopulations such as Th1, Th2, Th17, or Treg cells [4]. As a result, they allow for naive T-lymphocytes to contact with peripheral antigens that they would not otherwise encounter. Once the invader has been removed, the body continues to develop acquired immunity against a second attack by the same organism [63].

General opinions assume that dendritic cells exist in the body in two basic functional stages – as immature DCs, which induce self-tolerance, and mature DCs, which induce immunity to foreign antigens, but only mature ones are able to control clonal T cell expansion and prime immune responses [46].

DC ontogenesis and DC cells subsets

All cellular blood components are derived from haematopoetic stem cells ($CD34^+$) in a process called haematopoiesis [8]. $CD34^+$ stem cells found in the bone marrow can differentiate into lymphoid or myeloid progenitor cells, which have the potential to develop into both myeloid and lymphoid lineages [14, 30]. T- and B-lymphocytes, NK cells (Natural Killer cells) differentiate from a common lymphoid progenitor (CLP), while megakaryocytes, erythrocytes, granulocytes, monocytes and macrophages belong to the myeloid lineage and differentiate from common myeloid progenitor (CMP) [25, 26, 68].

Conventional lymphoid progenitors can differentiate into all types of lymphocytes under physiological conditions without visible myeloid potential; similarly, conventional myeloid progenitors can lead to all classes of myeloid cells with no or very low levels of B-cell potential [26]. DCs represent a "single" cell type that is not clearly clustered in lymphoid or myeloid lineage because, under certain conditions, they can arise from either CLP or CMP [33, 67].

Thus, DCs are derived from $CD34^+$ stem cells, but represent a heterogeneous group of specialized APCs [48], where it is important to realize that key growth factors are required for their differentiation, as their differentiation process strongly depends on the cytokine microenvironment such as for example Flt3-L (fms-like tyrosine kinase 3 ligand), GM-CSF (Granulocyte-macrophage colony-stimulating factor) and M-CSF (Macrophage-colony stimulating factor) or TGF-β (Transforming growth factor-beta) [6, 21, 38].

As DCs represent heterogeneous cell type, they are classified into different subsets. With no standardized approaches for dendritic cell classification in the past, they

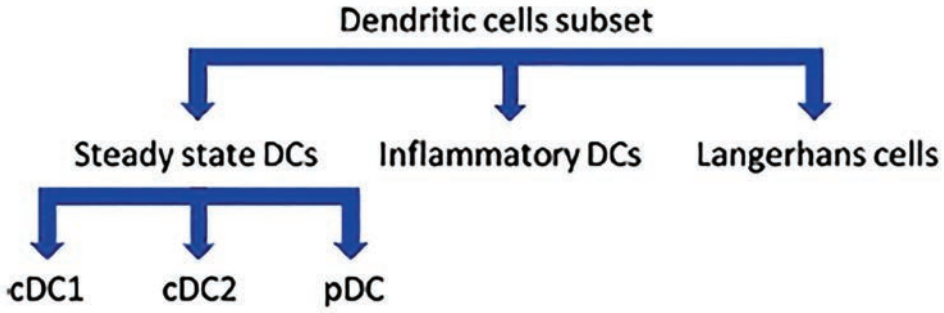

Fig. 1. Simplified classification of dendritic cell subsets by flowchart [6]

were roughly classified based on their origin, tissue location, phenotype, or cytokine profile [6]. Current experimental studies prove that there are several dendritic cell lineages. These include the originally identified so-called conventional cDCs, which belong to steady-state DC divided into cDC1, cDC2, and plasmacytoid DCs (pDCs), Langerhans cells (LCs), and monocyte-derived DCs (moDCs) (Fig. 1.) [2, 17, 37].

The identified DC populations differ in basic modules such as expression of various markers, transcription factor requirements, recognition of patterns from the environment, and specialized functions in initiating and modulating T-lymphocytes. As a result of this process, different DC populations acquire different phenotypes and also colonize different tissues performing different functions [2, 5, 37].

Immunological tolerance

Dendritic cells formed in the bone marrow are released into the bloodstream, where they circulate and serve as effective immunological surveillance. Immature DCs circulating in peripheral blood are characterized by low potential for T cell activation but high endocytic activity, they continuously capture proteins from the environment, digest them to short peptides, and display on their surface [61, 63].

The term mature DC is commonly used as a phenotypic characteristic to label those cells which are expressing high levels of MHC molecules and CD40, CD80, CD83, and CD86 on their surface. It is generally assumed that DCs that are mature according to phenotypic criteria are also functionally mature, so the expression of these molecules often correlates with the ability to prime T cells [46].

The study by A u s t y n et al. [5] showed that DCs migrating from peripheral blood to the spleen, or Langerhans cells (LCs) migrating from skin grafts, mature during migration and lose the ability to phagocytose antigens. The phenotypic analysis of mature and immature DCs is in line with the previous statements. Freshly isolated immature LCs from the skin expressed multiple receptors for antigen uptake (such as receptors for IgG (FcγRs)), and fewer receptors for T cell activation (including MHC molecules, the integrin LFA-1 (Lymphocyte function-associated antigen 1) and the co-stimulatory molecules) than cultured mature LCs obtained from the skin. The mature one expressed on their surface a much lower number of receptors for antigens, but a higher number of receptors needed for T cell activation [28, 46, 50, 56].

DCs play an indispensable role in building self-tolerance through their ability to present their own antigens to evolving T cells in the thymus, thereby inducing negative T cell selection or differentiation of T regulatory cell (Treg) population [44]. Immune tolerance has been described in immature DCs that have low numbers of surface MHC, costimulatory and adhesive molecules, such as CD80 and CD86 [13]. In contrast, mature DCs are characterized by the presence of high expression of CD80/86 [58]. Thus, tolerogenic DCs often exhibit an immature or semi-mature phenotype, which is characterized by low expression of costimulatory and MHC molecules. The immunosuppressive mechanisms of tolerogenic DCs further show decreased secretion of IL-12 and, conversely, increased secretion of immunomodulatory cytokines such as IL-10 and TGF-β, leading to naive T-lymphocytes differentiating into Treg rather than into T-effector cells [27, 45]. These facts suggest that the induction of tolerance depends on the state of development and activation of DCs, as well as on the surrounding cytokine microenvironment and growth factors [13].

Porcine MoDC

The human and the porcine IS have many similarities with regard to DC biology [3]. The porcine model has found application in many biomedical industries such as wound healing (skin) models [51], renal anatomy models [32], or cardiac cell therapy [59], thus, there is a growing interest in porcine immunology due to the potential of the pigs as a donor in xenotransplantation [12, 23, 35, 49]. Gnotobiotic miniature pigs represent presumably the most convenient model for human immunology study tool, thanks to their cDC2 gene expression signature which is the closest to the human counterpart [3, 15, 24].

The *in vitro* generation of DC represents an advantageous model for studying DC biology. These differentiated cells have been widely used [18], mainly bone marrow-derived dendritic cells (BMDCs) cultured with cytokine cocktail containing GM-CSF and IL-4 for generating BMDCs [24].

Currently, the two most common protocols are used to generate DC from peripheral blood monocytes [39]. Porcine monocyte-derived DCs (MoDCs) can be generated by the so-called fast method (within 48 hours), but it must be taken into account that the function and phenotypic characteristics of these MoDCs are only partially developed [70]. The classical method lasts at least 7 days, when the generated MoDCs are more reliable and effective for investigating the host-pathogen interactions [29, 64]. As was mentioned before, it is well known that the biological and functional properties of immature and mature DCs are far different. From this point of view, the maturation stage of MoDCs is crucial for experimental interpretations [39]. It is important to mention that the classical method has also some limitations, frequent manipulation with harvested cells can cause undesirable DC maturation by physical and pressure stimulation and this method also requires a large amount of peripheral blood mononuclear cells [7].

In various studies, blood-derived monocytes are used for further DC studies. For instance, the Transwell co-culture system was used as an *in vitro* model for intestinal submucosal immune system (GALT) studies. This co-culture platform was established from a cell culture of porcine intestinal IPEC-J2 cells and from porcine MoDCs, which served as a model for testing the effect of enterotoxigenic Escherichia coli (ETEC) [31]. Intestinal epithelial cells and DCs located in the lamina propria by communicating with each other and releasing different soluble molecules, coordinate the immune response in the presence of intestinal pathogens, so this co-culture model made it possible to assess pro-inflammatory signalling pathways induced by ETEC.

Another study used human (hMoDC) and porcine (pMoDC) MoDCs for a better understanding of Flavivirus species tropism and innate immune responses. MoDCs were selected based on their role as early target cells for several Flaviviruses. In comparison, viral infectivity and replication between hMoDC and pMoDC were minor, but some of the tested Flaviviruses were found particularly strong replication in hMoDC with Usutu virus, meanwhile, the Japanese encephalitis virus showed a stronger tropism for pMoDC [16].

Clinical implications—DC vaccines for cancer immunotherapy

Dendritic cells currently represent the most attractive platform for anti-tumour cell vaccine preparations. Their unique ability to activate naive T-lymphocytes and elicit effective specific immune responses, make them the most important candidates in this field [9]. Cancer vaccines are designed to "educate" patient's own immune system to generate effector T cells specifically for the detection and destruction of tumour cells. The goal of a customized cancer vaccine is to focus on patient-specific tumour antigens and reduce the side effects by protecting normal tissue and maintaining the longest possible regulation of immune memory [20]. Dendritic cell vaccines represent an attractive choice for immunotherapy due to their low toxicity profile, non-invasiveness, and their potential to elicit long-term effects through immunological memory [9, 53].

The first clinical studies testing dendritic cells as vaccines in anti-tumour therapy are dated back to the late 1990s. Dendritic cells were obtained from peripheral monocytes, which were subsequently loaded with tumour antigens in various ways and used to promote immunity against tumour-specific T cells [52]. These immunostimulatory vaccines have been used for the treatment of various types of cancers such as B cell lymphoma [19], melanoma [11, 36, 40], myeloma [47], hepatocellular cancer [11] or acute myelogenous leukaemia [65]. These first experiments showed that DC vaccines were both safe and immunogenic [52].

The opportunity of preparing dendritic cells from peripheral monocytes ex vivo (MoDCs) has led to intensive

scientific work in order to prepare an effective antitumor vaccine from dendritic cells for various types of cancer. Although MoDCs are immunogenic they lack adjuvant capacity and immune-activating properties of natural DCs, such as cross-presentation and optimal production of IL-12 and type I interferon [48, 55]. The preparation process of anti-tumour cell vaccines uses defined tumour-associated antigens in the form of proteins, peptides, and RNA or viral vectors as a source of antigens in order to achieve their expression in the cytoplasm of DC and thus ensure efficient presentation by MHC molecules [41, 69]. DCs loaded with the desired antigens are administered to patients in a number of ways. In one approach, autologous antigen-specific T cells are expanded ex vivo and then re-infused into patients. Another approach is through vaccination; that is, the provision of an antigen together with an adjuvant to elicit therapeutic T cells *in vivo* [42].

CONCLUSIONS

Dendritic cells as the most effective APC in the body represent a essential part of the immune system necessary for the initiation and modulation of T cell immune responses *in vivo*. They are the only ones in the body able to directly activate antigen-specific cytotoxic $CD8^+$ T cells and T cell-dependent antibody production, thus they serve as crucial component of our immune system, critical for the initiation and regulation of adaptive immune system. Their antigen presentation ability and immune-regulatory capacities hold great promise for the treatment of autoimmune diseases, cancer, and the prevention of transplant rejection.

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A FIELD STUDY EVALUATING HUMORAL IMMUNITY IN CALVES VACCINATED WITH MULTIVALENT BOVINE RESPIRATORY PATHOGEN VACCINES

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ABSTRACT

Bovine Respiratory Syncytial Virus (BRSV), Bovine Parainfluenza 3 (BPI3) and *Mannheimia haemolytica* (*Mh*) are major respiratory pathogens in the bovine respiratory disease complex. It is important to optimize passive and active immunity to these pathogens early in life to reduce clinical and subclinical productivity losses. The administration of inactivated, adjuvanted and multivalent vaccines, such as Bovilis® Bovipast RSP (Bovipast), and Bovalto® Respi 3 (Bovalto) to calves, may enhance cellular and humoral immunity against BRSV, BPI3 and *Mh*. A field trial evaluated the immune responses to these three agents in the first year of life in 12 Bovipast and 13 Bovalto vaccinated calves, and 5 negative control calves. Calves were vaccinated starting at 2 weeks of age and revaccinated 4 weeks later (primo vaccination). A booster vaccination was given at approximately 10 months of age. Serum samples were taken at time intervals up to 6 months after primo vaccination and up to 1 month after the booster vaccination. BRSV serum titres were evaluated using a serum neutralisation assay (SN), and BRSV, BPI3 and *Mh* titres were evaluated using a commercial enzyme linked immunosorbent assay (ELISA) test. Serum antibodies after primo and booster vaccinations in the individual calves were evaluated by calculating the areas under the curve (AUC) of the Log2 transformed BRSV SN titres and the optic density measures of the ELISA tests for BRSV, BPI3 and *Mh*. Multivariate general linear models were used to evaluate the influence of the vaccination on the AUC of the serum measures within 6 months after the primo vaccination. Similarly, models evaluated the AUC of the serum measures after the booster vaccination. The Bovipast vaccinated calves had significantly higher SN and ELISA titres AUC following the primo vaccination and booster vaccinations compared to the negative control calves and the Bovalto vaccinated calves. The Bovalto vaccinated calves did not have a significantly different BRSV SN and ELISA titres AUC response after the primo or booster vaccinations compared to the negative control calves. The serum antibody responses to BPI3 and *Mh* in the vaccinated calves were less pronounced than the Bovipast BRSV antibody response. Bovipast and Boval-

to vaccinated calves mounted a significantly higher AUC ELISA OD for both BPI3 and *Mh* and the highest AUC was measured in the Bovipast vaccinated calves. This study indicated that early vaccinations of calves with multivalent adjuvanted inactivated BRD vaccines, such as Bovilis® Bovipast RSP can elicit a humoral response with a cellular-mediated memory effect as indicated by the booster vaccination.

Key words: Bovine Respiratory Disease; BPI3; BRSV; comparison; field study; *Mannheimia haemolytica*; neutralization; respiratory; vaccination

INTRODUCTION

Bovine respiratory disease (BRD) in calves is a complex and multifactorial disease, causing huge financial losses to the dairy and beef industry worldwide in veterinary treatment costs and reduced performance [3, 4, 13]. Bovine Respiratory Syncytial Virus (BRSV), Bovine Parainfluenza 3 virus (BPI3), bovine herpesvirus type 1 (BHV-1), bovine coronavirus (BCoV), *Mannheimia haemolytica* (*Mh*) and *Mycoplasma bovis* are considered as major or primary agents able to initiate BRD pathology. Other agents like *Pasteurella multocida*, *Histophilus somni* are generally considered as secondary or opportunist pathogens. With increasing threats of antimicrobial resistance due to antimicrobial prophylactic, metaphylactic and therapeutic BRD treatments, multivalent BRD vaccines are of increasing importance.

The bovine respiratory syncytial virus (BRSV) is a major pathogen within the BRD complex and cattle populations around the world are infected. Seroprevalences in infected herds can reach nearly the entire herd and many countries report that most herds are seropositive. BRSV infection predispose calves to secondary bacterial infection, resulting in the BRD complex [18].

BRD is mostly affecting young calves from the first days of life and BRSV-associated respiratory disease is most pronounced in calves less than 6 months of age. Therefore, it is important to optimize passive and active immunity to these pathogens early in life to reduce clinical and subclinical productivity losses. Pregnant cows could be vaccinated to transfer specific immunity by colostrum and calves may be vaccinated early in life to develop their own immunity quickly. However, some vaccines may be adversely affected by interference from maternal antibodies or unfavourable nutritional conditions [2]. As BRD may affect young calves after the decline of the maternal antibodies, vaccination against BRD pathogens should target calves in the first weeks of life, when maternal antibody levels are still reasonably high.

It is difficult to draw general conclusions about the immunity and protection induced by BRD vaccines. Differences in strains and adjuvant composition, or other differences in formulation, such as antigenic mass, the inactivation process, may be responsible for induction of disparate immune responses by 2 apparently similarly adjuvanted vaccines [9].

The multivalent, adjuvanted and inactivated respiratory disease vaccines Bovilis® Bovipast RSP (Bovilis Bovigrip: product name in France/ Bovigrip® RSP plus: product name in Germany) (Bovipast) from MSD Animal Health and Bovalto® Respi 3 (Bovalto) from Boehringer Ingelheim can be administrated to calves from two weeks of age and these contain inactivated BRSV, BPI3 and *Mh* strains. For Bovipast it has been demonstrated that protection against BRSV, BPI3 and *Mh* infection is provided even in the presence of maternal antibodies. The Bovalto vaccination efficacy has only been proven in seronegative animals, and the efficacy of the vaccination has not been demonstrated in the presence of maternal antibodies. Consequently, the level of antibody response after Bovalto administration may be reduced by maternal antibodies. It is difficult to find and keep calves seronegative for these respiratory pathogens in commercial farms since most dairy cows have been vaccinated or exposed to the viruses and transmit colostral antibodies to the calf. The BRSV seroprevalence in infected herds can reach nearly the entire herd and many countries have reported the prevalence of infected herds of 80 % or higher. Also, a BPI3 seroprevalence above 50 % is commonly reported in European countries [18]. Serum antibody response may be used to evaluate vaccination response and clinical risk of a respiratory challenge with BRSV has been shown to be correlated with neutralising antibody titres [25]. Virus neutralisation is a technique that makes it possible to objectively evaluate and compare the neutralisation capacity of the serum from vaccinated animals.

The objective of this field study was to evaluate the seroneutralising antibody responses against BRSV and specific humoral (IgG) response to BRSV, BPI3 and *Mh* in

young calves vaccinated with either Bovipast or Bovalto and non-vaccinated calves between 15 to 30 days of age, and after revaccination 9 to 11 months later.

MATERIALS AND METHODS

This study was performed on a dairy farm officially free from bovine viral diarrhoea (BVD) and infectious bovine rhinotracheitis (IBR) in Maine-et-Loire, France. The farm had no previous history of BRD in calves and no BRD vaccinations had been performed. Thirty calves born at the farm during the fall season of 2016 were included. All calves received at least three litres of colostrum during the first 6 hours of life according to farm protocols and were healthy at the time of the enrolment. All calves were raised under the same conditions as regards management, housing and feeding throughout the study. The calves were raised in individual igloo type hutches separated by at least a 40 cm space, thus not allowing nose to nose contact until after weaning at 2.5—3 months of age. After the first colostrum feed, the calves were fed farm whole fresh milk. After weaning between two to three months the calves were placed in group housings of 4—5 calves per group for a few weeks. Thereafter the calves were mixed in larger groups of approximately 20 calves in semi-enclosed buildings providing protection from rain and wind. There was no introduction of calves from other farms during the study period.

Healthy calves were randomly allocated to either vaccine groups Bovipast or Bovalto, or non-vaccinated Control group during each vaccination visit by the local practitioner who performed the vaccinations. Control calves were included in the first 5 vaccination visits to evaluate farm background levels of exposure to the respiratory agents. The calves were sequentially allocated to treatment and were group housed mixed together after the individual hutch period. The Bovipast group consisted of 12 calves, the Bovalto group of 13 calves and the control group of 5 calves. Both vaccines are composed of three inactivated pathogens. The Bovipast vaccine contains the BRSV strain EV908, PI3 strain SF-4 Reisinger and *M. haemolytica* A1 strain M4/1-IRP. The Bovipast vaccine used were in 50 ml bottles and the vaccine dose was 5 ml. The Bovalto vaccine contains the BRSV strain BIO-24, PI3 strain BIO-23 and *M. haemolytica* A1 strain DSM 5283. The Bovalto vaccines used were in 10 ml bottles and the vaccine dose was 2 ml. The study calves were in contact with non-study calves in the group housing pens.

The calves in the two vaccine groups were primo vaccinated at an age of 14—28 days (T0) and a second time 1 month later (T0+1m) and booster vaccinated at 9—11 months of age (T10). Blood samples were taken at T0, and thereafter every 2 weeks until 3 months after T0 (T0+0.5m, T0+1m, T0+1.5m, T0+2m, T0+2.5m, T0+3m). Thereafter, monthly blood samples were taken between 4 and 6 months after the first vaccination (T0+4m, T0+5m, T0+6m). Serum samples were taken at the booster vaccination (T10), and thereafter 2 weeks (T10+0.5m) and 1 month (T10+1m) after T10.

The serum was collected and transported cooled to the laboratory. All samples were analysed with ELISA kit BioX K 369 MULTISCREEN for serodiagnosis of BRSV, BPI3, and *Mh* (BioX Diagnostics SA, Rochefort, Belgium). The tests were 96-well microtitration plates sensitized with monoclonal antibodies specific to BPI3 and BRSV and purified lipopolysaccharide (LPS) of *Mh.* The seroneutralisation assay previously described was adapted for BRSV seroneutralising antibody detection [5]. Heat inactivated serum samples were two-fold diluted in minimum essential medium (1:2 to 1:4096) in 96-well plates. One hundred TCID 50.50 μl^{-1} of the 220/69 virus strain (from Belgium) suspension were added to each well. After homogenization, plates were incubated one hour at 35 °C ± 1 °C and 5 % CO_2. A bovine foetal kidney cell suspension containing 100 000 cells.ml^{-1} was added in each well. After homogenization, plates were incubated 4 to 5 days at 35 °C ± 1 °C and 5 % CO_2. Cell culture control, positive and negative control sera were added in each assay. Virus strain suspension used in the assay was also titrated. Conformity of all these parameters was checked for validation of the assay. The end-point titre was determined as the inverse of the highest dilution of serum that inhibited viral cytopathogenic effects. Seroconversion was defined as a four-fold increase in BRSV titres. The optic density (OD) measures of the ELISA tests and Log2 of SN titres were used as outcomes.

The results were entered into a spreadsheet by the study investigator. The data were verified and subsequently analysed using the statistical software SAS 9.4. The number and % of calves in the respective groups that had SN titres ≥ 16 (4Log2) were evaluated. Univariate analysis was performed of the outcomes and the calves respective AUC

values to evaluate their distributions for further statistical modelling [15]. To determine the effect of the level and duration of immunity, the area under the curve (AUC) was calculated for the period T0+1.0m to T0+6m (AUC-primo), and for the period T10m to T10+1m (AUC-booster) using Log2 transformed BRSV serum titres and ELISA OD measures of the BPI3 and *Mh*. General linear models (GLM) were used for the three agents, to evaluate the influence of the vaccination on the AUC of the serum measures within 6 months after the primo vaccination (AUC-primo) with age at vaccination (T0-age) and AUC of serum measures at the time of the primo vaccination (AUC-base) as covariates. Similarly, general linear models evaluated the influence of the vaccination on the AUC of the serum measures after the booster vaccination (AUC-booster) with the serum titres at the time of the booster vaccination (T-booster) as covariates.

RESULTS

All of the enrolled calves completed the first 6-month follow-up period of the primo vaccination. Two calves in the Bovalto group were lost to follow up in the booster vaccination. From each calf, ten serum samples were obtained following the primo vaccination and 3 serum samples were obtained following the booster vaccination. No BRD associated diseases were recorded during the study. The mean serum titres per calf group are described in Table 1 and Figures 1 through 4.

The number and percentage of calves with serum SN titres for BRSV ≥ 16 is described in Table 2. Over the complete study period, after the primo vaccination, 83 % of Bovilis Bovipast RSP® vaccinated calves, 31 % of Bovalto 3® vaccinated calves and 20 % of control calves had seroneutralisation titres ≥ 1 : 16 (4Log2) at one or several sampling times. The Bovalto vaccinated calves and the control calves

Table 1. Mean Log 2 serum neutralisations (SN) titres for Bovine Respiratory Syncytial Virus (BRSV) and ELISA optic density (OD) measures for BRSV, Bovine Parainfluenza 3 (BPI3) and *Mannheimia haemolytica* (*Mh*)

Agent	Group	Months after T0. Mean Log2 SN titres for BRSV												
		T0	T0+0.5	T0+1	T0+1.5	T0+2	T0+2.5	T0+3	T0+4	T0+5	T0+6	T10	T10+0.5	T10+1
BRSV	Bovipast	1.7	1.9	1.3	2.9	2.6	2.8	2.7	2.6	2.5	1.9	1.3	5.3	4.2
BRSV	Bovalto	2.8	2.4	1.9	1.9	1.8	1.5	1.3	0.8	0.7	0.7	0.5	1.2	1.2
BRSV	Control	3.4	2.0	1.4	1.8	0.8	0.6	0.2	0.0	0.0	0.4	0.6	0.8	0.2
Agent	Group	Months after T0. Mean ELISA OD for BRSV												
		T0	T0+0.5	T0+1	T0+1.5	T0+2	T0+2.5	T0+3	T0+4	T0+5	T0+6	T10	T10+0.5	T10+1
BRSV	Bovipast	45.6	35.7	32.6	87.9	78.2	76.0	75.9	64.2	50.2	38.4	45.9	106.1	109.1
BRSV	Bovalto	50.6	41.3	42.3	60.2	45.4	37.9	40.0	37.2	27.6	20.5	34.6	86.8	85.3
BRSV	Control	64.1	48.1	36.3	33.2	26.8	16.1	22.7	11.3	18.3	12.0	17.8	16.1	12.9
Agent	Group	Months after T0. Mean ELISA OD for BPI3												
		T0	T0+0.5	T0+1	T0+1.5	T0+2	T0+2.5	T0+3	T0+4	T0+5	T0+6	T10	T10+0.5	T10+1
BPI3	Bovipast	56.6	60.9	77.0	131.0	123.3	115.4	108.7	89.1	73.3	62.2	100.9	130.8	123.3
BPI3	Bovalto	72.5	71.4	73.1	115.8	102.0	82.4	78.7	73.3	68.4	56.4	99.7	116.8	118.3
BPI3	Control	69.9	58.2	50.0	34.0	34.0	23.4	42.1	70.2	60.3	40.2	105.4	115.7	106.7
Agent	Group	Months after T0. Mean ELISA OD for *Mh*												
		T0	T0+0.5	T0+1	T0+1.5	T0+2	T0+2.5	T0+3	T0+4	T0+5	T0+6	T10	T10+0.5	T10+1
MH	Bovipast	59.6	47.0	41.1	42.5	41.3	46.0	54.2	138.3	183.2	210.1	80.8	99.1	87.3
MH	Bovalto	74.1	67.9	42.3	34.8	31.7	44.1	48.5	120.2	162.2	174.3	78.3	91.8	79.4
MH	Control	57.1	48.5	37.5	33.0	25.2	27.1	24.1	42.5	117.4	114.1	64.9	69.7	60.8

Table 2. Number of calves tested and percentage of calves that had serum neutralisations (SN) titres) ≥ 16 to Bovine Respiratory Syncytial Virus (BRSV)

Group	N	T0	T0+0.5	T0+1	T0+1.5	T0+2	T0+2.5	T0+3	T0+4	T0+5	T0+6	N	T10	T10+0.5	T10+1
Bovipast	12	8%	17%	0%	50%	33%	33%	50%	42%	25%	17%	12	0%	83%	66%
Bovalto	13	31%	31%	23%	23%	15%	0%	0%	0%	0%	0%	11	0%	0%	0%
Control	5	60%	20%	20%	20%	0%	0%	0%	0%	0%	0%	5	0%	0%	0%

Table 3. Least square Means (LSM) from General Linear Models (GLM) of Area Under the Curve (AUC) 1 months to 6 months after primo vaccination with multivalent BRD vaccines using serum Log 2 SN titres for BRSV and ELISA OD for BRSV. BPI3 and *Mh*

Serum test	Value	LSM	S.E	P-value	Lower CI	Higher CI	Sign diff
BRSV SN	Control	2.3	2.4	ref.	-2.5	7.0	b
BRSV SN	Bovalto	5.6	1.4	0.25	2.8	8.3	b
BRSV SN	Bovipast	12.9	1.5	< 0.01	9.9	15.9	a
BRSV ELISA	Control	76.3	40.6	ref.	–3.2	155.8	b
BRSV ELISA	Bovalto	180.4	23.3	0.03	134.7	226.1	b
BRSV ELISA	Bovipast	322.9	40.6	< 0.01	243.4	402.4	a
BPI3 ELISA	Control	220.5	50.6	ref.	121.2	319.7	c
BPI3 ELISA	Bovalto	387.4	29.3	< 0.01	330.0	444.9	b
BPI3 ELISA	Bovipast	485.0	32.0	< 0.01	422.3	547.7	a
***Mh* ELISA**	Control	293.1	64.6	ref.	166.5	419.8	b
***Mh* ELISA**	Bovalto	479.7	38.1	0.02	405.2	554.3	a
***Mh* ELISA**	Bovipast	521.7	41.7	<0.01	440.0	603.5	a

had elevated titres only during the first month after vaccination, and no antibodies detected thereafter, indicating that the titres might have been residual colostral antibodies. After the booster vaccination, 83 % of the Bovipast vaccinated calves seroconverted, whereas no calves in Bovalto or control group seroconverted.

The baseline serum titres for BRSV SN, BRSV ELISA, BPI3 ELISA and *Mh* ELISA (AUC-base) at the time of the first and second injection of the primo vaccination (Table 1) were not significantly different between groups, and there was no significant influence of age at vaccination (T0-age) on AUC-base (analysed separately in 4 separate GLM models, models not shown, can be obtained upon request).

The primo vaccination resulted in varied vaccination response in the groups in the period T0+1m to T0+6m (Table 1) for the various agents. Four separate GLMs evaluated the AUC-primo with AUC-base and T0-age as covariates (Table 3). BRSV Log2 SN AUC-primo was significantly higher in Bovipast vaccinated calves (12.9) compared to Bovalto vaccinated calves (5.6) and control calves (2.3). Similarly, BRSV ELISA AUC-primo was significantly higher in Bovipast vaccinated calves (322.9) compared to Bovalto vaccinated calves (180.4) and control calves (76.3). BPI3 ELISA AUC-primo was significantly higher in Bovipast vaccinated calves (485.0) compared to Bovalto vaccinated calves (387.4) and both were significantly higher than control calves (220.5). *Mh* ELISA AUC-primo was significantly higher in Bovipast vaccinated calves (521.7) and Bovalto vaccinated calves (479.7) compared to control calves (293.1).

The booster vaccination (AUC-booster) GLMs accounted for the titre at the booster vaccination (T-booster) (Table 4). BRSV Log2 SN AUC-booster was significantly

Table 4. Least square Means (LSM) from General Linear Models (GLM) of Area Under the Curve (AUC) 1 month after booster vaccination with multivalent BRD vaccines using serum Log 2 SN titres for BRSV and ELISA OD for BRSV. BPI3 and *Mh*a

Serum test	Value	LSM	S.E	P-value	Lower CI	Higher CI	Sign diff
BRSV SN	Control	0.8	0.4	ref.	-0.1	1.7	b
BRSV SN	Bovalto	1.2	0.3	0.40	0.6	1.8	b
BRSV SN	Bovipast	3.7	0.3	<0.01	3.2	4.3	a
BRSV ELISA	Control	23.4	5.4	ref.	12.9	33.9	b
BRSV ELISA	Bovalto	74.1	3.4	<0.01	67.5	80.7	a
BRSV ELISA	Bovipast	87.9	3.4	<0.01	81.3	94.5	a
BPI3 ELISA	Control	109.9	2.6	ref.	104.8	115.0	b
BPI3 ELISA	Bovalto	113.2	1.8	0.30	109.8	116.6	b
BPI3 ELISA	Bovipast	121.5	1.7	<0.01	118.2	124.8	a
***Mh* ELISA**	Control	74.1	4.7	ref.	64.9	83.4	b
***Mh* ELISA**	Bovalto	84.5	3.1	0.08	78.3	90.6	ab
***Mh* ELISA**	Bovipast	89.1	3.0	0.01	83.2	95.0	a

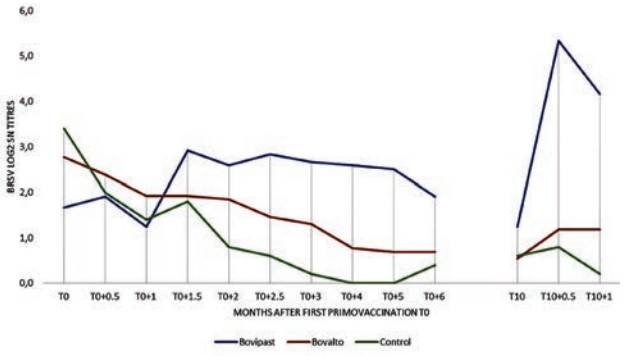

Fig. 1. Log 2 serum neutralisation titres against Bovine Respiratory Syncytial Virus in serum from calves vaccinated with multivalent bovine respiratory disease vaccines

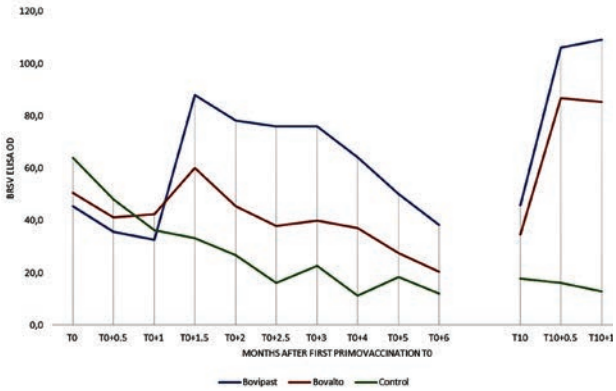

Fig. 2. Bovine Respiratory Syncytial Virus ELISA optic density measures in serum from calves vaccinated with multivalent bovine respiratory disease vaccines

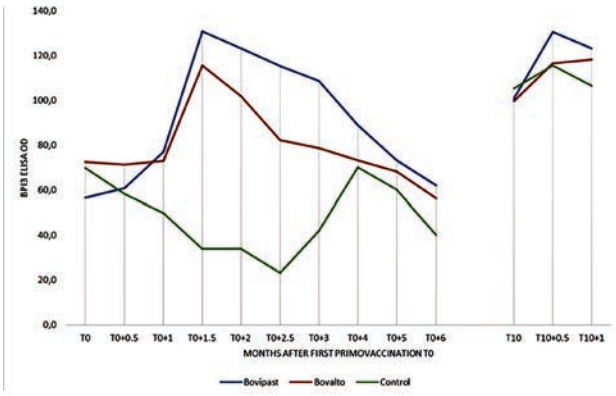

Fig. 3. Bovine Parainfluenza 3 ELISA optic density measures in serum from calves vaccinated with multivalent bovine respiratory disease vaccines

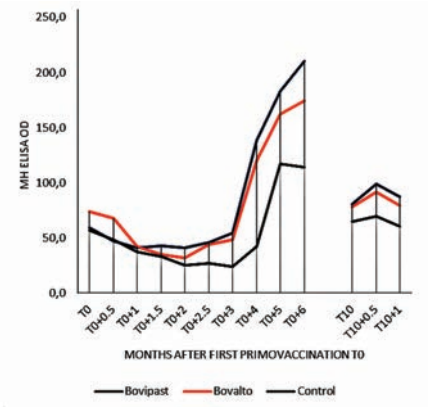

Fig. 4. *Mannheimia haemolytica* optic density measures in serum from calves vaccinated with multivalent bovine respiratory disease vaccines

higher in Bovipast vaccinated calves (3.7) compared to Bovalto vaccinated calves (1.2) and control calves (0.8). For BRSV ELISA AUC-booster, both Bovipast vaccinated calves (87.9) and Bovalto vaccinated calves (74.1) were significantly higher than control calves (23.4). BPI3 ELISA AUC-booster was significantly higher in Bovipast vaccinated calves (121.5) compared to Bovalto vaccinated calves (113.2) and control calves (109.9). *Mh* ELISA AUC-booster was significantly higher in Bovipast vaccinated calves (89.1) compared to control calves (74.1), whereas Bovalto vaccinated calves (84.5) were not significantly different from either group.

DISCUSSION

During the past few years, an increasing number of vaccines against BRD have been available on the European market and good availability of vaccines is a high priority in the European Union [10]. As most BRD vaccines have similar indications, it is not easy for veterinary practitioners and farmers to make evidence-based vaccine choices [8]. Meaningful comparisons between vaccines can only be done if the vaccines are tested under similar conditions. The Federation of Veterinarians in Europe has emphasized that vaccines are important to reduce antimicrobial use, but that there are lacking studies performed in dairy calves and lacking good field trials with natural exposure to pathogens [12]. In this study, 2 multivalent inactivated vaccines with similar pathogen spectra and adjuvants have been tested under field conditions as this is the most representative for the real situation.

The overall lack of respiratory disease in the calves in this study did not allow for evaluation of reduction in respiratory diseases between the vaccines and controls. Vaccines can be defined as generally effective if they prevent the disease which can only be proven by clinical and immunological efficacy studies. These studies are relatively expensive and are not fully in line with the Replacement, Reduction, Refinement principle of performing animal experiments [21]. However, serum antibody titres can be quite easily measured and the seroneutralisation assay used in this study for BRSV can to a certain extent indicate biological activity of the antibodies as this assay indicates that infectious agents are truly neutralized. Neutralizing antibodies are an integral part of the immune system and provide protection against many disease pathogens. Measurement of neutralizing antibodies provides an indication of the level of protection present in an animal and has been correlated with disease protection [14]. A four-fold serum antibody titre increase is often used as an indication of an appropriate vaccination response. However, this rule of thumb may seriously impact vaccination response in individuals with a high titre at the start of vaccination, and therefore a more flexible regression modelling approach may be preferable [1]. These SN tests may be preferable to evaluate immunity compared to simply quantitative tests such as the enzyme-linked immunosorbent assay (ELISA) that are not all specific for neutralizing antibodies or specific vaccines antigens. We were unable to find a reliable SN test for BPI3. Thus, in this study we used SN for BRSV, and the commercially available ELISA test K369 (BioX) for the 3 pathogens. Other facets of the immune system, particularly those related to cell-mediated immunity, may be of equal or greater importance in the control of viral pathogens. However, measuring cell-mediated immunity is expensive and cumbersome in field conditions with large numbers of animals and logistic limitations.

This field study indicated that BRSV SN response was different between the two vaccines and over time. Seroconversion indicated that higher percentage of Bovipast vaccinated calves seroconverted compared to the Bovalto vaccinated calves and the negative control calves. Only the Bovipast vaccinated calves show a high seroconversion rate after the booster vaccination. A reason for the failure of the seroconversion in the Bovalto group at the booster vaccination at 9–11 months of age may be the inhibition of priming by maternal antibodies in the passively immune calves [7]. Since seroconversion as measured at a single time may not well describe the temporal immunity of calves following vaccination and it is based upon an arbitrary cut point, this outcome was not further analysed statistically.

For further statistical analysis, the area under the curve from the second dose of primo vaccination until 6 months after the primo vaccination and one month after the booster vaccination was used. The AUC analysis indicated significantly higher BRSV antibody response in Bovipast vaccinated calves compared to Bovalto vaccinated calves and the negative control calves. The Bovalto vaccination was not significantly better than control. It may be claimed that there was a possible influence of colostral antibodies on the vaccination results as the BRSV titre at the first injection

was numerically higher in the Bovalto group compared to the Bovipast group. However, the statistical model shows that the AUC base titres were not significantly different between groups. Furthermore, the general linear model of AUC-primo was not significantly associated with AUC-base titres or age of calf at the primo vaccination.

Overall, this field study indicated elevated neutralising and humoral immunity to BRSV in Bovipast vaccinated calves after the primo and booster vaccinations, whereas Bovalto vaccinated calves had no significantly elevated titres compared to negative control calves. The humoral immunity to PBI3 was elevated after the primo vaccination in Bovipast and Bovalto vaccinated calves compared to negative control calves, however the humoral immunity decreased over the 6 months post-vaccination to levels of negative control calves and a significant elevation of humoral BPI3 immunity was detected only in Bovipast vaccinated calves after the booster vaccination. However, the BPI3 titres of the negative control calves also increased between 2.5 and 4 months, possibly indicating a natural infection. The humoral immunity against *Mh* was gradually increasing 2 months post-vaccination in Bovipast and Bovalto vaccinated calves most likely in response to a natural infection as a similar increase is also happening in the control group (Diagram 4). The absence of increasing titres for *Mh* after the primo vaccination may be explained by 2 reasons. The first hypothesis is a total or partly inhibition of the primo vaccination due to a high level of passive immunity received via colostrum. The second hypothesis is a difference in antigens between the ELISA BioX kits and the vaccines without cross detection. The latter hypothesis is most probably the most plausible as from previous testing (not published) and information from the developer of the BioX test kits (personal communication BioX), we know that ELISA BioX K369 and K139 are appropriate diagnostic kits to detect a natural infection but not always to detect responses to vaccination. The ELISA BioX kits are based on an LPS virulence factor of *Mh* which is most probably not present as antigen in the vaccines. This has been confirmed with an ELISA test that has been specifically developed to detect IRP antigens present in Bovipast. When analysing the samples of the Bovipast vaccinated animals with this specific ELISA in-house test, *Mh* primo vaccination and booster humoral significant responses could be confirmed (supplementary data can be obtained from corresponding author upon request). As for Bovipast, a specific ELISA test detecting antigens present in Bovalto could give a similar result. Based on these *Mh* results, it is very important to underline those commercial kits, BioX K369 and K139, are not able to detect if animals have been vaccinated and to estimate or compare vaccine responses. Additionally, these results indicate that it is important to know which antigens are in the vaccine as well as in the ELISA kit. Some *Mh* vaccines are based on capsular antigens, whereas some others on specific virulence factors (IRP, leukotoxins).

At the booster vaccination (AUC-booster), there was evidence of a memory response for BRSV, BPI3 but not for *Mh* for both vaccines. However, the lack of *Mh* increasing titres at booster vaccination may again be explained by the used ELISA kit as explained before (based on specific LPS *Mh* antigen). On the other hand, for the BRSV seroneutralisation response at booster, there was a significant lack of response in the Bovalto group. This can be explained by several hypotheses:

1. No cross neutralization response between vaccine strain and lab test strain. It may be interesting to repeat the seroneutralisation tests with several current strains for which BRSV and BPI3 cross protection is expected [23];
2. Denaturation of immunogenic antigens during the inactivation process of the vaccine strain [24];
3. Inhibition of priming humoral immunity and long cell-mediated memory in field condition by maternal antibodies;
4. Antigen mass and adjuvant quality and quantities are not appropriate to give a detectable response in the current conditions.

The antigen origin and mass in both vaccines are different. The 2 adjuvants in the vaccines (Quil A and Aluminium hydroxide) are the same, however the concentration and absolute volume per dose is different, e. g., Quil A adjuvant can activate both the cell mediated and the antibody mediated immune responses to a broad range of viral, bacterial, parasitic and tumour antigens [22]. One dose of Bovipast contains more Quil A than one dose of Bovalto, which may contribute to overcoming the inhibitory effects of maternally derived antibodies on the vaccine immune response.

There are limited studies documenting the efficacy of calf immunization in the presence of maternal antibody protection. It has been described that passive immunity might interfere with the induction of an active immune response after vaccination [2, 16]. However, some vaccines

are effective in young calves, even in the presence of maternal antibodies [19, 24]. One study evaluated virus excretion and seroconversion following calves with maternal BRSV immunity receiving single dose of either live vaccine, inactivated vaccine, or negative control [19]. It was noticed that the virus excretion was reduced, and seroconversion was highest in the calves having received the inactivated vaccine. Another study indicated that, despite the lack of BRSV seroconversion following the administration of an inactivated vaccine, the vaccination of calves with maternal antibody protection can results in lower virus shedding and clinical signs of disease [20]. A good response following Bovipast vaccination in the presence of maternally derived antibodies has been described where a single vaccination with Bovipast was able to break through the maternal antibody immunity, prime the cellular immune response and induce partial protection in young calves [24]. This study further confirms that Bovipast vaccination can create a neutralising immune response to BRSV in the presence of maternal antibodies.

No significant BRSV neutralizing response was observed in conventional calves vaccinated with Bovalto despite the presence of the maternal antibodies at vaccination. However, vaccination with Bovalto might still provide partial clinical protection as it might induce cellular immunity. The latter has not been measured in this study as explained before. It has been described that in presence of the maternal antibodies, intranasal vaccines or natural infections can induce cellular immunity without neutralising antibodies; reduce excretion and prime neutralizing response when a homologous or heterologous booster is done with an injectable vaccine or at new natural exposure [9, 11, 17]. A fairly new approach in comparative vaccinology is the heterologous prime-boost method of immunization, using different forms of an antigen, administered by different routes to broaden and extend immune responses [17]. Heterologous boost with adjuvanted inactivated parenteral BRSV vaccines may provide a better boosting response for mucosal primed calves with an intranasal attenuated vaccine [6, 11].

From this and other studies, it is clear that Bovipast primo vaccination in young calves could induce a long memory and regenerate quickly neutralizing antibodies when a booster is done: 9 to 11 months in this study, until 12 months in another study [19] or in case of natural exposure which often occurs in the field.

CONCLUSIONS

Seroneutralisation tests are able to evaluate a part of protective immunity in contrast to some ELISA kits that could detect or not specific neutralizing antibodies (false positive) or specific vaccine antigens. Under field conditions, it is hard to evaluate and compare immunity induced by vaccines based on diverse ELISA kits that are currently on the market. There is a need to develop more appropriate tests to measure vaccine immunity in the field.

In addition, significant differences were found in serological values after Bovilis® Bovipast RSP vs. Bovalto® Respi 3 vaccination, supporting the notion that despite containing antigens targeting the same pathogens, immunogenicity of vaccines can be very different.

ETHICAL CONSIDERATION

The study was carried out in compliance with the relevant animal welfare legislation. The authors declare no animal ethical approval was needed. This study was carried out on one commercial dairy farm, so on "private lands". Based on an accurate description of the study's objectives and its design, the farm owner gave permission to the herd veterinarian to conduct the study on their farm. Except for this informed consent, no other specific permission was required. No calves were sacrificed for the purposes of the study.

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DECLARATION OF INTEREST

Thibault Jozan and Geert Vertenten are employees of MSD Animal Health, the company that markets one of the vaccines that have been used in the study reported herein. Anna Catharina Berge is an independent consultant at Berge Veterinary Consulting BV, performing consultancy for MSD-AH. Camille Levesque is employed at Laboceae, where serum analysis was performed.

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ULTRASOUND APPLICATION IN PHYSICAL THERAPY OF DOGS: COMPARATIVE STUDY

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ABSTRACT

The application of ultrasonic methods in veterinary medicine, especially in the therapy of dogs, is the main objective of this study. We compared the frequency of therapeutic ultrasound using in rehabilitation as well as in microsurgical interventions of dogs in Slovakian and Hungarian veterinary practices. Regarding to the evaluation of survey realized in restricted regions, the ultrasound therapies and interventions are currently used in Slovakia less than in Hungary. Our study could start a change in this unfavourable aspect in Slovakian veterinary medicine and contribute to a better promotion of ultrasound application in the therapy of animals.

Key words: dogs; questionnaire; rehabilitation; ultrasound therapy

INTRODUCTION

Mechanical waving field includes the waves in a broad frequency range namely from 2 Hz to 200 MHz. The range is divided in relation to human audibility into three approximate intervals: infrasound, audible or acoustic sound, and ultrasound. The ultrasound (frequencies above 20 kHz) is an essential tool in medicine in both diagnostic as well as therapeutic applications. Recently, it has become obvious that no human or veterinary clinic and hospitals exist without a sonography which is a widespread diagnostic method based on ultrasound waves. Short ultrasound waves are spreading directly through the environment, which enables an application of their reflection on tissue boundaries as the basic phenomenon in sonography [13].

Diagnostic information is obtained by capturing, processing, and displaying of ultrasound signals reflected from tissue interfaces. These signals are subsequently received by an ultrasonic probe [3]. The information about the investigated tissues, their depths, and possible artefacts found within the body is given by evaluation of reflected ultrasound waves [1].

Nowadays, the ultrasound field has a broad application in human and veterinary medicine. Besides using sonography for diagnostics, there are a lot of applications in therapy and surgery. Figure 1 presents a brief comprehension of ultrasound utilization in medicine. Therapeutic effects

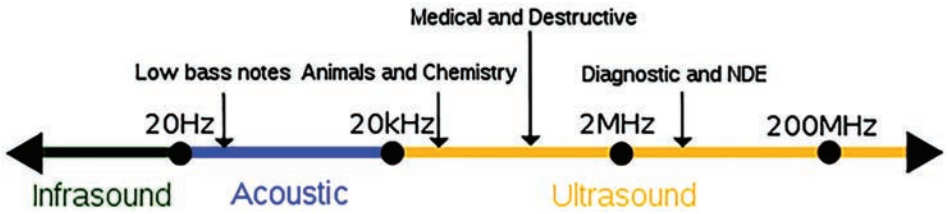

Fig. 1. Spectrum of mechanical waves
NDE—non-destructive effects

of ultrasound can be divided into destructive effects and non-destructive effects (Fig. 1).

Ultrasound frequencies above 100 kHz and below 2 MHz have destructive effects; they can be used in many medical applications. For example, Extracorporeal Shockwave Lithotripsy (ESWL) is a sophisticated method using for fragmentation and removing of urinary, bile or kidney stones. Other prospective methods such as releasing of drugs from their delivery systems (liposomes, microspheres) or High Intensity Focused Ultrasound (HIFU) which is used for the ablation of tumours are based on the utilization of frequencies from the above mentioned range. Phacoemulsification in ophthalmology and cleaning of teeth in dental hygiene are methods based on the destructive effects of ultrasound as well. Recently they apply predominantly in human medicine with the exception of tartar removing, which is a common method in veterinary dental medicine.

In general, ultrasound evokes primary and secondary effects when it interacts with biological organisms. The majority of ultrasound effects are presented by the transformation of mechanical energy to thermal or chemical energy within tissues. Non-thermal effects are called cavitation: vibration of tissues causes a formation of microscopic bubbles – they stimulate cell membranes and enhance the cell-repair effects of the inflammatory response. It is clear that these effects are utilized predominantly in physiotherapeutic procedures in physical therapy or rehabilitations [3].

Therapeutic ultrasound is a physiotherapy technique that aims to accelerate the healing of damaged muscles, tendons or ligaments by reducing inflammation. Physical therapists have made use of therapeutic ultrasound for the therapy of human patients since the 1940's, and veterinarians have used it on animals since the 1970's. It is applied in various fields of veterinary medicine such as surgery, dentistry, inhalation therapy, or physiotherapy. In medical practice, therapy by ultrasound has many benefits such as non-invasive intervention in the patient's body leading to faster recoalescence, increasing blood flow in the treated area and thereby accelerating the treatment process, alleviation of oedemas and heat production in the tissues that help in reducing pain and muscle spasms [4].

In our study, we would like to point out the possibility of ultrasound application in the treatment of dogs. We summarized and compiled the current state of ultrasound utilization as therapeutic method in Slovakia (from the perspective of dog owners and veterinarians) and compared it with the trend abroad (specifically in Hungary).

Application of ultrasound in physical therapy

The purpose of physical therapy (physiotherapy, rehabilitation) is to accelerate the healing of damaged muscles, joints, tendons, and ligaments by reducing their inflammation. Excellent results have been obtained in acute injuries as well as in chronic lesions [14]. The rehabilitation effect of ultrasound is caused by the absorption of ultrasound waves by individual molecules and macromolecules within the tissue.

When the tissue contains a lot of protein macromolecules, after the ultrasound, the absorption given by absorption coefficient is higher and vice versa: the absorption coefficient decreases when water content within tissues increases. This fact has various beneficial effects including increased blood flow (which promotes healing), reducing swelling, and acceleration the comprehensive healing process. Penetration depth which is the maximum depth when a rehabilitation effect is expected, has reached in the frequency interval 0.9—3 MHz with few $W.cm^{-2}$ of ultrasound intensity [9, 11]. Currently, there is not enough published scientific clinical trials focused on ultrasound physiotherapy and the used exposures (doses) are largely empirically determined. Most physiotherapy devices of-

fer a probe emitting ultrasound intensities in the range of 0.2—3 $W.cm^{-2}$ and discrete frequencies 1—3 MHz for deep treatment (to depth of 8 cm) and 3 MHz for surficial treatment. Ultrasound physical devices allow either discrete intensity (pulsed regime) settings or continuously (continuous regime) variable control.

Setting of the work cycle parameters applied to the patient depends on diagnosis and the fact whether the problem is acute or chronic. Usually, the acute diseases need to adjust lower intensities because of the excessive heating of the tissue (only 60 % of the intensity, which is necessary for the treatment of chronic diseases) [6, 10].

Physiotherapy by using the ultrasound is a very simple procedure which must be repeated several times (2 to 3 weekly sessions with 10—20 minute applications are needed depending on the patient's condition) for achievement of a satisfied therapeutic effect. The total duration of the treatment depends on the particular patient and diagnosis [4, 12]. The ultrasound produced by special therapeutic probes is coupled directly into the patient through a thin layer of coupling medium (e. g. aqueous gel or mineral oil) [5]. It is necessary to keep the ultrasound probe in the continuous contact with the skin during the therapy. Insufficient contact absorbs a large amount of energy resulting in a lower intensity of ultrasound wave motion in the tissues. The therapeutic unit works in so-called scan mode, which represents the longitudinal movement of the probe along the muscle [4, 14].

Each treatment form has also its undesirable effects. The unfavourable effects of ultrasound physical therapy include a status worsening of inappropriate dosing (intensity, duration of application), overall weakness, short term temperature rise, insomnia, vomiting, and extension at the application side [3]. The ultrasound rehabilitation is not recommended for contraindications such as an orthopaedic implant or any metallic components in the patient's body (overheating and subsequent damage to the surrounding tissue may occur). It also is not to be used in patients with cancer, infected areas of the skin over the foetus during pregnancy, growth platelets in young animals, open spine after surgery, eyes or over uncastrated male testicles [4].

Application of ultrasound in surgery, micro-interventions and dental medicine

Surgical therapy has developed more slowly in view of its technical difficulties and possible larger risks for the patient. The ultrasound with intensity up to 20 $W.cm^2$ and frequency from interval 100 kHz—2 MHz (Fig. 1) is used to destroy pathological tissues that could also damage the surrounding healthy tissue or organs when it is applied incorrectly [3]. The ultrasonic aspirator is the most popular ultrasound instrument used in general surgery. It belongs to the contact surgical instruments. Oncologists most often use it for removing the primary tumours and metastases of the liver, kidney, breast, bone, uterus, and pancreas. The advantage of this method is that ultrasound quite hardly disturbs rigid and elastic tissues with high collagen content (blood vessels, bronchi, and various ligaments). Surrounding soft tissues are disturbed by ultrasound and then can be easily aspirated away, thereby tissues with higher levels of collagen are exposing. The ultrasonic aspirator also has a thermo-coagulation effect which reduces the bleeding from small blood vessels [7, 8].

Dental tartar is one of the most commonly diagnosed dental problems in animals, especially in dogs. Ultrasonic units used in dentistry are currently available in two basic types; their mechanism of action is different. Magnetostrictive units operate between 18 and 45 kHz using flat metal strips or metal rod attached to a scaling tip and the tip movement is elliptical. Piezoelectric units operate in the 2—50 kHz range and are reactive by dimensional changes in the crystals housed within the hand piece and the tip movement is primarily linear in direction [2].

MATERIALS AND METHODS

To monitor the current state of using the ultrasound therapy in veterinary practice (especially in the therapy of dogs), we have chosen two forms of questionnaire. The first form was addressed to the dog owners in Slovakia and it was published on the internet. The second one was assigned to veterinarians, it was sent via e-mail, and the doctors were contacted by phone. We also monitored the application of ultrasound therapy abroad, specifically in Hungary, by sending questionnaires to Hungarian veterinarians. We do not know the total number of dog owners contacted as it is not possible to know how many saw the questionnaire on the internet but did not reply to us. The number of veterinarians to whom we either sent the questionnaires by e-mail or contacted by telephone was 209 (153 from Slovakia and 56 from Hungary).

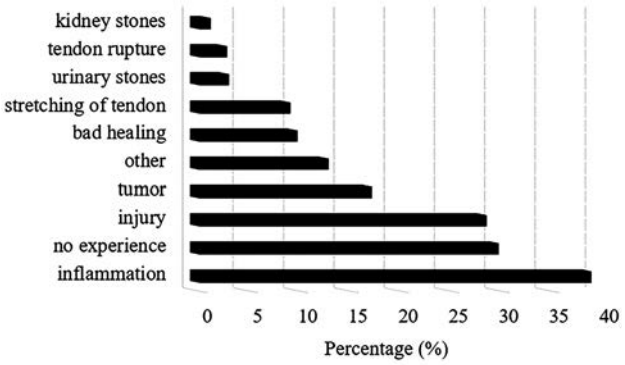

Fig. 2. Experiences of dog owners with individual types of diseases

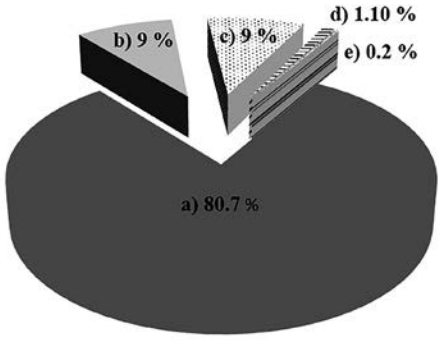

Fig. 3. The possibility of ultrasound rehabilitation offered by a veterinarian; a) no, b) I don't remember, c) the therapy is not available, d) no injury of the dog, e) yes

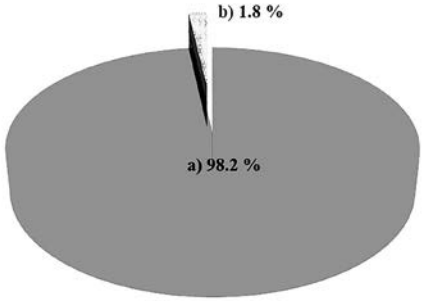

Fig. 4. Real experience of dog owners with ultrasound therapy; a) no experience or the owners confused ultrasound therapy with diagnostics, b) positive experience

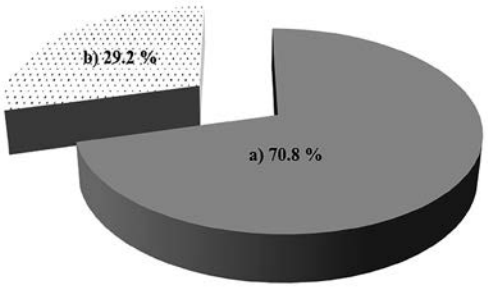

Fig. 5. Awareness of dog owners about ultrasound rehabilitation; a) no, b) yes

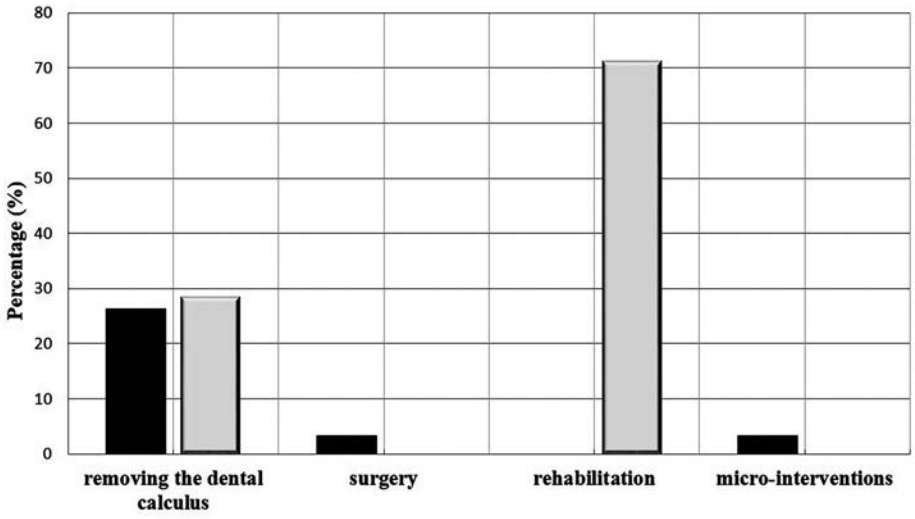

Fig. 6. Comparison of ultrasound therapy applications in Slovakia and Hungary; Slovakia—black, Hungary—grey

Questionnaire for dog owners

The questionnaire for dog owners consisted of nine questions. It contained three structured questions (the owner had the choice of several answers), three open questions (the respondent was allowed to answer individually), and the three semi-closed questions (the possibility to choose an answer or answer individually). General information such as their age or sex were considered in the questionnaire. The questionnaire was preferentially focused on the owners' experiences with ultrasound therapy.

Questionnaire for veterinarians

The questionnaire addressed to the veterinarians was designed by a veterinarian was more concise and contained two open and two closed questions. All of questions that were proposed have led to the collection of information about veterinarians' knowledge, use, and experiences with ultrasound therapy applications on dogs' patients.

RESULTS

Evaluation of the questionnaire done by dog owners

Four hundred and forty-five dog owners coming from Slovakia were included in the study. The average age of the respondents was 32 years, of which 55 were men and 390 were women. The age of their dogs ranged from 2 months to 17 years with the average age being 6 years.

In question number six, we asked the dog owners if they had experience with any of these types of diseases (injury, tendon strain, urinary and kidney stones, tumour, tendon rupture, inflammation or poor healing). Up to 38.9 % of dog owners had experience with inflammation, 28.5 % with injury, 17.1 % with tumour, 9.7 % with poor healing, 9 % with tendon extension, 2.9 % with urinary stones, 2.7 % with tendon rupture and 1.1 % with kidney stones. Almost a third of the owners (29.7 %) answered that they have no experience with any disease of their dog. 12.8 % of respondents mentioned the possibility of „another" (they did not specify the type of disease) (Fig. 2).

In another question, we were interested in whether dog owners were offered the possibility of ultrasound rehabilitation by veterinarians, if the patient's condition required rehabilitation. 80.7 % of respondents were not offered such a possibility by a veterinarian, 9 % stated that they did not remember it, and 1.1 % of owners answered that their dog did not have an injury, did not need such a form of therapy. Only a very low percentage (0.2 %) answered that they were offered the option of ultrasound rehabilitation (Fig. 3). Even today, there are surgeries that do not have the therapeutic ultrasonography.

The eighth question of the questionnaire focused on the personal experiences of dog owners with ultrasound therapy, specifically with ultrasound rehabilitation. However, the majority of responses (98.9 %) were negative, as respondents had no experience with this type of therapy. Only 1.1 % of owners had the positive experience with ultrasound rehabilitation.

In processing the answers to this question, we found that a very large percentage of dog owners (Fig. 4) confused ultrasound therapy with ultrasound diagnostics. Respondents most often stated that they encountered ultrasound when diagnosing diseases or detecting pregnancy in their bitch.

"Have you ever heard about ultrasound form of rehabilitation?", this was the ninth question of our questionnaire. 70.8 % of respondents confirmed that they have not heard about this method yet and 29.2 % of interviewees knew about this method (Fig. 5).

Evaluation of the questionnaire done by veterinarians

Thirty veterinarians from Slovakia and seven veterinarians from Hungary responded to the questionnaire intended for them. For comparison, we present together the results of a questionnaire obtained from both countries, Slovakia and Hungary.

In the first question, we were interested in whether veterinarians have and use an ultrasonograph. Of the randomly selected outpatient clinics throughout Slovakia, they have and utilize 100 % diagnostic ultrasound. The situation is the same in Hungary, all surgeries we contacted are equipped with the ultrasonography and use it.

In the next question, we focused on medical procedures in which veterinarians use ultrasound. Approximately one-third (32.9 %) of the surveyed veterinarians in Slovakia apply ultrasound in addition to diagnostics but also for the removal of the tartar (26.7 %), in surgery (30.3 %) and in micro-interventions (30.3 %) (Fig. 6). Hungarians use ultrasound for therapy much more, namely, 71.4 % for rehabilitation and 28.6 % for tartar removal in dog patients.

From Fig. 6, it can be seen that Hungarian veterinarians utilize ultrasound as an effective tool for rehabilitation of

dogs; with the difference to Slovak vets, they do not use ultrasound for rehabilitation at all. Almost 72 % of Hungarian vets used ultrasound rehabilitation on the therapy of arthrosis, neurological problems, lymph stimulation, metabolism acceleration, tendinitis, bursitis, and joint diseases.

DISCUSSION

Ultrasound therapy is one of the newer therapeutic methods, so we were interested in the knowledge and experiences associated with application of this therapy in dogs, their owners, and the veterinarian in their communities. Although our study does not depict a large sample of veterinarians and dog owners, the results are more than surprising. From the above-mentioned questionnaire, it is clear that among the most common dogs' difficulties in which their owners look for professional veterinary help include inflammation, injury, and cancer. Less often, there occurs kidney and urinary stones, stretching or rupture of tendons. Up to 80 % of owners mentioned that if rehabilitation was needed, they were not offered the opportunity to apply ultrasound therapy. As can be seen from Fig. 6, the Hungarian veterinarians utilize ultrasound as an effective tool for dog rehabilitation more frequently than Slovak veterinarians. Almost 72 % of Hungarian vets use ultrasound rehabilitation on the therapy of arthrosis, neurological problems, lymph stimulation, metabolism acceleration, tendinitis, bursitis, and joint diseases. Slovak vets are predominantly concerned with micro interventions by ultrasound, such as removing tartar and micro surgery.

When we have processed and evaluated the results, we also found that approximately 98 % of dog owners exchange ultrasound therapy with sonography. This phenomenon is especially widespread in Slovakia. We suppose that it is related to the low promotion of application, benefits, efficiency and effectiveness of ultrasound therapy, and especially rehabilitation in the lay public. We were pleasantly surprised by the fact that the lay public as demonstrated in the questionnaire has the ambition to obtain more information and knowledge about possibilities of using ultrasound therapy in health problems to alleviate and improve the welfare of their pets. It may be advisable to organize a series of popular lectures designed for laypersons/dog owners focusing on the benefits and effectiveness of ultrasound therapy. This would certainly help to boost the awareness of veterinarians contributing to increased interest and enhanced use of effective non-invasive method such as ultrasound therapy. Increased interest in this form of therapy by owners could assist veterinarians overcome barriers (higher financial contributions, profitability) that are likely to prevent them from using ultrasound therapy and rehabilitation to a greater extent.

CONCLUSIONS

Innovative therapeutic methods in veterinary medicine include ultrasound therapy. Rehabilitation by ultrasound leads to faster recovery, accelerates the healing process, reduces oedema, pain and occurrence of muscle spasms. In this study, we wanted to provide a consistent overview on the ultrasound application in dog therapy; specifically rehabilitation in Slovakia. By using a questionnaire, we have compiled the current state of using ultrasound therapy in practice. We compared the results with the situation in Hungary. It is clear from our study that Slovakia lags behind the current trend, which dominates abroad. Most veterinarians use ultrasound only for diagnostics as assistive technique in surgery, in catheterization or elimination dental tartars in dog patients. No participating doctors use ultrasound for rehabilitation treatments. The trend prevailing abroad, especially in Hungary, shows a tendency for an increased number of ultrasound applications in arthrosis, rehabilitation, tendon injuries, dampening pain and inflammation, and oedema reduction.

Finally, we can conclude that ultrasound therapy is currently very little used in Slovakia, which is obstructive to its positive aspects. Our study could contribute to a better promotion of this expanding rehabilitation method.

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GENES OF CONGENITAL DERMATOLOGIC DISORDERS IN DOGS—A REVIEW

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ABSTRACT

This article presents an overview of up-to-date identified genes responsible for congenital canine skin diseases of dogs and the characteristics of these diseases. Congenital skin diseases constitute a specific group of dermatologic disorders that plays an important role in breeding of purebred dogs. They include primary seborrhoea, ichthyosis, hereditary nasal parakeratosis, dermatomyositis, colour dilution alopecia, skin mucinosis, dermoid sinus, lethal acrodermatitis, acral mutilation syndrome, keratoconjunctivitis sicca, ichthyosiform dermatosis, bullous epidermolysis, exfoliative dermal lupus erythematosus, congenital footpad hyperkeratosis and sebaceous adenitis. In the majority of cases, their occurrence is linked to particular breeds. In more than half of these diseases a specific defective gene variant responsible for the disease has been identified. Genetic tests for identification of the relevant defective genes serve as an important tool in the diagnostics of diseases in veterinary practice and in breeding of purebred dogs.

Key words: congenital dermatologic disease; dog; gene; skin

INTRODUCTION

The group of dermatologic disorders of dogs consists of diseases of different origin (bacterial, parasitic, autoimmune, hormonal, viral, metabolic, hereditary, etc.) and, typically, they have very similar clinical signs. The differential diagnostics in veterinary dermatology is very complicated and for various reasons, incorrect diagnosis has frequently been made [20].

Congenital skin disorders constitute a specific type of skin diseases. They are not common, or sporadic, and play an important role in the breeding of purebred dogs. Many congenital diseases developed first as spontaneous mutations that by cognation, or very limited selection of individuals used for breeding, were gradually induced in the genome of many descendants. The majority of congenital skin disorders are monogenic as they are caused by one defective gene and there is still no cure for them. In the ma-

jority of cases, autosomal recessive inheritance is involved and the simple Mendelian way of inheritance is applicable [17]. Congenital canine skin diseases include: primary seborrhoea, ichthyosis, hereditary nasal parakeratosis, dermatomyositis, colour dilution alopecia, skin mucinosis, dermoid sinus, lethal acrodermatitis, acral mutilation syndrome, keratoconjunctivitis sicca, ichthyosiform dermatosis, bullous epidermolysis, exfoliative dermal lupus erythematosus, congenital footpad hyperkeratosis and sebaceous adenitis [2, 4, 14, 18, 21, 22, 27, 33, 35, 40, 42, 47, 48, 52]. The congenital skin diseases are mostly linked to specific breeds or their direct crossbreds. More than half of these diseases have been identified genetically and the variant of the gene responsible for the disease were confirmed (Tab. 1) [5, 10—14, 16, 17, 23, 25, 27, 32, 36, 38, 46, 51]. Standardized genetic tests based on PCR were developed to identify the particular defective genes. For some congenital diseases the defective genes have not yet been identified or for the time being there is available only information about their presumptive localisation in certain chromosome [4, 22, 47, 48]. The clinical picture of an individual disease may differ depending on the specific affected breed, but also within the breed, the signs may vary considerably among the individuals [17]. The introduction of genetic tests into a veterinary practice can prevent many erroneous diagnoses of skin diseases. Genetic tests may constitute an important contribution to the breeding of purebred dogs, whether in planning of parental pairs or elimination of individuals unsuitable for breeding; because not all congenital skin diseases may be confirmed by genetic tests and due to a broad range of severity of clinical signs of individual diseases, biopsies of skin and the subsequent histopathologic examination play an important role [20, 22].

The aim of this article was to present an overview of up-to-date identified genes responsible for congenital canine skin diseases of dogs and characteristics of these diseases.

Table 1. Congenital dermatologic disorders of dogs—genes responsible for the disease and the most frequently affected dog breeds [5, 10—14, 16, 17, 23, 25, 27, 32, 36, 38, 46, 51]

Congenital dermatologic disorders	Defective variant of the gene	The most frequently affected dog breeds
Hereditary nasal parakeratosis	*SUV39H2*	Labrador Retriever, Greyhound
Colour dilution alopecia	*MLPH*	Doberman, German Pinscher, Dachshund, Greyhound, Whippet, Great Dane, Labrador Retriever, Yorkshire Terrier, German Shepherd, Chow
Lethal acrodermatitis	*MKLN1*	Bull Terrier, Miniature Bull Terrier
Acral mutilation syndrome	*GDNF*	English Cocker Spaniel, English Pointer, English Springer Spaniel, French Spaniel, German Shorthaired Pointer
Congenital keratoconjunctivitis sicca and ichthyosiform dermatosis	*FAM83H*	Cavalier King Charles Spaniel
Junction bullous epidermolysis	*LAMA3*	German Shorthaired Pointer
Epidermolysis bullosa simplex	*PLEC*	Eurasier dog, Chesapeake Bay Retriever
Dystrophic epidermolysis bullosa	*COL7A1*	Central Asian Shepherd
Exfoliative cutaneous lupus erythematosus	*p.Pro480Thr*	German Shorthaired Pointer, Hungarian Vizsla, Braque du Bourbonnais
Hereditary footpad hyperkeratosis	*FAM83G*	Irish Terrier, Cromforland Dog, Bordeaux Dane, French Mastiff, German Jagdterrier
Non-epidermolytic ichthyosis	*PNPLA1* *SLC27A4* *NIPAL4* *TGM1*	Golden Retriever German Dane American Bulldog Jack Russell Terrier
Epidermolytic ichthyosis	*KRT10*	Norfolk Terrier

Hereditary nasal parakeratosis

Hereditary nasal parakeratosis is described as an autosomal recessive condition. It is unique to the Labrador Retriever and English Greyhound. Mutation ("N324K missense variant") in the *SUV39H2* gene has been described as the genetic cause of the development of hereditary nasal parakeratosis. This genetic variant *SUV39H2* is associated with an inactive enzyme SUV39H2 [6, 23, 49]. It has been assumed that the *SUV39H2* gene affects a great number of cell processes, such as: proliferation and differentiation of keratinocytes and the synthesis of some specific proteins. The unequivocal role of the *SUV39H2* gene in skin has not yet been completely elucidated. The proteins specific for the terminal differentiation processes include loricrin, invulocrin and filagrin. It is likely that the loricrin may play a key role in the hereditary nasal parakeratosis [1]. In the healthy dog, loricrin is the main constituent of the epidermal keratinised coat where it accounts for 70—85 % of the proteinaceous components [9], while the amount of loricrin detected in the *planum nasale* of dog suffering from hereditary nasal parakeratosis is insufficient. It has been assumed that the deficiency of loricrin seriously disturbs the final phase of differentiation and results in impaired *stratum corneum*. Besides loricrin, changes involve also keratin, involucrin and other proteins therefore it is not possible to identify clearly the ways and causes of the development of this disorder [1].

The main and exclusive sign is a non-pruritic hyperkeratosis of the *nasal planum* in otherwise healthy dogs. The first visible changes are in the muzzle and can be observed in 6 to 12 weeks old puppies, but the clinical signs do not appear until the age of 6 to 24 months. The signs may be mild, in the form of the dry crusty layer on the dorsal portion of the muzzle that cannot be detached, or they may be more serious with the occurrence of erosions and fissures [40, 44]. Only one study described intercurrent signs that affected the entire dorsal part of the nose, ears and paws [26]. Mandatory genetic testing of dogs in breeding centres may prevent the development of hereditary nasal parakeratosis and significantly reduce its incidence in the future.

Colour-dilution alopecia

Colour-dilution alopecia is associated with a generally called dd phenotype (colour-dilution genes) that changes the hair colour from black or brown to blue, fawn or taupe. A characteristic feature of this colour-dilution phenotype is the defective transport of melanosomes leading to large clumps of pigment in melanocytes. This syndrome is most common in Dobermans (Blue Doberman Syndrome) in which the blue-grey colour may develop due to inhibition of full pigmentation. Dilution of hair colour is caused by mutations in *MLPH* (the melanophilin gene). In dogs, the *MLPH* gene is associated with the D locus and three variants, c.-22G > A (d^1), c.705G > C (d^2), and c.667_668insC) (d^3) [51]. This colour-dilution alopecia affects also other breeds that possess the colour-dilution genes, for example, German Pinschers, Italian Greyhounds, Greyhounds, Whippets, Yorkshire terriers, German Shepherds, Chow Chows, Silver Labrador terriers and many other breeds [8, 26, 37, 42, 45].

Puppies with defective genes are born without signs of this disease. The first loss of hair usually occurs between 6 months to three years of age, but some even later. The signs such as dry split hair, scales, folliculitis, hypotrichosis development of comedones and alopecia are observed in the areas of blue coloured hair or hair with other colour dilution. Histopathologically, the epidermis is relatively normal but may be hyperplastic. Hair follicles are atrophied and deformed. Melanin clumps are observed in follicles, epidermis and dermis. The disease is not accompanied with pruritus, but the loss of hair cannot be affected or treated. The treatment by supportive preparations can improve only the skin status. This syndrome is undesirable but the dog can lead a normal life in the household of his owner [26, 37].

Lethal acrodermatitis

Lethal acrodermatitis is an autosomal recessive hereditary disease which affects Bull Terriers and Miniature Bull Terriers. The examination of the genome of affected dogs identified a variant in the *MKLN1* gene that encodes the intracellular protein muskelin 1. The various cellular functions that have been postulated for muskelin 1 include roles in intracellular transport processes, cell morphology, cell spreading, and cell adhesion. However, the exact pathogenesis of acrodermatitis is still unclear [5].

The clinical signs of this disease appear in puppies shortly after birth. The principal sign is the progressive acrodermatitis with typical lesions at the marginal parts of the body. The characteristic skin lesions such as erythema, scales, erosions and scabby ulcerations appear mostly on the extremities and the muzzle. Later, the disease

progresses to hyperkeratosis on paws, onychogryposis, paronychia and pyoderma. The growth of the affected animals is retarded and they often suffer from diarrhoea and bronchopneumonia. The clinical signs may include diluted hair colour in the pigmented skin areas [33, 34]. An abnormally arched hard palate in the mouth and the presence of foul smelling residues of food in the mouth may serve as a marker for the diagnosis of this disease. Lower plasma zinc levels (supplementation of zinc did not result in improvement) were detected in the pups and lymphocytes in the T-lymphocyte region of the lymphoid tissue were significantly reduced [50]. The mean age of survival is 7 months and the maximal 2 years, or even more, and the animals die mostly due to health complications related to infections or must be euthanized due to serious lesions at the extremities that do not allow them to live normally. If they live up to the age of one year, their body weight usually reaches only one half of that of the dog of a similar age. The decrease in the incidence of lethal acrodermatitis can be achieved only by identification of carriers of this disease by genetic testing and their elimination from breeding [5, 24].

Acral mutilation syndrome

Acral mutilation syndrome (AMS) is caused by one variant—mutation within the regulatory region of the gene for the Glial Cell-derived Neurotrophic Factor (GDNF), located 90 kb upstream of the *GDNF* gene. GDNF is the factor that strongly promotes development of new neurons, growth of axons and the survival of adult neurons. AMS causative mutation disturbs the expression of *GDNF* gene causing a decrease in the level of GDNF which leads to progressive degeneration and abnormal development of sensory neurons in the spinal cord and peripheral nerves. This disorder causes insensitivity to pain in the distal extremities. The pathological process affects only primary sensory neurons. This disorder affects most commonly: English Cocker Spaniels, English Pointers, English Springer Spaniels, French Spaniels and German Shorthaired Pointers [2, 41, 46].

The disease can affect only one limb or more and the involvement of the pelvic limbs are more serious. Dogs lick, chew and bite distal parts of their limbs which results in various erosions and damage and in the worse cases even auto-amputation of parts of them (claws, digits, footpads). Paws are swollen and bleeding, present are skin abscesses, ulcerations, infections, hyperpigmentation and fibrosis. Insensitivity to pain in the extremities is a typical symptom of this disorder. Dogs walk normally on mutilated limbs without feeling the pain or limping. Puppies affected by AMS appear smaller than healthy pups in the litter. The first signs of limb biting are observed when they are 3.5 to 12 months old. There is no cure for this disease. It is necessary to limit the access of animals to their limbs by various bandages, protective sleeves or collars, or limit the mutilation by application of preparations with unpleasant taste. The disease is not life threatening but requires much patience and care from the owner and the outcome is not always successful. Even after all the efforts, the disease sometimes ends by euthanasia. Affected animals must not be used for breeding. Genetic tests for the identification of carriers and their elimination from breeding is recommended [2, 41].

Congenital keratoconjunctivitis sicca and ichthyosiform dermatosis

Congenital keratoconjunctivitis sicca (KCS) and ichthyosiform dermatosis present as one disease that affects only the Cavalier King Charles Spaniel breed. Dog breeders refer to this disease as "dry eye curly coat syndrome". In 2012 it was revealed that this condition is caused by deletion of one base pair in gene *FAM83H* [16].

The signs of this disorder appear at an early age and the affected individuals are smaller than their healthy siblings, they have rough curly coat on their back and after eyelid opening they suffer from KCS. Reduced production of tears and qualitative abnormality of tear film led to serious cases of sticky mucoid or mucopurulent eye discharge and ulceration of the cornea. In addition to a curly coat of poor quality along the dorsal spine, scales and alopecia appear in this region. Footpads are hyperkeratinized with nail growth abnormalities and intermitted sloughing is observed, leading to pain and limping. Dogs suffering from this disease show increase in dental diseases, extensive production of dental calculus and serious gingivitis which often results in teeth extraction. Symptomatic treatment has been used to relieve the symptoms but due to early diagnosis in puppies and unfavourable prognosis the breeders frequently decide to euthanize the affected individuals. Congenital keratoconjunctivitis sicca and ichthyosiform dermatosis is inherited by an autosomal recessive mode and can be confirmed by genetic testing [3, 16, 21].

Epidermolysis bullosa

Epidermolysis bullosa is a rare autosomal recessive dermal disease characterised by the loss of epidermal-dermal integrity, extreme fragility of skin and mucous membranes, development of blisters and erosions as a response to the smallest mechanical trauma. This disease is caused by gene mutations and frequently ends in death or euthanasia of the affected dog. The phenotypic changes are due to abnormalities in specific structural proteins within the epidermis and basement membrane zone. According to localisation of the subcellular defect, three main types of epidermolysis have been recognised in veterinary medicine: simplex, junctional and dystrophic [35].

The German Shorthair Pointer is affected by junctional bullous epidermolysis caused by a variant of the *LAMA3* gene and is typical by the development of defects in *lamina lucida*. These defects are caused by mutations affecting either extracellular matrix protein laminin 5 (essential for keratinocytes), its cellular receptor integrin α6β4, or collagen type XVII. Signs appear shortly after birth and the animal may die soon. The skin and mucous membranes are very fragile, susceptible to tearing and massive non-healing erosions, blisters, ulcers and deep wounds develop. The condition is very painful. The most frequently affected regions include: muzzle, footpads, elbows, knees, flanks, genitals and ears, but any other region can respond by damage at a small touch. Erosions can appear also in the oral cavity, nasopharynx, pharynx and other parts of the digestive and respiratory systems which causes considerable problems with accepting food or breathing. There is no cure for this disease [25, 39, 43]. Currently, the reduction in the number of affected individuals, the carriers, can be achieved by not using them for breeding which is feasible as they can be identified by commonly available genetic tests. Recently, the disease was confirmed in an Australian Shepherd litter where there was identified a single protein-changing variant, *LAMB3*: c.1174T> C. This is a "human variant" that was confirmed in the animal kingdom for the first time [25].

Bullous epidermolysis simplex is the most superficial form and includes damage or loss of proteins in the cytoskeleton of basal or suprabasal keratinocytes. This type was described in the Eurasier dog and is caused by the variant in the gene *PLEC*, that resulted in defective production of plectin. Plectin is a ubiquitous linker protein that acts to connect the components of the cellular cytoskeleton to proteins in the dermis, nervous system and skeletal muscles. A special form classified as suprabasal form of epidermolysis bullosa simplex is called Ectodermal Dysplasia-Skin Fragility Syndrome (ED-SFS) and affects Chesapeake Bay Retrievers known as Chesapeake. It can be confirmed by genetic testing [32].

The disorder associated with dystrophic bullous epidermolysis involves the superficial layer of the dermis. A serious form of dystrophic bullous epidermolysis was detected in a Central Asian Shepherd. It is caused by the defective gene *COL7A1*. The variant results in a premature stop codon and likely absence of the functional protein in the basement membrane of the skin in the affected dogs [38].

Exfoliative cutaneous lupus erythematosus

Exfoliative cutaneous lupus erythematosus is a serious form of a dermal disorder called lupus erythematosus. It occurs as a hereditary disease particularly in German Shorthaired Pointers but was diagnosed also in Braque du Bourbonnais. It is an autosomal recessive hereditary disease caused by a homozygous mutant variant *p.Pro480Thr* that binds to chromosome 18 and affects the C-terminal end of UNC93B1, limiting the TLR7 mediated autoimmunity by means of interaction with syndecan binding protein (adhesive role receptors). UNC93B1 is a transmembrane protein positioned in the endoplasmic reticulum and endolysosomes, necessary for correct functioning with several Toll-like receptors (TLR-transmembrane receptors play a key role in an innate immune response and constitute the primary recognition system of the body) [27].

The first skin lesions are easily visible at the age of 4 to 6 months but appear also in young adult dogs. The signs of lesions usually proceed from the face region (particularly the dorsal part of the muzzle and ears) to trunk (the neck is affected less commonly), tail, scrotum and limbs. Skin manifestations start as extensive scaling, followed by erythema with or without depigmentation, erosions, exudative ulcers, adherent crusts and gradual alopecia without growth of new hair. Secondary infections of skin and ears may appear. Over time, arthralgia develops in dogs manifested by limping, hunched posture and reluctance to move. Neither *post mortem* examination nor histological examination of joints, spinal cord and peripheral nerves shows any pathological changes that would explain the symptoms concerning the locomotory apparatus. The de-

velopment of a progressive infertility was recorded in dogs of both sexes (oligospermy, later azospermia in males and irregular or absent oestrous cycle in females), haematological abnormalities (lymphopenia, thrombocytopenia), generalised peripheral lymphadenopathy, enterocolitis and others. Treatment with immunomodulatory drugs are frequently insufficient to ensure long-term control of this disease. Dogs with progressive disease must be frequently euthanized [15, 30].

Hereditary footpad hyperkeratosis

Hereditary footpad hyperkeratosis, originally referred to as digital hyperkeratosis, causes palmoplantar hyperkeratosis in the breeds such as: Irish Terriers, Cromforland Dogs, Bordeaux Danes, French Mastiffs and German Jagdterriers. The disease is caused by a mutation in the gene expressing protein FAM83G. The family of FAM83 proteins consists of 8 known members, FAM83A to FAM83H. Another mutation in the gene *FAM83H* in dogs causes hereditary diseases: keratoconjunctivitis sicca and ichthyosiform dermatosis in the breed Cavalier King Charles Spaniels that presents with some phenotypic manifestations similar to digital hyperkeratosis [14].

The first manifestations of this disease appear from the age of 10 weeks up to one year and involves all footpads. The surface of the footpads are very hard and deep cracks often develop that open into sensitive living cell layers. Similar cracks may develop also in healthy dogs due to external factors, but they heal spontaneously in contrast to dogs with hyperkeratosis. The affected dogs have problems with walking on irregular surfaces and refuse to move due to the pain. The cracks may lead to the development of infections which intensifies the pain. The claws are very hard and it seems that they grow faster. The seriousness of the symptoms may differ between individual dogs; even between the footpads of the same dog. The available therapy is only symptomatic. In a differential diagnosis, it is necessary to eliminate idiopathic hyperkeratosis which develops for unknown reasons. Hereditary footpad hyperkeratosis can be identified by a genetic test [14].

Ichthyosis

Ichthyosis belongs to the group of genetically conditioned dermatoses that present by excessive scaling and peeling of the scales over the entire body. The scaling is caused by a defective gene that disturbs a certain phase of the formation of the *stratum corneum* [11]. The process of the *stratum corneum* formation is complicated and each step of this process is controlled by genes. In case of the presence of the defective genetic information and disturbance in one step of the process of formation of this skin layer, the formation of the healthy *stratum corneum* is impossible. The change in any step results in disturbances in the barrier function of the *stratum corneum* and in an attempt to repair them. This leads to excessive regulation of lipids in order to replenish the *stratum corneum* and as a result of this the epidermis becomes hyperplastic [18, 28].

Ichthyosis has been recognised as a disease for a long time but only in 2012 was the defective gene identified in the genome of a Golden Retriever [17]. Two basic forms of ichthyosis have been characterised in veterinary medicine, i.e., epidermolytic ichthyosis typical of Norfolk Terriers and a non-epidermolytic one that affects various breeds. It should be noted that individual breeds mostly possess mutations in different genes that result in different clinical pictures of ichthyosis in the affected breeds [17, 19, 20].

Epidermolytic ichthyosis in Golden Retrievers is caused by a mutation in the *PNPLA1* gene. It is not completely clear whether the *PNPLA1* gene is the only gene involved in the development of ichthyosis in Golden Rretrievers or other genes or environmental factors play also some role and therefore ichthyosis is sometimes classified as heterogenic disease. In Golden Retrievers ichthyosis, it is presented with adherent scales of various size (from small to large) and a wide range of colouring. Clinical signs of fully manifested disease include skin lesions, exfoliation and hyperpigmentation [17, 18]. In Great Danes, ichthyosis is caused by the mutant variant of the *SLC27A4* gene. Expression of this gene results in non-uniform distribution of lipids in the *stratum corneum* and disturbances in the formation of the dermal barrier. Ichthyosis in this breed is manifested by strong wrinkles in the head and legs area in puppies. The face of these puppies looks like that of Shar-Pei dogs. The skin on the head and legs, particularly around the nose and eyes have an oily appearance. Fine white to yellow scales begin to appear all over the body in the coat. The skin is dry, inelastic and lichenified [36]. In American Bulldogs ichthyosis is caused by a molecular defect in the *NIPAL4* gene responsible for production of non-functional shortened protein ichthyin, necessary for the formation of the healthy *stratum corneum*. It is manifested by the development of larger or smaller scales [11,

31]. Ichthyosis in Jack Russel Terriers is caused by insertion in the gene *TGM1* [13]. In German Shepherds the ichthyosis was diagnosed as *de novo* missense variant in the gene *ASPRV1*. This novel variant has arisen by mutation that must have occurred either in one of the parental germlines or during early embryonic development of this individual [7]. In English Springer Spaniels, Labrador Retrievers and West Highland White Terriers, the cases of ichthyosis were confirmed, but molecular identification has not been documented [29]. Epidermolytic ichthyosis caused by mutation in the keratin encoding gene *KRT10* occurs in Norfolk Terriers. Generalised and pigmented hyperkeratosis with epidermal fragility developed in the affected dogs [12].

CONCLUSIONS

Congenital dermatological disorders of dogs play an essential role in the breeding of purebred dogs. Individual types of these diseases are mostly associated with specific breeds although in some cases, they can develop also sporadically in any individual as a result of spontaneous mutations. Owing to the advances in genetics in the past decade, the cause of more than half of all congenital dermal diseases has been identified genetically and the relevant genetic tests for diagnosis of these diseases contributed greatly to veterinary practice and to dog breeding.

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OCCURRENCE OF BITING MIDGES (DIPTERA: *CULICOIDES*) ON DAIRY FARMS IN EASTERN SLOVAKIA IN RELATION TO ABIOTIC FACTORS

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ABSTRACT

Within the scope of our research, we have performed 59 trapping sessions and collected 15,756 biting midges from 20 species at four farms (Kluknava, Ostrov, Turňa nad Bodovou and Zemplínska Teplica), The most frequent types of captured insects were representatives of the *Avaritia subgenus*, *C. obsoletus/C. scoticus*, representing on average 85.1 % (13,295 individuals) of the fauna of the biting midges, with the exception of the farm in Ostrov where this group represented only 41.7 % of the fauna. At this particular farm, the most frequently trapped insects belonged to the *Culicoides* subgenus (54.1 %), in particular the *C. bysta, C. lupicaris, C. newsteadi, C. Pulicaris* and *C. Punctatus* species. During the trapping sessions, we monitored factors affecting the number of trapped biting midges, such as the temperature, relative air humidity and airflow rate: the air temperature during the trapping of the biting midges ranged from 9.8 to 26.2 °C; the relative air humidity ranged from 35.1 to 100 %; and the air flow rate ranged from no wind to a wind velocity of 8.2 m.s^{-1}. However, in the final evaluation, we failed to observe a statistically significant correlation between the air flow and the number of trapped biting midges. The largest amounts of biting midges were trapped at temperatures ranging from 15.8 to 24.6 °C and at a relative air humidity ranging from 54.2 % to 68.6 %. While monitoring the seasonal dynamics of the physiological conditions of biting midge females at the selected farms, we confirmed that during the period from June to August, the most frequently trapped females were parous (50.1 %; 7,826 individuals). In addition, nulliparous females comprised 43.8 % (6,842 individuals) and were continuously trapped throughout the season (April—November).

Key words: abundance; biting midges; relative humidity; species composition; temperature

INTRODUCTION

Biting midges (Diptera: *Culicoides*) are very small hematophagous diptera, which are important for mammals

because they transmit pathogens, primarily viruses and parasites, especially microfilaria of the *Onchocerca* genus [22]. There are 1,347 species of biting midges identified so far, and they are categorised into 31 subgenera [5, 20]. Out of them, 589 species were identified in Europe and 64 particularly in Slovakia [37, 38]. Over the last 15 years, we have witnessed a significant increase in the interest in monitoring the occurrence and investigating the species composition of biting midges, primarily due to their ability to transmit the bluetongue virus (BTV) and the Schmallenberg virus (SBV) [1, 19, 39]. Extensive studies were launched in 2006, when BTV caused marked financial losses to breeders of ruminants in the Netherlands and Belgium, and later in Germany, Luxembourg and Northern France [7, 13, 35, 41]. Despite the fact that no primary focus of infection was confirmed in Slovakia at that time, a certain region of Slovakia was classified as a prohibited zone due to the presence of foci of infections in the neighbouring countries (Hungary and Czech Republic) [25]. This region has therefore become subject to extraordinary emergency measures ordered by law, as well as the requirement to perform quarantine examinations of animals and protect them, which had negative effects on the costs of breeding. Considering the epizootological aspect of the spread of this disease and the recurrent new foci of infection in Europe, in particular in Germany, Belgium and France [14, 16], it is necessary to monitor a relationship between a causal agent of the disease, a vector and a susceptible host. Obviously, the parasite-host relationships must be evaluated while monitoring local abiotic factors, such as a temperature, relative humidity and air flow, as they significantly affect the development and spread of biting midges, as well as the replication of viruses and the development of infective stages of parasites [9, 29, 40]. In addition to the factors listed above, the occurrence of biting midges is also undoubtedly affected by the presence of a sufficient number of hosts [33]. The available literature contains a number of references indicating that beef cattle in particular are very attractive for biting midges. This is primarily a result of a high production of carbon dioxide, acetone and other phenol derivatives, which are highly attractive for these Diptera [4, 24, 27].

Therefore, we have been conducting an entomological survey of the fauna of biting midges in cattle breeds for more than ten years, especially in eastern Slovakia.

MATERIALS AND METHODS

Field trapping

This entomological survey was carried out in 2016 (Kluknava and Zemplínska Teplica) and 2019 (Turňa nad Bodvou and Ostrov) (Figure 1).

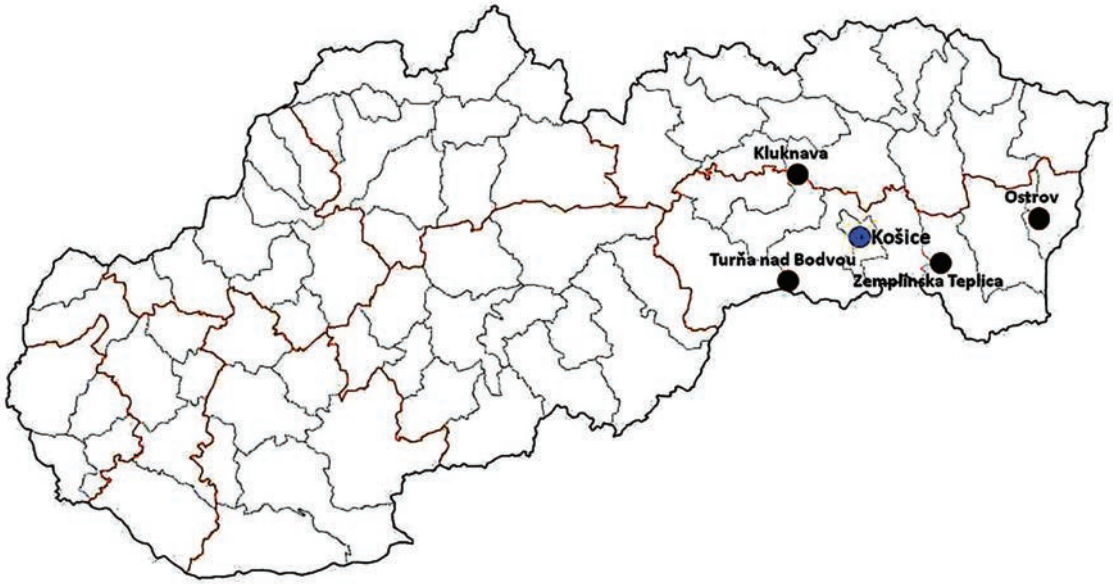

Fig. 1. Biting midges trapping sites in Slovakia

Kluknava (48.923475, 20.932527)

This is a village in the Gelnica District, located in the south-east part of the Spiš Region, in the Kluknava Valley; the pastures are located on the western hillsides of the Čierna Hora Hill on both banks of Dolinský Brook and on the northern hillsides of the Slovak Ore Mountains.

Ostrov (48.719501, 22.165222)

This village is located in the Sobrance District, the Zemplín Region. It lies in the north-east part of the Eastern Slovakia Lowland.

Turňa na Bodvou (48.61095, 20.87444)

This village is located in the Košice-vicinity District, the Abov Region, on a border between the Turnianska Hollow Basin (Slovenský kras) and the Košice Plane (Košická kotlina). North of the village, there is Turňa Castle Hill that belongs to the Zádielska Plateau.

Zemplínska Teplica (48.384596, 21.342568)

It is a village in the Trebišov District, located in the Southern Zemplín Region, near the eastern roots of the Slanské Mountains in the Podslanská Highlands.

Biting midges were collected using the CDC Miniature Light Trap 1212 (John Hook Company, USA). The collected insects were processed in a laboratory by applying a standard procedure [18], put in test tubes containing 70 % ethanol, and stored in a fridge at a temperature of 4—6 ^{0}C.

Morphological determination of biting midges

The primary morphological determination was carried out using the Zeiss-Stemi DV-4 microscope. Using the rapid diagnostic key [18], biting midges were divided into four complexes: *Obsoletus, Pulicaris, Nubeculosus* and the other *Culicoides* spp. Subsequently, the biting midges were identified based on typical morphological features visible under a binocular magnifier, such as wing spots, a chest colour, a shape of the 3rd segment of the palp, a ratio of the length of 10th segment to the length of the 11th segment of the antenna, etc. The morphological identification was carried out using available standard diagnostic keys [10, 17] and the interactive key [28].

Female age grading

Within the species determination, we divided female midges into four categories based on their abdominal pigmentation and their physiological condition [12]:

Nulliparous—young females which have never sucked blood and their abdomens were of a white or off-white colour;

Parous—older females which had at least once sucked blood of a host and their abdomens contained grains of the Burgundy Red Pigment, which were formed during the 1st gonotrophic cycle and persisted even after the oviposition;

Engorged—females with fresh host's blood of a deep red colour in their abdomens;

Gravid—females with abdomens filled with eggs.

Measurement of temperature and air humidity

A temperature and air humidity were measured using a digital datalogger Log 32. Dataloggers were launched at the same time as the light traps were installed. Following each trapping, a record was saved in the PDF version.

Statistical evaluation of the results

The statistical evaluation was carried out using the Pearson's correlation coefficient in order to evaluate the effects of a temperature and air humidity on the fauna of biting midges. The data were processed into a point chart.

Ethical statement

The Ethics Committee of the University of Veterinary Medicine and Pharmacy has approved the present research in accordance with applicable national and international animal welfare legislation. No animals were killed for the purpose of this study.

The authors declare no conflict of interest.

RESULTS

Within our research, we have performed 59 trappings and collected 15,756 biting midges of 20 species (99.1 %) (Table 1). The most frequently captured individuals were the representatives of the *Avaritia* subgenus, *C. obsoletus/C. scoticus*, representing 85.1 % (13,295 individuals) of the fauna of biting midges, except at the Ostrov farm where they amounted to only 41.7 % of the fauna. At this particular farm, the most frequently trapped midges belonged to the *Culicoides* subgenus (54.1 %), in particular *C. bysta, C. lupicaris, C. newsteadi, C. Pulicaris*, and *C. Punctatus*

Table 1. Species composition of biting midges collected in beef cattle during one season

Subgenus	Species	Biting midges trapping sites – beef cattle/dairy cows			
		Kluknava	Ostrov	Turňa nad Bodvou	Zemplínska Teplica
Avaritia	*C. chiopterus*	–	–	–	–
	C. dewulfi	–	–	–	–
	C. obsoletus/C. scoticus	2,484	1,131	4,551	5,129
Beltranmyia	*C. circumscriptus*	–	26	6	–
	C. manchuriensis	–	1	–	–
	C. salinarius	–	8	–	–
Culicoides	*C. bysta*	–	2	7	–
	C. lupicaris	3	92	65	31
	C. newsteadi	–	853	217	–
	C. pulicaris	8	14	70	3
	C. punctatus	–	505	70	12
Monoculicoides	*C. nubeculosus*	11	–	–	29
	C. riethi	–	–	32	–
	C. stigma	–	3	17	13
Pontoculicoides	*C. slovacus/C. tauricus*	–	6	3	–
Silvaticulicoides	*C. subfasciipennis*	–		1	–
Unclassified	*C. clastrieri*	–	1	–	–
	C. festivipennis	–	67	6	–
	C. furcilatus	–	1	125	–
	C. vexans	17	–	–	–
Total females		2,523	2,710	5,170	5,221
Including: Nulliparous		1,034	1,255	2,099	2,454
Parous		1,287	1,236	2,588	2,715
Engorged		151	113	154	17
Gravid		51	106	329	35
Unidentified		18	–	–	14
Males		3	2	31	1
Damaged		26	13	8	16
Number of trappings in a season		10	10	25	14
Total		**2,570**	**2,725**	**5,209**	**5,252**

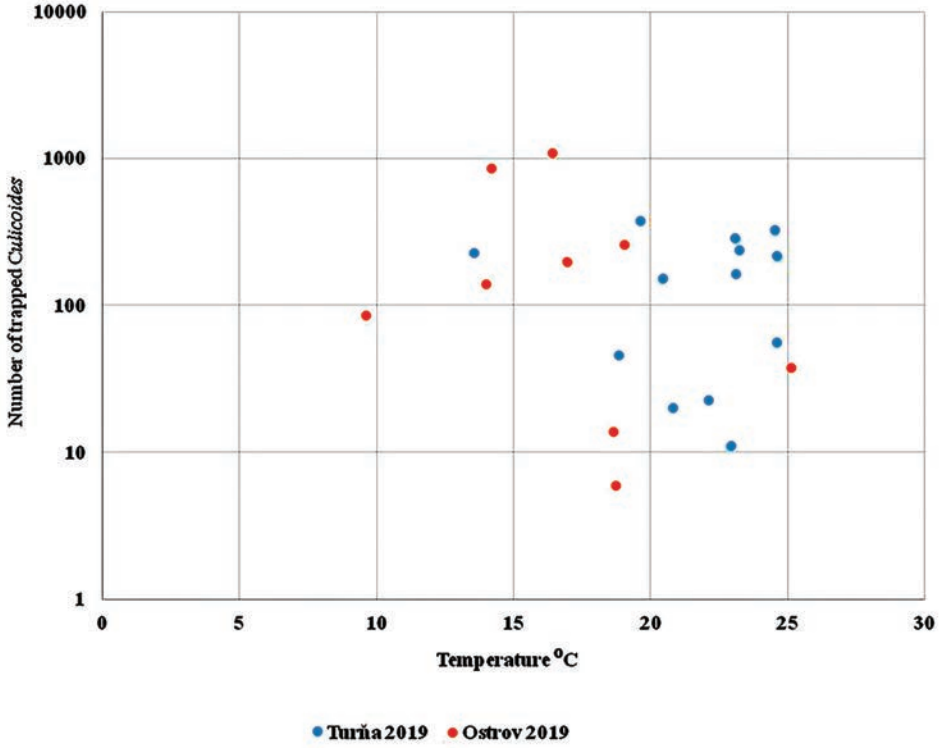

Fig. 2. Effect of temperature on the abundance of the trapped biting midges

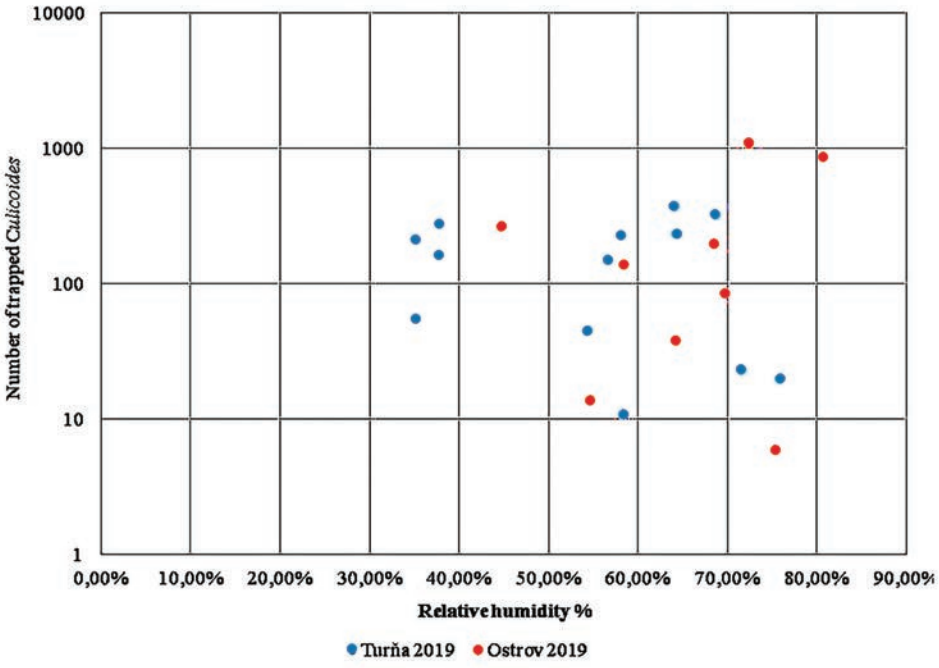

Fig. 3. Effects of humidity on the abundance of trapped biting midges

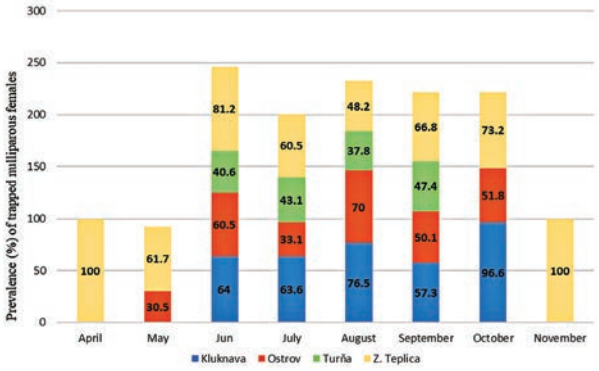

Fig. 4. Seasonal dynamics of the trapped nulliparous females

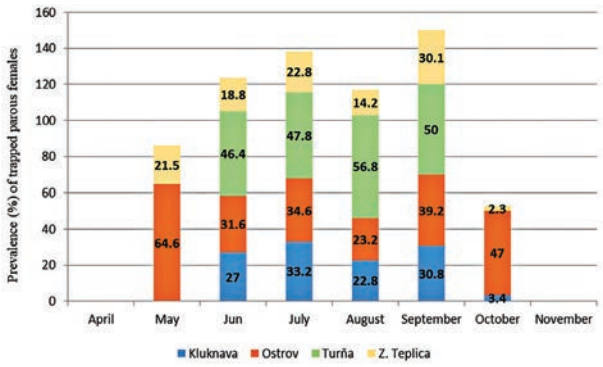

Fig. 5. Seasonal dynamics of the trapped parous females

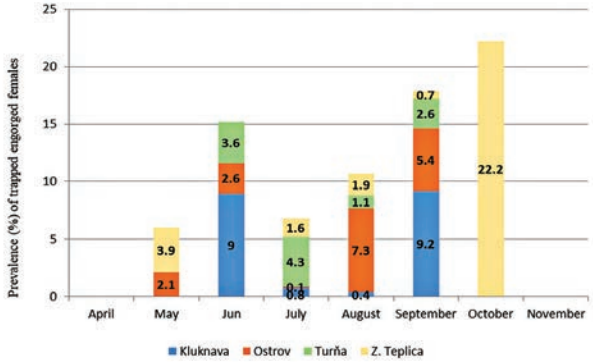

Fig. 6. Seasonal dynamics of the trapped engorged females

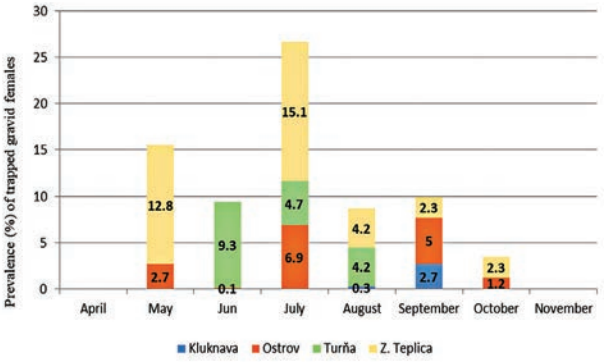

Fig. 7. Seasonal dynamics of the trapped gravid females

species. On the pastures of the farm in Kluknava, we have captured 5 species and a similarly low number of species, i. e. 8, were collected in the cattle-ranges at the farm in Zemplínska Teplica, although the amount of the collected biting midges was the highest at this particular farm (Table 1).

As for the species composition, 14 species at the farm in Ostrov and 13 species at the farm in Turňa nad Bodvou were identified. The factors which were monitored during the trappings included the temperature, relative air humidity and the air flow rate, as they affect the number of the trapped biting midges. During the trappings, a temperature ranged from 9.8 to 26.2 °C; relative air humidity ranged from 35.1 to 100 %; and the air flow rate ranged from no wind to a wind velocity of 8.2 m.s^{-1}. Intensive measurements of abiotic factors in a correlation to the number of trapped biting midges were carried out at the farm in Turňa nad Bodvou and at the farm in Ostrov. We found out that the optimal air velocity was 0.1—0.3 m.s^{-1} due to the number of midges caught; however, the final evaluation revealed that the air flow rate was not a statistically significant factor for changes in the number of the collected biting midges. The effects of a temperature and relative air humidity on the abundance of biting midges are presented in Figures 2 and 3.

The largest amounts of biting midges were trapped at temperatures ranging from 15.8 to 24.6 °C and at the relative air humidity ranging from 54.2 % to 68.6 %.

The investigation of the seasonal dynamics of the physiological condition of females of biting midges at the farms revealed that there are two peaks of their activity, in June and in August (Figures 3 through 7). The first nulliparous females were trapped at the farm in Zemplínska Teplica in April, but also as late as in early November. They were found in the traps throughout the entire collection season at every farm, and their occurrence reached peaks in May, June and September. These nulliparous females represented 43.8 % (6,842 individuals) of all the collected females. The most frequently trapped females were older females

(parous)—they represented 50.1 % (7,826 individuals) and occurred at farms in the period from late June to September. Females with fresh host's blood in their abdomens (435 individuals) and gravid females (521 individuals) represented 2.8 % and 3.3 % of all females, respectively. Females of both groups were sporadically found in the collected material throughout the entire season, but primarily in August and September.

DISCUSSION

In epidemiological studies, it is necessary to perform the diagnostics of pathogen vectors. Our study provided information on the fauna of biting midges in eastern Slovakia at four farms where beef cattle were bred and where we confirmed the occurrence of 20 species of biting midges which were potential vectors of pathogens. The species with the highest occurrence were *C. obsoletus/C. scoticus*; they represented on average 85.1 % (13,295 individuals) of the fauna of biting midges and belong to the most frequent species in the Palaearctic region. These species are potential vectors of the catarrhal fever virus and the Schmallenberg virus in Europe, as well as filariae. Haematophagous females of biting midges parasitise on a wide range of vertebrates [27]. Some studies claim that the range of suitable hosts from which *Culicoides* biting midges may choose is limited by the availability of such hosts in their environment [33]. On the other hand, such an anthropogenic activity facilitates the spread of new pathogens among humans. The anthropogenic impact changes the landscape through various agricultural interventions, irrigation and production of manure and silage, where hatching sites for biting midges are formed. Moreover, climate changes that increase the frequency of storms and floods, as well as the sea level rise, increase the availability of brackish hatching sites used by, for example, *C. newstedi, C. halophilus, C. circumscriptus, C. salinarius* and *C. maritimus* [41]. Biting midges of *C. newsteadi* were the second most frequent species diagnosed at farms in Ostrov and in Turňa nad Bodvou. In Portugal, the *C. newsteadi* biting midges were observed mainly in autumn months [34]; however, at the Slovak farms, their presence was detected in June. During the following months, the abundance of *C. newsteadi* biting midges decreased. It is known that the ideal conditions for the development of these biting midges are in shallow brackish waters, bordered with decomposing plants [15]. As for abiotic factors, the occurrence of biting midges is most significantly affected by the ambient temperature, which affects the geographical spread of the species, the sizes of populations and their survival rates, as well as the number of generations per season. The development from an egg to an imago accelerates with higher temperatures; however, at a certain temperature, survival rates begin to decrease. A shadowed and cooler biotope prolongs the development from an egg to an adult [9], and this results in a lower number of generations in a year. On the other hand, at higher temperatures, the period of hatching from eggs to larvae accelerates and it leads to a higher number of generations in a season. Also, the ambient temperature regulates the duration of a gonotrophic cycle, and hence also of the sucking frequency; as a result, the probability of pathogen transmission increases too. A temperature affects also the replication of infectious agents in the bodies of biting midges [31].

Recently, the impact of higher temperatures has been monitored within the investigation into climate changes and global warming. An increase in the average annual temperature by 2 °C corresponds to a shift of the northern border of the insect-spread area by approximately 200 km [23]. The research conducted by B i s h o p et al. [3] indicated that an increase in temperature by 2 °C in winter prolonged the season of the presence of *C. brevitarsis* by 0.7 month per year. Another risk related to increased temperatures in winter months is that warmer winter makes it possible for adults, as well as pathogens they carry, to survive. Our observations indicated that the highest amounts of individuals were collected at temperatures ranging from 15.8 to 24.6 °C. Higher air humidity positively affects the activity of biting midges, while the optimal relative air humidity ranges from 80 to 90 % [30]. At low relative humidity and low temperatures, the survival rate of biting midges is low [43]. During our trappings, the optimal relative air humidity ranged from 54.2 % to 68.6 %, at which we observed the highest amount of trapped biting midges. The air flow facilitates active or passive motion of biting midges, which actively fly and search for a host in windless conditions, but in windy weather the intensity of this search for a host decreases. According to L e h a n e [26], the wind with a velocity of 1—2 $m.s^{-1}$ may reduce their flying activity and the search for a host. W i t m a n, B a y l i s [42] stated that at wind velocities below 3 $m.s^{-1}$, biting midges actively fly,

whereas when a wind velocity exceeds 3 m.s^{-1}, they only float [29], and at wind velocities above 11 m.s^{-1}, they stay hidden in the groundcover [42]. Biting midges move actively, but they are also passively moved by the wind for as far as 700 km [21]. The flying activity and dispersion of biting midges is one of the factors that condition the introduction and spread of pathogens, especially the viruses they transmit [36]. Biting midges which fly to higher altitudes may be easily carried away by the flowing air for dozens of kilometres; this explains the fast expansion of arboviruses many kilometres away from a source, while probably a single sucking by an infected biting midge is sufficient for the transmission of a virus to a susceptible host [2]. Most biting midges are active at dusk, and increased abundance is observed at the light intensity of 101—10,000 lux, which corresponds to dusk or cloudy weather [32]. *C. obsoletus* may also attack hosts during the day at the light intensity of 20,000 lux, optimally at ca 5,000 lux [17]. In our survey, especially at the farm in Ostrov, we diagnosed the biting midges primarily of the *Culicoides* genus (54.1 %), in particular the *C. bysta, C. lupicaris, C. newsteadi, C. pulicaris* and *C. punctatus* species. Similar results were reported by Colins et al. [8], who monitored the fauna of biting midges at farms in Ireland in 2014. These species are assumed to be potential vectors of the bluetongue virus [11]. The third most frequent species of biting midges observed in our study was *C. furcillatus* (2.34 %). Their presence was detected from June to August. This species has also been detected in years 2003—2006 at several farms in Switzerland, where mostly beef cattle and goats were bred [6].

CONCLUSIONS

With regard to the fact that biting midges cause enormous economic losses, the knowledge obtained during the entomological survey contributes to understanding the biology of biting midges, which will be beneficial in epizootology and in the implementation of preventive measures. However, climate changes bring constant changes in the spread of vectors and pathogens, and the present paper provides the latest information. The prospects presented by climatologists are alarming, and the current information does not necessarily have to be valid in a very near future. It is therefore necessary to conduct continuous investigation, not only in eastern Slovakia, but also elsewhere.

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SELECTION OF THE MOST RELIABLE METHOD FOR THE ANALYSIS OF INHIBITORY SUBSTANCES IN RAW AND SKIMMED MILK

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ABSTRACT

Milk used for human consumption must comply with the European Union legislative requirements for residues of inhibitory substances in milk, the values of which must not exceed the established maximum residue limit. In order to ensure the quality and safety of milk and milk products placed on the market, the presence of residues of inhibitory substances should be monitored and verified. The aim of our study was to select the most reliable method for the analysis of residues of inhibitory substances in milk. In the search for the most reliable method, a total of 49 milk samples were tested in the form of raw milk, skimmed milk and skimmed-milk powder throughout the agri-food chain. For comparison, the microbial inhibition tests Eclipse 50, Eclipse Farm, Explorer 2.0, Delvotest®, Premi®Test and the fast receptor screening test TwinSensor were used. The most relevant results were obtained by the Eclipse 50 and Eclipse Farm tests, the reliability of which were also confirmed by the Explorer 2.0 and Premi®Test tests. Moreover, according to the State Veterinary and Food Administration of the Slovak Republic, Eclipse 50 is an official reference method for the determination of residues of inhibitory substances in milk. Therefore, we can only state that of all the methods used, the Eclipse 50 seems to be the most reliable for routine control analysis of residues of inhibitory substances in all types of milk.

Key words: analysis; inhibitory substances; milk; analysis; screening tests

INTRODUCTION

Milk and milk products as a source of nutrients, probiotics and prebiotics, vitamins and minerals, are widely consumed worldwide. They have become a major part of a balanced diet and are important for people's nutrition and health. There are several different types of milk, e.g. raw milk, whole milk, semi-skimmed, skimmed milk, powdered milk like skimmed milk powder, etc. "Raw milk" means milk produced by the secretion of the mammary gland of farmed animals which has not been heated

to more than 40 °C or undergone any treatment that has an equivalent effect [15]. Skimmed milk is made by removing the fat components by centrifugation. To obtain skimmed milk powder, the process involves evaporating water and drying at 94 °C.

However, milk is exposed to and gets contaminated with various types of chemical contaminants. One of these undesirable contaminants in milk and milk products are residues of pharmacologically active substances. 'Residues of pharmacologically active substances' means all pharmacologically active substances, expressed in $mg.kg^{-1}$ or $\mu g.kg^{-1}$ on a fresh weight basis, whether active substances, excipients or degradation products, and their metabolites which remain in food obtained from animals [14]. Inhibitors, especially antibiotics, are currently widely used in humans as well as other animals.

The use of antibiotics in livestock is of great importance in the treatment of bacterial infections, but also to ensure the quality of life of animals, but their use has a significant impact on the quality and safety of animal products. Approximately 63,000 tons of antibiotics are used in livestock worldwide each year [18]. They are used for therapeutic and prophylactic purposes [10], but also for growth promotion [2]. The global use of antimicrobials in animals is twice as high as in humans [1].

Many studies have shown that significant amounts (30—70 %) of antibiotics are released unchanged, i. e., with potential antimicrobial activity in the environment [10]. When released into the environment, most antibiotics are persistent and biologically active [17]. The main risks we should consider when using antibiotics are residues of inhibitory substances and the development of microbial resistance [7]. The World Health Organization [19] emphasizes that bacterial resistance to antibiotics is rising to dangerously high levels in all parts of the world. Globally, new mechanisms of resistance are emerging and spreading that threaten our ability to treat common infectious diseases. Various alternatives to antibiotics (plant-derived substances and probiotics) can be beneficial in the fight against bacterial resistance [3].

The residues of various antibiotics are associated with many types of allergic reactions, especially in the case of penicillin. Antibiotic residues have potential carcinogenic effects by interacting with DNA and RNA [5]; and a mutagenic effect that can cause mutation of the DNA molecule or damage to chromosomes [8]. Such mutations can manifest itself as infertility for an example [16]. In the newborn, various congenital anomalies may occur due to long-term exposure to antibiotic residues during pregnancy [5]. The use of broad-spectrum antibiotics can destroy a variety of bacteria in the gut, including non-pathogenic organisms, which can disrupt the normal intestinal microflora [16]. Last but not least, the existence of antibiotic residues in milk, even in very low concentrations, is very worrying in the dairy industry. Antibiotic residues can interfere with the fermentation process during cheese and yogurt production by inhibiting starter cultures [5].

Milk used for human consumption must comply with the European Union requirements for residues of inhibitory substances in milk, the values of which must not exceed a maximum residue limit (MRL). MRL is the maximum concentration of a residue of a pharmacologically active substance which may be permitted in food of animal origin [14]. On 15 March 2019, Commission Implementing Regulation (EU) No. 2019/627 laid down uniform practical arrangements for carrying out official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and measures for the monitoring of certain substances and their residues in live animals and animal products in accordance with Council Directive 96/23/EC of 29 April 1996. Council Directive 96/23/EC provides, inter alia, that antibacterial substances, including sulphonamides and quinolones, are to be monitored in milk. For this purpose, several methods are currently available, such as e.g., chromatographic, immunological, microbiological and others.

An effective screening method must be inexpensive and capable of high-throughput, and able to effectively identify potentially non-compliant samples from a large number of negative samples. Microbial inhibition tests were the first methods used to detect antibiotic residues and are still used. They are cost-effective and, in contrast to e.g., immunological or receptor-based assays, they have the potential to cover the entire antibiotic spectrum within one test. This format is commonly used in routine milk screening [13].

Microbial inhibition tests are available that can detect a wide range of antibacterial substances in milk, such as betalactams, tetracyclines, sulfonamides, macrolides, aminoglycosides and trimethoprim. These tests are available in tube (or vial, or ampoule) format and are easy to use, affordable and the test result is readable within two to three hours. The tubes contain an agar medium inoculated

with (spores of) a sensitive test bacterium, supplemented with a pH or redox indicator. At the appropriate temperature, the bacteria start to grow and produce acid, which will cause a color change. The presence of antimicrobial residues will prevent or delay bacterial growth, and thus is indicated by the absence or delay of the color change. This group of tests includes, e. g. Delvotest or Eclipse test. Immune-receptor tests are competitive tests involving specific receptors with high affinity to specific antibiotic groups. They usually comprise of a lateral flow strips which are read by eye or an incubator Reader. They are very fast and the test result is readable within 5 to 10 minutes. Therefore, they are suitable for initial rapid antibiotic screening. However, these assays are generally limited to the detection of certain antibiotics, such as beta-lactams or tetracyclines. From this type of tests are available, e.g. TwinSensor, BetaStar, AuroFlow, or BT Scan [11, 13].

In order to ensure and verify the safety of milk and milk products without the presence of antibiotic residues, the aim of our study was to select the most reliable method for the routine analysis of inhibitory substances in various types of milk.

MATERIALS AND METHODS

As part of the search for the most reliable method, a total of 49 cow's milk samples in the form of raw milk, skimmed milk and skimmed milk powder. The samples were provided to us by milk processing plant or were purchased from retail chains. The tested raw cow's milk was obtained from five different dairy farms from Eastern Slovakia. Samples were examined using microbial inhibition tests: Eclipse 50 (Zeulab, Zaragoza, Spain), Eclipse Farm (Zeulab, Zaragoza, Spain), Explorer 2.0 (Zeulab, Zaragoza, Spain), Delvotest® (DSM, Food Specialties, Delft, The Netherlands) and Premi®Test (R-Biopharm AG, Darmstadt, Germany), as well as TwinSensor (Unisensor s.a., Ougrée, Belgium) rapid receptor screening assay.

Preparation of samples

Raw and skimmed milk was stored in a freezer at –18 °C and immediately before testing heated to a temperature of 40 °C for 20 minutes. The skimmed milk powder was treated with demineralized water in a ratio of 1 : 5 before the analysis.

Procedure

- **Eclipse 50, Eclipse Farm, Explorer 2.0, Delvotest®, Premi®Test:** Milk samples were transferred to individual ampoules or microtiter plates as follows: 50 µl (Eclipse 50) or 100 µl (Eclipse Farm, Explorer 2.0, Delvotest®, Premi®Test). A negative control using a milk sample that does not contain any inhibitory substances was also used to check the test accuracy. The wells were thoroughly sealed with an adhesive foil provided by the tests and placed in an incubator (Labnet Accublock Digital Dry Bath D 1200, Labnet, Edison, USA). The samples were incubated at 65 °C ± 2 °C for 2 to 3 hours depending on the test. The colour change of the negative control was monitored. When the colour of the negative control changed to yellow, the incubation was terminated. All samples were tested twice to verify the test result.
- **TwinSensor:** The test protocol as set by the kit manufacturer was followed. Milk samples (200 µl) were applied to microwells containing the lyophilized reagent and incubated in a HeatSensor (Unisensor, SA, Ougrée, Belgium) for 3 minutes at 40 °C. After the first three minutes, the strips were placed in the incubator above the corresponding milk samples and incubate for another 3 minutes at 40 °C. At the end of the incubation, the test strips were prepared to evaluate the results.

Evaluation of results

Eclipse 50, Eclipse Farm, Explorer 2.0, Delvotest®, Premi®Test

The interpretation of the results is possible visually or using an e-Reader. The yellow colour of the solid medium indicates that no antibiotics are present above the detection limit of the test and the milk is suitable for further processing. The purple colour of the solid medium indicates the presence of antibiotics whose concentration exceeds the detection limit of the test. This milk must not be used for further processing. Yellow/purple coloration of the solid medium indicates the presence of antibiotics at the level of the detection limit of the test (Figure 1).

Instrumental reading with e-Reader

Explorer 2.0 and Eclipse Farm tests are also compatible with the equipment e-Reader (Zeulab, Zaragoza, Spain). The e-Reader eliminates any doubts and provides numerical and qualitative evaluation of the samples, thus auto-

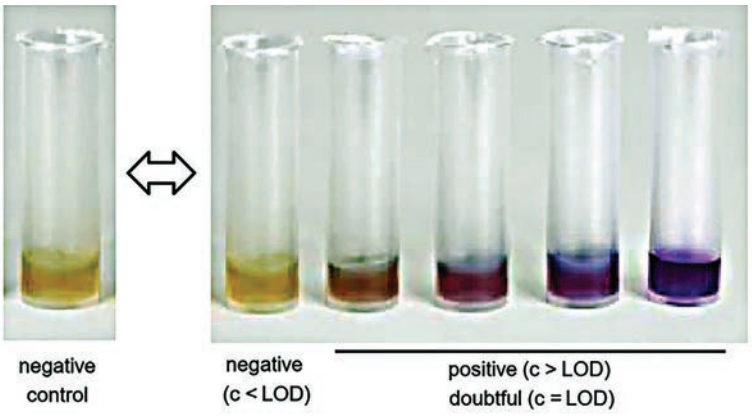

Fig. 1. Evaluation of microbial inhibition tests
https://www.bioscience.com.sg/testing-for-antibiotics-in-food-and-feed-industries/

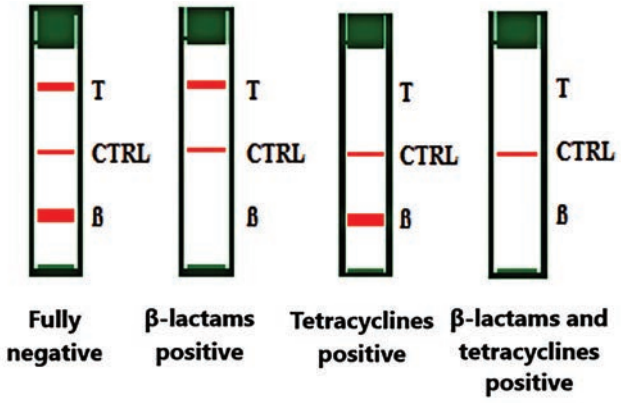

Fig. 2. Evaluation of TwinSensor

mating the process and standardised results. In addition, the instrument detects the colour change of the negative control by spectrophotometric measurement, determines the cut-off value and automatically terminates the incubation of the samples. The main advantage of combining tests with the e-Reader is the objectivity of the results, which ultimately saves time and reduces the number of erroneous (false positive/false negative) results.

TwinSensor

Test results can be evaluated visually or with the ReadSensor 2 device (Unisensor SA, Ougrée, Belgium). Visual interpretation of the result is obtained by comparing the red colour intensity of each test line with the control (CTRL) line. If the test line is darker than the CTRL line, the sample is considered negative for the antibiotic concerned, or it contains a concentration of antibiotic below the detection limit of the test. If the test line is of the same intensity, or if it is a lighter than the CTRL line, the sample is considered positive for the antibiotic concerned, and it contains an antibiotic concentration equal to, or above the detection limit of the test (Figure 2). ReadSensor quantifies, interprets, and records test strip results.

RESULTS

In the first round of testing, a total of 20 samples of raw cow's milk and a negative control sample were analysed using a broad spectrum microbial inhibition tests Eclipse 50, Eclipse Farm, Explorer 2.0, Delvotest®, Premi®Test and a narrow-spectrum test TwinSensor. Sample was tested in duplicate. As we can see from Table 1, Delvotest® (Fig. 3) and TwinSensor (Fig. 4) tests showed positive re-

Table 1. Results of testing 20 raw milk samples using 6 different tests

Sample ↓	Test name					
	Eclipse 50	Eclipse Farm	Delvotest®	Explorer 2.0	Premi®Test	TwinSensor
1	–	–	+	–	–	+
2	–	–	+	–	–	+
3	–	–	–	–	–	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
6	–	–	–	–	–	–
7	–	–	–	–	–	–
8	–	–	–	–	–	–
9	–	–	–	–	–	–
10	–	–	–	–	–	–
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	–	–	–	–	–	–
14	–	–	–	–	–	–
15	–	–	–	–	–	–
16	–	–	–	–	–	–
17	–	–	–	–	–	–
18	–	–	–	–	–	–
19	–	–	–	–	–	–
20	–	–	–	–	–	–
Negative control	–	–	–	–	–	–

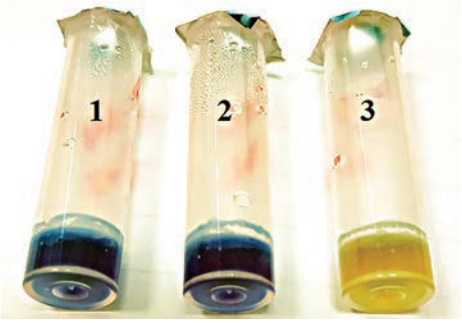

Fig. 3. Delvotest® result

sults in sample No. 1 and 2. The Eclipse 50, Eclipse Farm, Premi®Test and Explorer 2.0 tests showed negative results for all 20 examined milk samples.

As shown in Figure 4, the results of TwinSensor test indicated the presence of β-lactam antibiotics in both samples (No. 1 and 2). The test failed to confirm the presence of tetracyclines. The results of the visual evaluation were also confirmed by the ReadSensor 2 device (Figure 5).

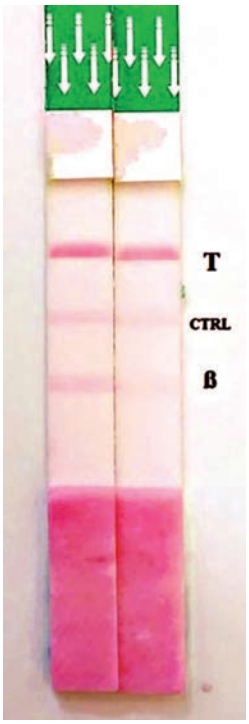

Fig. 4. TwinSensor test result

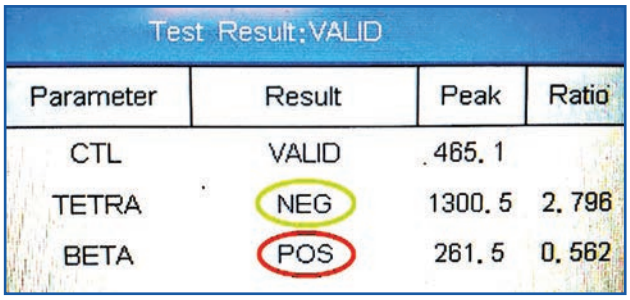

Fig. 5. TwinSensor result using the ReadSensor 2

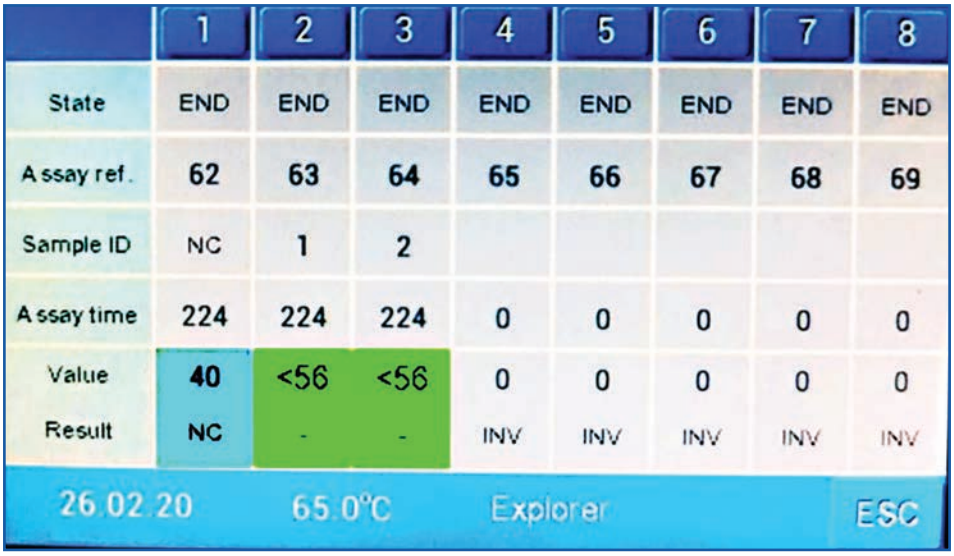

Fig. 6. Result of the Explorer 2.0 test using the e-Reader where No. 1 represents a negative control

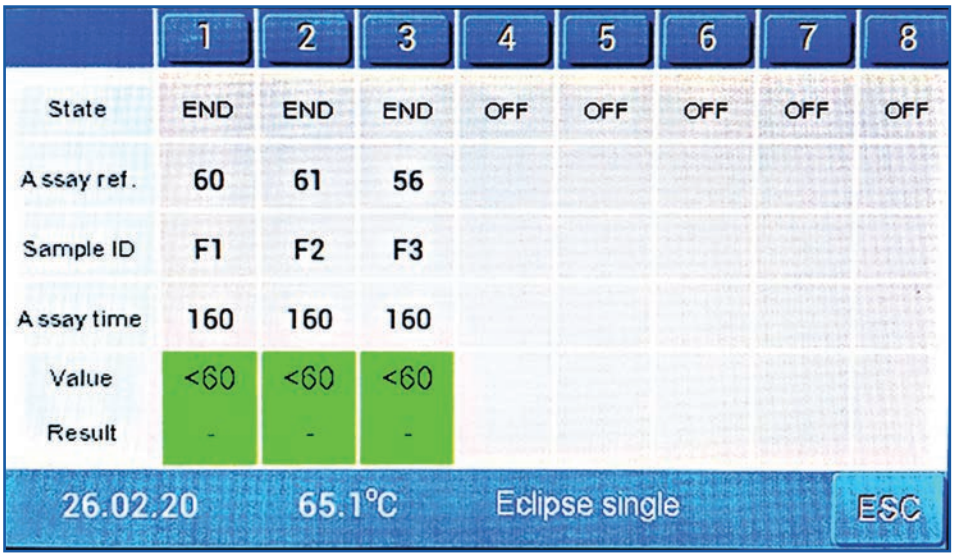

Fig. 7. Result of the EclipseFarm test using the e-Reader where No. 1 represents a negative control

Table 2. Eclipse 50 test results

Type of milk ↓	Farm 1		Farm 2		Farm 3	
	No.		No.		No.	
Raw milk	1	–	4	–	7	–
Skimmed milk	2	–	5	–	8	–
Skimmed milk powder	3	–	6	–	9	–
Negative control						
Demineralised water		–	**Rajo low-fat milk 0.5 %**		–	

In addition to the visual evaluation in the case of samples No. 1 and 2 (positive results), we subsequently used the e-Reader for the instrumental reading to confirm or refute the positive results. The following analysis was done by the Explorer 2.0 (Figure 6) and Eclipse Farm (Figure 7) test compatible with e-Reader. The results proved negativity in both samples results.

In the second round of testing, another 9 milk samples in the type of raw cow's milk, skimmed milk and skimmed milk powder provided to us by milk processing plant as control samples taken during routine milk processing operations, were analysed. From all tests used in the first round of testing and their results obtained, the Eclipse 50 microbial inhibition test was only used. Demineralized water and Rajo low-fat milk with a fat content of 0.5 % were used as a negative control. All samples showed a negative result, as we can see from Table 2.

For verification of the presence of residues of inhibitory substances in skimmed milk offered to consumers in the retail network, a total of 20 samples of skimmed milk with a fat content of 0.5 % were purchased from various retail chains and analysed by the Eclipse 50 test. In addition, according to the State Veterinary and Food Administration of the Slovak Republic, the Eclipse 50 test is the official reference method for detection of the presence of inhibitory substances in milk. When detecting residues of inhibitory substances in skimmed milk with a fat content of 0.5 %, the Eclipse 50 test confirmed our expected results. None of the 20 examined skimmed milk samples contained residues of inhibitory substances above the permitted MRL. We are very pleased with this test result, which indicates the required safety of milk that is sold for human consumption.

DISCUSSION

The European Food Safety Authority report [9] summarises the data collected in 2019 on the presence of residues of veterinary medicinal products and certain substances in live animals and animal products in the European Union, Iceland and Norway. A total of 671,642 samples were reported to the European Commission by the 27 out of the 28 EU Member States, Iceland and Norway. They consisted of 368,594 targeted samples and 5,016 suspect samples reported under Council Directive 96/23/EC, and of 2,342 samples collected at import and 295,690 samples collected in the framework of programs developed under the national legislation. The majority of countries fulfilled the minimum requirements for sampling frequency laid down in Council Directive 96/23/EC and in Commission Decision 97/747/EC. Overall, the percentage of non-compliant samples in 2019 (0.32 %) was comparable to the previous 11 years (0.25 %—0.37 %). Slight decreases were noted in 2019, for resorcylic acid lactones, prohibited substances, antibacterials, anticoccidials, and dyes, compared to 2017 and 2018. For Group B1 (antibacterials), 0.14 % of the samples analysed under the Directive 96/23/EC monitoring were non-compliant.

B a c h i et al. [16] in their survey "Antibiotic residues in milk: past, present and future" found that residues of inhibitory substances in milk are often and largely detected worldwide. The world's highest number of publications on the detection of residues of inhibitory substances in milk come from Europe: 105 (46.88 %), followed by Asia: 77 (34.38 %), South America: 18 (8.04 %), North America: 16 (7.14 %) and Africa: 8 (3.57 %). Within Europe, Spain

leads with 30 (13.39 %) publications and Germany with 11 (4.91 %) published works. Cow's milk is most often tested, in a total of 193 publications (86.16 %), followed by sheep's milk: 9 (8.48 %) and goat's milk: 14 (6.25 %). Residues of β-lactam antibiotics were detected to the highest extent, in 133 (36.54 %) cases, tetracyclines: 51 (14.01 %), fluoroquinolones: 49 (13.46 %), sulfonamides: 46 (12.64 %) and aminoglycosides: 38 (10.44 %). The data suggest that the use of β-lactam antibiotics in dairy cows is increasing every day. In 16.96 % of detection cases, microbiological methods were used to determine residues of inhibitory substances in milk. Over the last 10 years, the number of publications has increased by almost 30 %, which indicates a growing trend in the detection of antibiotics in milk.

There are several studies on the use of microbial inhibition tests for the screening of antibiotic residues in cow, sheep and goat milk. B e l t r á n et al. [4] tested the performance of the current microbial tests, such as the BRT MRL (Analytik in Milch Produktions-und Vertriebs-GmbH, Munich, Germany), Delvotest MCS SP-NT (DSM Food Specialties, Delft, the Netherlands), Delvotest MCS Accelerator (DSM Food Specialties), and Eclipse 100 (Zeulab, Zaragoza, Spain) for the screening of antibiotics in milk. They confirmed that the microbial inhibition tests are efficient to detect β-lactams and other non-beta-lactam drugs such as neomycin, tylosin, sulfadiazine and sulfadimethoxine in raw milk from small ruminants. However, in spite of the improvements made in these tests in the last decade, they continue to be inefficient for the detection of other drugs, such as quinolones and tetracyclines, at safety levels. Therefore, the periodic use of more sensitive tests towards these substances would be convenient to widen the detection range in screening and guarantee the quality of milk and dairy products from small ruminants.

B i o n et al. [6] evaluated Delvotest® T for its ability to detect 27 antibiotic residues in raw cow's milk, skimmed milk and whole milk powder. The test showed a false negative result for nafcillin, oxytetracycline, tetracycline, rifaximin and sulfadiazine compounds.

In the research of M a t a, S a n z, R a z q u i n [12], Eclipse Farm test was verified in combination with an e-Reader. Detection limits for penicillins, cephalosporins, tetracyclines, sulfonamides, macrolides and aminoglycosides were evaluated. All residues were detected at or below the MRL. It was also shown that the course of the method was not affected by changes in the procedure (sample volume, incubation temperature, test dose). Usability has been demonstrated in raw cow's milk regardless of fat and protein content or high somatic cell content. Eclipse Farm in combination with e-Reader has proven to be a valuable tool for screening a wide range of antimicrobial residues in raw milk. The test is sensitive and easy to use. e-Reader simplifies analysis and increases the accuracy of results, interprets results in an objective way.

In our study we used Eclipse 50, Eclipse Farm coupled to e-Reader, Explorer 2.0, Delvotest® and Premi®Test as the microbial inhibition tests, and TwinSensor coupled to ReadSensor 2 as the immune-receptor test for analysis of residues of inhibitory substances in a total of 49 samples of raw milk, skimmed milk and skimmed milk powder. We considered that the most relevant and uniform results were obtained by the Eclipse 50 and Eclipse Farm tests, the reliability of which was also confirmed by the Explorer 2.0 and Premi®Test.

All these microbiological tests combine the principle of agar diffusion tests with colour change of a pH indicator from blue/violet to yellow due to the active metabolism of the test strain Bacillus stearothremophillus var. calidolactis at the absence of inhibitory substance in the sample. Bacillus stearothermophilus var. calidolactis is generally considered the most susceptible microbial strain for broad-spectrum analysis of antibiotic residues in various food matrices of animal origin such as milk, but also meat, eggs, fish, shrimps and honey.

All these tests are able to detect a broad-spectrum of antibiotics such as b-lactam, aminoglycosides, tetracyclines, sulphonamides, macrolides, lincosamides and quinolones at the level of MRL. This ability clearly favours them over tests that have a narrow spectrum of activity, but the importance of the competitive immune-receptor tests with a narrow spectrum of activity cannot be questioned, whereas it is precisely these tests which are the tests of first choice for verifying the presence of residues of a specific group/groups of antibiotics in milk.

CONCLUSIONS

The aim of our study was to select the most reliable method for the analysis of residues of inhibitory substances in various types of milk. In terms of cost, efficacy, spectrum of detectable substances and time consuming, rapid receptor screening tests or microbial inhibition tests are

mostly used on the dairy farm and in the milk processing plant. From all tests used, only two tests, the Delvotest® and the TwinSensor detected positive results in the same two raw milk samples with the positivity for beta-lactam antibiotics. Further testing of both positive samples with the Eclipse 50 and the Eclipse Farm coupled to instrumental reading with an e-Reader do not confirmed the positive result obtained. Positive results probably occurred due to the higher sensitivity of both methods used compared to other tests. Such over-sensitivity of screening tests is not desirable for residue control purposes, as such screening tests should detect the presence of a substance or class of substances at the level of interest (compliance with EU MRL) without the risk of false-positive results. The Eclipse 50 and Eclipse Farm tests coupled to e-Reader proved to be the more relevant tool in residue analysis. e-Reader provides an objective and accurate result in an automatic way, but the analysis is instrumentally demanding and expensive. Due to the technical and economical aspect of residue testing in milk, the use of Eclipse 50 is more practical and cost-effective for the routine control of residues of inhibitory substances in all types of milk.

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EFFECT OF NUTRITION ON THE MORPHOMETRIC MARKERS IN SPAYED DOGS

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ABSTRACT

Obesity and overweight have been frequently observed in dogs in recent years as in humans. The compositions of fatty acids in the accumulated lipids in tissues of obese animals may have important roles in the process and mechanisms related to the onset of metabolic disorders. The purpose of this study was to evaluate the effects of a vegetarian diet, which contained a higher proportion of unsaturated fatty acids on individual morphometric parameters in spayed dogs. Seven mongrel dogs without ideal body condition scores (average: 7.42) were fed vegetarian dog kibbles and received the daily amount of energy calculated with the following formula: 130 kcal × weight (kg) 0.75. The animals were evaluated on days 0 and 60 in relation to the body measurements, such as: body weight, metabolic body weight, body fat percentage, nape, rib, tail base, chest, and abdomen, using a plicometer. Also determined were the body condition scores (scale from 1—thin to 9—obese), canine body mass index, and their waist circumference. These data were analysed by the Student's *t*-test. The body measurement variation of body fat percentage, waist circumference, body condition score and thickness values of the abdomen differed significantly ($P < 0.01$).

Key words: body condition score; body fat; dog; obesity; overweight

INTRODUCTION

Increased demand for vegetarian animal feeds has raised a response from some companies to produce dry or preserved feeds exclusively from plant components. There are several dry and wet dog foods on the market where only plant components have been used. Feeding with commercial feeds offers more advantages than feeding with home-prepared diets, for example, better nutritional balance of feed rations, time-saving preparation, price and comfort associated with it. Despite these benefits, there are owners who prefer home-prepared diets. These dog owners reported that they feel better when they know what components they use to prepare a feed ration and what their origin is [25].

There are studies where the relationships between metabolic parameters and condition are described. On the other hand, there are only a few studies on dogs dealing with the relationship between castration and condition [7]. Oestrogen plays an important role in the regulation of energy and fat metabolism and affects the condition of dogs. According to P a n [24], castration is one of the significant factors influencing obesity and overweight in dogs. It should be added that there are factors that affect the condition of dogs and factors which need to be taken into account when observing the relationship between sex hormones and the condition of individuals (for example, energy expenditure, type of feed ration, digestibility, age, food intake, breed, reproductive status, and so on) [4, 5, 9, 16, 20]. In addition to the benefits, castration also carries an increased risk of certain diseases [1, 26, 29]. Veterinary researches show that castration increases the risk of being overweight or obesity and this risk is higher if individuals are castrated at an early age (less than six months of age) [13, 15]. Bitches after ovariohysterectomy fed *ad libitum* significantly increased their body weight than bitches after ovariohysterectomy, whose feeding dose was controlled by the exact daily amount [12, 18].

The aim of this study was to provide general information about vegetarian or vegan nutrition in dogs, how to achieve adequate intake of nutrients only from plant components, control of nutritional status in spayed bitches fed this way and monitoring of individual morphometric parameters.

MATERIAL AND METHODS

Animals, diets and experimental design

The project was conducted in 2017—2018 on the total number of seven healthy household spayed bitches which were brought to private veterinary clinic for routine consultations, whose age ranged from 3 to 7 years. These individuals were dewormed. All spayed bitches were crossbreds of medium height (10.3—27.3 kg). All the animals were submitted to a physical examination and considered free of underlying pathologies. Body composition was determined with the dogs fasting on days 0 and 60. Body condition score (BCS) of each dog was recorded (nine-point rating) [17]. The canine body mass index (CBMI) was calculated according to M ü l e r et al. [22] using the following equation for the calculation:

$$\text{CBMI} = \text{body weight [kg]}/\text{height [m]}^2 \quad \text{(Fig. 1)}$$

Measurements were taken from the atlanto-occipital joint to the hind leg limit with the animal in a steady position. Body fat percentage (BF %) was calculated according to B u r k h o l d e r, T o l l [2] using the following equation:

$$\begin{aligned} \text{BF \%} &= \text{BF in females [\%]} \\ &= -1.7\,[\text{LRH cm}] + 0.93\,[\text{WC cm}] + 5 \end{aligned}$$

where LRH is the length of the right posterior limb from the tuberosity of the calcaneus to the medium patellar ligament and WC is the waist circumference (from the middle point

Fig. 1. Measurement of the body height [22]

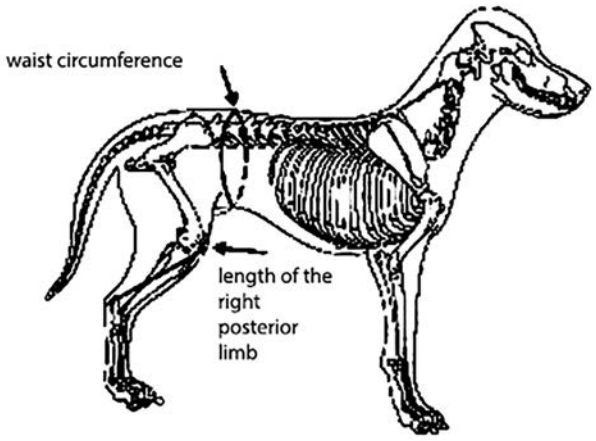

Fig. 2. Anatomic sites for measurement of morphometric variables for dogs [2]

between the iliac crest and the last thoracic vertebra, measured with the dog in standing position) (Fig. 2).

Subcutaneous fat was measured using a mechanical caliper at five locations on the body: in the neck, chest, base of the tail, neck and abdomen in mm [28]. All these morphometric measurements and weighings were performed for each dog on the first and last day of the trial period. Commercial vegetarian feed (Table 1) was administered twice daily according to NRC [23] at a daily dose of 130 kcal × weight [kg] 0.75. The diets were given to the dogs for 60 days at fixed schedule twice a day. The basic ingredients of the feed were: wheat, soybeans, corn, sunflower seeds, wheat semolina, minerals, brewer's yeast. All feed ingredients came from controlled organic farming. Water was administered *ad libitum*.

ETHICAL CONSIDERATIONS

The owner of the dogs gave permission to conduct this study. The relevant animal welfare legislative provisions were met while handling the experimental animals.

Table 1. Chemical composition of the experimental diets (% dry matter)

Item	Composition
Dry matter	92.68%
Crude protein	20.4%
Fat	12.2%
Crude fibre	7.0%
Ash	7.4%
Calcium	1.2 $g.kg^{-1}$
Phosphorus	0.9 $g.kg^{-1}$
Metabolizable energy	3670 $kcal.kg^{-1}$

Statistical analysis

The results were reported as means ± SD (standard deviation). The differences between means were determined according to the paired *t*-test (Graph-Pad Prism program, version 5, USA). By conventional criteria, the differences $P < 0.05$ were considered to be of statistically significance.

RESULTS

In this study, we observed a decrease in the body fat and waist circumference in dogs fed the selected diet ($P < 0.01$). As these values decreased, a decrease in BCS from 7.42 to 6.28 was also noted ($P < 0.01$). No statistical significance was noted between the two measurements at body and metabolic weight ($P > 0.05$) (Table 2). For body condition score, body fat, abdominal circumference and subcutaneous fat in the abdomen, statistical significance was recorded between the two measurements ($P < 0.01$) (Table 2). When measuring subcutaneous fat in the neck, neck, chest and base of the tail, the difference was not statistically significant ($P > 0.05$) (Table 2).

DISCUSSION

The aim of this research was to investigate morphometric markers in spayed dogs fed by vegetarian diet which contained a higher proportion of crude fibre. Fibre is commonly used in commercial dry dog and cat foods due to their several important physiological functions, such as improvement of gastrointestinal health and function, reduction in the nutrient digestibility and energy value of the food and alterations in digesta mean retention time [14, 19, 21]. F r i t s c h et al. [10] pointed out in his study that

Table 2. Average of body measurements on days 0 and 60 observed in dogs fed vegetarian feed

	BW	MW	CBMI	BCS	BF	WC	Nape	Rib	Chest	AB	TB
Day 0	14.80	7.45	14.82	7.42	27.93	51.42	9.00	6.28	6.35	6.85	10.07
Day 60	14.25	7.22	14.34	6.28	26.34	49.71	8.57	5.00	5.00	3.42	8.57
P	0.18	0.17	0.38	0.004	0.003	0.003	0.48	0.17	0.24	0.005	0.28
SEM	0.36	0.14	0.74	0.26	0.33	0.35	0.57	0.83	1.05	0.81	1.27

BW—body weight; MW—metabolic weight; CBMI—canine body mass index; BCS—body condition score; BF—body fat; WC—waist circumference; AB—abdomen; TB—tail base; SEM—standard error of the mean; P—significant differences at $P < 0.05$

the condition of dogs fed with a higher fibre content in the diet, they lose weight more easily than individuals with ideal BCS, because they have a higher % of subcutaneous fat to mobilize and compensate for energy deficiency. The diet contained a higher proportion of fibre, because it was a feed composed exclusively of plant components. The addition of fibre in the diet formulated for dogs can affect the viscosity of food intake, peristalsis of digestion in the digestive tract, the feeling of satiety, digestibility of nutrients, can affect the bacterial population of the colon. A higher proportion of fibre in the feed ration may be useful in the prevention and treatment of diseases such as diabetes and obesity [6]. Fibre can be classified based on the way it reacts with water (soluble or insoluble) or based on the fermentability (high, medium and low) associated with the degradation of the substrate by anaerobic bacteria [8]. Soybeans, as a source of insoluble fibre, can affect faecal consistency, digestion rate in the digestive tract, reduce the incidence of constipation, and reduce nutrient absorption [3].

The BCS is an important tool to determine the nutritional status of dogs in clinical cases [27]. The BCS assessment was performed using the validated 9-point BCS scale, where 1—3 was considered underweight, values between 4-5 ideal, values between 6-7 as overweight and values between 8—9 were evaluated as obesity [17]. The use of techniques such as BCS measurement, subcutaneous fat measurement using a mechanical caliper (in the neck, chest, base of the tail, neck and abdomen) and anthropometric measurements (stem circumference) are the most common evaluation methods for calculating and determining subcutaneous fat [11]. If 15 to 25 % body fat is accepted as optimal for dogs and cats, then an animal with a BCS of 4—5 out of 9 (4—5/9) should have about 20 % body fat. Dogs with body fat more than 25 % are considered as overweight or obese [2]. In our study we recorded average body fat 27.93 % what we can consider as overweight.

CONCLUSIONS

When monitoring morphometric indicators before and after the administration of vegetarian feed in spayed bitches, we observed statistically significant changes in body condition score, waist circumference, % of body fat and subcutaneous fat thickness in the abdomen. We did not record statistically significant changes in the other indicators. These simple measurements are used to objectively assess the nutritional status not only in dogs but also in cats.

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THE ROLE OF LYMPHATIC MARKER *PROX-1* IN RELATION TO BRAIN TUMOURS

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ABSTRACT

The homeobox gene, *Prox-1* is a transcription factor essential for lymphatic development (lymphangiogenesis) during embryogenesis. It also performs different functions in various tissues such as: retina, lens, liver, pancreas and the central nervous system. Intense expression of *Prox-1* has been demonstrated in the developing spinal cord and brain. In adulthood its expression continues in the hippocampus and cerebellum. In adult tissues the process of lymphatic vasculature formation is accompanied under certain pathological conditions such as inflammation, tissue repair and tumour growth. *Prox-1* expression is typical for lymphatic vessels; thus it belongs to one of the most specific and widely used mammalian lymphatic endothelial marker in the detection of lymphangiogenesis and lymphatic vessel invasion in oncogenesis. It has been shown that *Prox-1* is involved in cancer development and progression. It's tumour suppressive and oncogenic properties are proven in several human cancers, including brain tumours. Among all body cancers the brain tumours represent the most feared tumours with very limited treatment options and a poor diagnosis. The aim of this paper was to show the current knowledge of the gene *Prox-1* with an emphasis on brain tumours, especially in gliomas.

Key words: brain lymphatic system; brain tumours; gliomas; lymphatic markers; *Prox-1*.

INTRODUCTION

The brain represents a significant part of the central nervous system (CNS), and since it controls all of the body functions, it is considered as an exceptional organ of the body [5]. Despite its small size, it is characterized by high metabolic activity and surprisingly consumes 25 % of the total body's energy. Thus it produces metabolic waste at a higher rate than any other organ. For this reason, the brain drain is a critical factor for its proper functioning. The transport is necessary for the delivery of nutrients and other materials needed for cell repair and renewal. The transport of molecules is an integral part of cerebral waste

clearance, which is an essential link in many physiological and pathological processes of the brain. The transport is necessary for the delivery of nutrients and other materials needed for cell repair and renewal. Furthermore, the fluid flow in the brain has great impact in delivery of therapeutics and the movement of biomarkers to the peripheral body for detection [29].

In peripheral organs, lymphatic vessels are those, which eliminate waste products from the tissues [27]. The brain parenchyma does not contain lymphatics, however it has its own lymphatic drainage system. This unique system of lymphatic drainage has been developing over the last 50 years. It includes communication between brain parenchyma, extracellular space, perivascular spaces, perineural space, subarachnoid space, meningeal lymphatic vessels (MVL) and cervical lymph nodes (CLNs). The existence of lymphatic drainage routes presents an effective tool for cleaning the brain of harmful substances. Dysfunction of the brain lymphatic system has a crucial impact on brain function and the pathogenesis of neurovascular, neurodegenerative, neuroinflammatory diseases, as well as brain injury and tumours [33].

The aim of this paper was to show the current knowledge of the gene *Prox-1* with an emphasis on brain tumours, especially in gliomas.

Prox-1

The homeobox gene, *Prox-1*, was cloned in humans by homology with the Drosophila gene Prospero and mouse *Prox-1* [36, 40]. In vertebrates, *Prox-1* expression is fundamental for organ development during embryogenesis. It plays an essential role in a variety of tissues, including: the lens, cochlea, heart, liver, pancreas, and skeletal muscle. Intense expression of *Prox-1* has been reported in developing spinal cord and brain [26, 31]. During the CNS development, *Prox-1* regulates progenitor cell differentiation and starting neurogenesis, and is found in some regions of the brain like in the cerebral cortex, cerebellar cortex, in lateral ventricle wall and dentate gyrus as a part of hippocampal formation [7]. In adulthood the expression of *Prox-1* continues in hippocampus and cerebellum. Wherein, the hippocampus represents a significant amount of adult neurogenesis and exhibit one of the radical pathways in this process [19, 35].

Moreover, *Prox-1* is necessary for the induction and development of the lymphatic system by regulating the proliferation of lymphatic endothelial cells [17]. Its overexpression in blood endothelial cells will transform these cells to lymphatic endothelial cells and is identified as a master switch in the program specifying lymphatic endothelial cell fate [12]. Therefore, *Prox-1* belongs to reliable and widely used mammalian lymphatic endothelial marker in the detection of lymphangiogenesis and lymphatic vessel invasion in oncogenesis.

The molecules which were recently established, such as vascular endothelial growth factor receptor 3 (*VEGFR-3*), lymphatic vessel endothelial hyaluronic acid receptor 1 (*LYVE-1*), prospero-related homeobox-1 (*Prox-1*) and podoplanin (*PDPN*) [37] have facilitated key scientific advances and brought novel data into the molecular mechanisms that control lymphatic development and function. The following breakthrough findings include the identification of specific genetic defects in certain hereditary diseases that are associated with lymphatic hypoplasia and dysfunction, and evidence that malignant tumours can directly activate lymphangiogenesis and lymphatic metastasis [16, 32]. These four specific markers of lymphatic endothelial cells (LECs) are important for the detection of lymphangiogenesis and lymphatic vessel invasion in variety of cancer types and provided modern insights into the biology of malignant tumours [17].

Lymphangiogenesis is understood as a formation where the new lymphatic vessels are form from pre-existing lymphatic vessels [9]. It is a dynamic process during embryogenesis but is largely absent under normal physiological postnatal conditions [14]. In adult tissues is associated with some pathological conditions such as inflammation, tissue repair and tumour growth [38]. Generally, it has been found that the solid tumours frequently induce angiogenesis and lymphogenesis [28].

It has been demonstrated that *Prox-1* is involved in cancer development and progression and has both oncogenic and tumour-suppressive functions. The function of *Prox-1* in tumour metastatic growth is caused by its impact on migration and invasion of cancer cells. *Prox-1*'s contribution to cancer cell metastasis presumably resides on its role as a main regulator of the lymphatic system [7]. Tumour progression involves the phase of tumour initiation and promotion, and is characterized by increased proliferation and/or invasiveness of tumour cells [30]. Decreased expression of *Prox-1* has been found in some tumours, signifying that it has a possible role as a tumour suppressor

gene [39]. Accordingly, high expression of *Prox-1* mRNA was found in: neuroblastoma, glioma, small cell lung carcinoma, colon cancer, small intestine adenocarcinoma, liver carcinoma and rhabdomyosarcoma [7]. The expression of other lymphangiogenesis markers was detected by J i a n g et al. in primary and recurrent glioma tumours [22]. Consequently, its functions in different types of tumours depends on cell type [39].

Prox-1 is differentially expressed in human gliomas of different malignancy grades. A positive correlation was demonstrated between *Prox-1* protein expression and a higher degree of glioma malignancy [7]. Particularly, *Prox-1* is abundantly expressed in high-grade malignant astrocytic gliomas, what is related with its effects on cell growth, tumourigenesis and invasiveness [39]. Raising levels of *Prox-1* protein in malignant gliomas may reveal the presence of a subset of cancer cells with the phenotype of early neural progenitors. Even though intense expression of *Prox-1* is in high-grade gliomas, on the other hand it is also up-regulated in a subset of diffuse low-grade gliomas with worse prognosis and probably to progress more rapidly to high-grade tumours. It has been shown that low-grade gliomas with a high proportion of *Prox-1* immunoreactive cells represent a more advanced, less differentiated phenotype than their counterparts with low *Prox-1* expression. These low-grade gliomas seem to represent tumours that are further advanced on the evolution to anaplastic gliomas [7].

According to available study, where they did immunohistochemical staining of *Prox-1* in cell of astrocytomas, they investigated that *Prox-1* expression in this type of gliomas reveals a stepwise increase correlating with tumour grade. *Prox-1* expression was limited to the cell nuclei and was the most evident in areas of tumour cell crowding. The researchers found out in peritumoural normal-appearing tissue, which was close to the high-grade tumour areas much more *Prox-1* expressing cells than in non-neoplastic control samples. The *Prox-1* cells were randomly scattered in peritumoural tissue with no preference for perivascular areas. It was also proven that overexpression of *Prox-1* significantly increased the proliferation and colony formation of glioblastoma cells. For high-grade malignant glioma is typical tumour induced angiogenesis [6].

Even if, *Prox-1* was highly distributed in endothelial cells of blood vessels and capillaries within glioma tissues, the glioma vasculature itself does not express *Prox-1* protein [22]. Tumour vessels in malignant gliomas have a lymphatic-like phenotype. During lymphangiogenesis *Prox-1* mediated podoplanin and *VEGFR-3* expression that elevated levels of *Prox-1* in aggressive gliomas which are responsible for the apparent illegitimate induction of target genes involved in the process of lymphatic vessel invasion [7]. Vascular endothelial growth factor C (*VEGF-C*) and vascular endothelial growth factor D (*VEGF-D*), which are ligands for *VEGFR-3*, may promote tumour lymphangiogenesis and lymphatic metastasis. In addition, *VEGF-C* induces protection againts glioblastoma and requires lymph drainage to the deep cervical lymph nodes [17].

Definition of brain tumours

Brain tumours are kind of intracranial neoplasm and represent a heterogeneous group of tumours whose common feature is the formation of abnormal tissue mass. The cells in this mass grow and multiply uncontrollably, seemingly unchecked by the mechanisms that control normal cells [18]. More than 150 different brain tumours have been documented. The world Health Organization (WHO) classified the brain tumours into two main groups: primary and metastatic. Primary are those that originate from the tissues of the brain, they are categorized as glial or non-glial, benign and malignant. Glial tumours called gliomas are malignant brain tumours. They arise from the cells of glia (supporting cells of the brain) and include: astrocytomas, ependymomas, glioblastomas, medulloblastomas and oligodendrogliomas. Astrocytomas are distinguished into 4 grades that are correlated with clinical outcome: Grade I pilocytic astrocytoma, Grade II astrocytoma (low-grade diffuse), Grade III anaplastic astrocytoma and Grade IV glioblastoma. Glioblastoma (GBM) has the highest incidence rate, and there is a considerable heterogeneity among the different types of glioma [22]. They diffusely infiltrate the brain parenchyma and are badly differentiated tumours with proven cellular polymorphism, high proliferative activity, necrosis and neovascularization [7]. Metastatic brain tumours arise in other organs of the body and spread to the brain usually through bloodstream or lymph system. They are considered as malignant. Both groups of brain tumours are among the most feared of all forms of cancer with very limited treatment options and a poor prognosis. It has been known that brain tumours can invoke massive antitumour immune responses. The most prevalent type of adult primary brain tumours are gliomas,

which account for 80 % of the malignant brain tumours [13]. They occur in the cerebral cortex with the highest percentage developing in the frontal lobe (26 %). The other brain tumours locations are in the temporal lobe (19 %), parietal lobe (12 %), cerebellum (5 %), brainstem (4 %) and the occipital lobe (3 %) [11]. The survival time is different between patients with glioma, depending on grades of glioma, and the prognosis is worst for patients with GBM. In addition, GBM has a high probability of recurrence even in the case successful initial surgical resection [22].

Although the brain tumours comprise only 2 % of all cancers of the body, they represent a significant source of morbidity with a high mortality rate worldwide [11]. In past decades, the incidence of brain tumours has been increasing. Generally, the lowest incidence is in Africa, and the highest is in northern Europe. It is reported, that in 2016 at the global level, there were 330 000 incident cases of CNS cancer, and 227 000 deaths [8].

Nevertheless, the exact causes of brain tumours are still unclear, one of the causes that leads to their occurrence is the dysfunction and impairment of the brain lymphatic system [33].

The brain lymphatic drainage

The lymphatic system plays a crucial role in waste drainage pathway of the body and is the essential component of the immune system [25]. It is considered as a body's sewer system and first line of defence against disease [1]. It's discovery was preceded by a long and fascinating history. Although literary sources date its investigation to the 17th century, when the lymphatic circulation was correctly described by Olof Rudbeck, the first insights were contributed by the Hippocratus School [15]. The ancient anatomist (Hippocrates, Aristoteles) reported the presence of lymphatic vessels and lymph nodes without any accurate knowledge of their true functions [34].

The lymphatic system develops parallel with the blood vascular system, but unlike the cardiovascular system, the lymphatic system is not a closed system. In the body, a network of lymphatic vessels starts as blind-ended capillaries that drain excess fluid and protein from the interstitial fluid surrounding tissues and most organs. The interstitial fluid containing molecular waste is delivered from lymphatic vessels to lymph nodes for filtration and then to the venous circulation where it is carried to the liver and kidneys for further processing [3]. Moreover, the key function of lymphatics is to maintain fluid homeostasis, lipid absorption and perform the important role in the proper functioning of the immune system and the immune surveillance of the whole body [2, 28]. The lymphatic vessels are not normally present in avascular structures such as epidermis, hair, nails, cartilage and cornea [10].

The researchers always wondered how the brain effectively removes its waste, mainly because of its high metabolic rate. The questions about the existence of lymphatic vessels in CNS has been studied for many decades, but the specific markers for lymphatic endothelial cells (LECs) were discovered about a decade ago [24]. The brain was very long time considered as organ lacking lymph vessels. Even if, the first evidence of lymphatics in the brain meninges was published in 1787 by Paolo Mascagni an Italian physician, but his claim was discredited and forgotten [23]. New findings utilizing cellular, molecular and neuroimaging techniques show that functional lymphatic drainage does exist in the brain and its failure is a central feature of neurodegenerative disease and post traumatic brain injury [33]. The brain lymphatic drainage assists in maintaining the water and ion balance of the interstitial fluid (ISF), removing the metabolites, reabsorption of macromolecular solutes and modulate immune surveillance and responses of the brain [20]. Insufficient and reduced clearance from the brain could cause the accumulation of protein aggregates into insoluble clumps, which is a common feature in patients with amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases [29]. Contemporary scientists indicate that some of the brain's waste products enter the cerebrospinal fluid, before being disposed of via the bloodstream [1]. The first who discovered how the CSF and brain tissue fluids are exchanged and named the lymphatic pathways, was N e d e r g a a r d et al. in 2012 [22]. CSF circulates around the brain and spinal cord, fills the brain ventricles and subarachnoid spaces and are mainly produced by choroid plexus; a collection of special endothelial cells that filter blood plasma to create the CSF. The composition of the CSF can be modulated by brain parenchymal cells. It is composed of about 99 % water with several dissolved ions, and has a much lower protein concentration than plasma [23]. The content of protein in CSF increases with aging and it was proven that is higher in patients with aging-associated dementia. The CSF is produced daily, its volume is approximately 500 ml, but only 100—150 ml fills the fluid spaces of the

brain and spine at any time, ensuring constant circulation and reabsorption [29]. The total volume of CSF is renewed about 4 times a day in healthy humans. CSF flow within the ventricles is pulsatile, being influenced by cardiac and respiratory pulsation.

The traditional concept of CSF homeostasis has been challenged by the latest reports when the existence of a network of true lymphatic vessels in the brain meninges was after more than 200 years reconsidered [23]. The meningeal lymphatic vessels (MLV) found within the mouse dura mater were rediscovered in 2015 by two independent groups using novel, specific lymphatic endothelial markers [24]. They declared that the MLV which are located both dorsally and basally beneath the skull have a direct access to the CSF and, under healthy conditions continuously drain both soluble molecules and immune cells from the subarachnoid space into the cervical lymph nodes (CLNs) [4]. The dorsal MLV undergo extensive remodelling in mice with intracranial gliomas or metastatic melanomas. Disruption of the dorsal MLV alone impaired intratumour fluid drainage and the dissemination of brain tumour cells to deep cervical lymph nodes. In contrast, the last study point that the MLV situated at the basal part of the skull appeared to be morphologically distinct and have stronger draining functions than dorsal MLV.

Like conventional LECs, MLV express all of the classic markers of LECs like as *CD31*, *VEGFR3*, *Prox-1*, *PDPN*, *LYVE-1* and *CCL21* [13]. On the other hand, the meningeal lymphatics in comparison with peripheral lymphatic vessels have a less ramified network of thin-walled initial lymphatic vessels that converge and exit the cranium along particular anatomical structures, such as along the retroglenoid vein and sigmoid sinus and along the meningeal portions of the pterygopalatine artery [23]. Overall, these observations show that the lymphatic vessels of the meninges are important players in the draining lymphatic route for the clearance of molecules from the brain. [13]. The most recent study of lymphatic elements in the human brain brought new knowledge, that the fine lymphatic structure was presented in membranes covering the brain, walls of vessels, perivascular spaces and among nerve fibres. Lymphatics in and around the cranial and spinal nerves are important in transporting neuronal end products out of the brain [24].

New era brought novel and extended discoveries in the field of lymphatic system in the brain, when many detailed and intensive research was realized, using modern techniques. The presence of unique brain lymphatic drainage pathways have been confirmed by using the different types of tracer dyes of Indian ink, Evans blues, radioactive protein tracers and various fluorescent tracers with different molecular structure [33]. Finally, according to all available studies the cleaning system of the brain can be divided into two compartments. The first one, described as glymphatic system is draining the interstitial space of the brain tissue and between ISF and CSF. The second compartment is responsible for cleaning CSF towards to extracranial lymphatic structures [21]. These functions of the brain lymphatic drainage system are regulated by aging, genetic phenotypes, sleep-wake cycle, cardiac pulsations, respiratory and body posture and the effectiveness of such cerebral removal is also influenced by different pathological conditions or diseases [33].

CONCLUSIONS

The homeobox gene *Prox-1* is essential in developmental processes such as the progenitor cell regulation in various mammalian organs. Apart from its role during embryogenesis, *Prox-1* expression is associated with a number of human cancers. It performs tumour suppressive or an oncogenic role in a variety of cancer types. *Prox-1* is involved in the different stages of tumour development. Moreover, it participates in metastatic tumour growth by its impact on migration and intervention of cancer cells.

Regarding the brain tumours, it has been demonstrated, that *Prox-1* was found in the tumour cells in all diffuse astrocytic tumours, and its expression increases with the tumour grade. For that reason, *Prox-1* can be used as a diagnostic marker to distinguish low-grade from high-grade gliomas. Its oncogenic role in GBM promotes cell proliferation and invasiveness. Therefore, *Prox-1* may represent a potential target for the treatment of GBM. Also it has been an independent prognostic factor for survival in patients with World Health Organization grade II gliomas.

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