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Contact: tel.: +421 915 984 669
e-mail: folia.veterinaria@uvlf.sk

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IMMUNOHISTOCHEMICAL LOCALIZATION OF ELASTIC SYSTEM FIBRES IN THE CANINE PROSTATE

Marettová, E.

Department of Anatomy, Histology and Physiology
University of Veterinary Medicineand Pharmacy, Komenského 73, 041 81 Košice
Slovakia

elena.marettova@uvlf.sk

ABSTRACT

The elastic fibres are particularly important for the structural integrity and function of the prostate. In this study, the elastic fibres of the normal dog prostate gland were identified by immunohistochemistry. In the capsule, the elastic fibres form membranes of different thicknesses-located mainly in the intermediate and deep zones. Large trabeculae which extend from the capsule contain elastic fibres with a prevalence in the longitudinal direction. Around blood vessels, the elastic fibres are concentrated and form annular structures. In the fine septa supporting the lobules,elastic fibres form a fine elastic meshwork. Between the secretory units of the prostate gland,the fine elastic fibres are located under the secretory epithelium. An increase of elastic fibres around the ducts entering the urethra was observed. An accumulation of elastic fibres in the capsule and stromal septa may participate in the releasing of secretory products during ejaculation.

Keywords: dog; elastic fibres; immunohistochemistry; prostate

INTRODUCTION

The presence of elastic tissue as a normal component of the stromal tissue has been reported ingenito-urinary organs. Most studies have been related to the urinary organs. In the female canine urethra,the elastic fibres system was described by Augsburger [1] and in the vesicourethral junction and urethra of the guinea pig by Dass et al. [2]. Murakumo et al. [11] in their scanning electron microscopic study,observed three dimensional arrangement of collagen and elastin fibres in the human urinary bladder. Elastin was also found in association with the human male membranous urethral sphincter [7].

Prostatic disorders are accompanied by extensive but poorly understood modifications of the cells and surrounding extracellular matrix. In the human prostate, the distribution of the elastic system fibres in prostatic disorders have been reported [15] and a positive correlation between the tumour progression and the presence of elastic fibres in the tumour stroma was found [8]. The elastin as a stromal component was increased in benign prostatic hyperplasia patients [12]. The elastic components in these cases were of

variable thicknesses and formed a three-dimensional network at the base of the epithelium. These results indicate, that prostate cancer cell invasion involves extensive remodelling of the fibres of the elastic system.

Studies on the occurrence of elastic fibres in the accessory glands in animals are scarce. By immunohistochemical methods elastic fibres were localized in the bovine vesicular gland [9].The presence of elastic fibres in the prostate is generally accepted, but their distribution is not well known. Dimitrov [4] described the elastic fibres in the prostate gland of the cat and Delella and Felisbino [3] in the rat. Little is known about the presence and arrangement of the elastic fibrillary components of the stroma of the normal prostate in the dog. Therefore, the aim of this work was to study the distribution of elastic fibres in the supporting tissue of the dog prostate by immunohistochemical means.

MATERIALS AND METHODS

Tissue samples of the prostate glands from five clinically healthy adult dogs (German Shepherd, age 3 to 4 years) have been used in this study. Samples of the tissue were fixed in 10% neutral formalin in 0.2 mol phosphate buffer for 24 h, then washed, dehydrated, cleared in xylene, and thereafter routinely embedded in paraffin for histological and immunohistochemical studies.

Immunohistochemistry

Sections 5 mm thick were collected on silanized glass slides, dewaxed with xylene, and rehydrated in a descending ethanol series. Immunohistochemistry was performed by the streptavidin-biotin peroxidase method. Endogenous peroxidase activity was blocked by treating the tissue sections with 3% hydrogen peroxide in methanol for 30 min. Nonspecific protein–protein interactions were blocked by incubating the sections with 3% Bovine Serum Albumin (BSA) (Sigma) in phosphate-balanced salt solution (PBS) containing 0.1% Tween 20 for 1 hour. Monoclonal antibodies against elastin (Sigma, 1:5000) were used. The primary antibodies were diluted in 1% BSA in PBS, and incubated overnight at 4 °C. The sections were then washed and incubated with a secondary peroxidase-conjugated antibody. After washing with PBS, the peroxidase activity was visualized with 0.05% 3´.3´-diaminobenzidine and 0.03% v/v H_2O_2. The slides were counterstained with Mayer's haematoxylin, air dried,and mounted in Entellan (Merck, Darmstadt, Germany). Negative controls were performed by omitting the primary antibodies.

RESULTS

Histologically, the prostate gland is a tubuloalveolar gland and consists of two portions: the external (compact) and internal (disseminate). The capsule and stromal septa of the prostate consists of fibrous connective tissue in which smooth muscle cells, fibroblasts, collagen and elastic fibres have been identified.

Immunohistochemically, in the capsule elastic tissue reactive to elastin antibody occupy the intermediate and inner zones and consists of elastic membranes and less of fibres (Fig. 1).In the large septa arising from the capsule and those between the lobules the elastic fibres form a dense elastic meshwork (Fig. 2) with a prevalence of the fibres in the longitudinal direction. Elastic fibres in different amounts are in the interlobular septa and in increased number around the blood vessels (Fig. 3). Between the secretory units of the prostate gland the elastic fibres were seen to form an elastic meshwork, whereas between the secretory alveoli elastic fibres they are arranged longitudinally (Fig. 4). In the wall of stromal arteries, the elastic fibres were barely present. A concentration of elastic fibres was found around the ducts and in the area of the urethra (Fig. 5).

DISCUSSION

The stroma of the prostate gland is dynamic tissue which directly influences the behaviour of the epithelium and is activated in response to injuries [6, 13, 14]. Previous studies have suggested that elastic tissue is a normal component in the genito-urinary region [1, 11]. Our results showed that the capsule of the dog prostate contain a large number of elastic membranes of different thicknesses forming one thick layer and partially integrated in the muscle layer of the capsule. In the stroma of the canine prostate, like in the cats [4], the elastic fibres were observed with almost equal density in the different parts of the glandular interstices. The elastic fibres and membranes were observed mainly in large interlobular septa, where they were intermingled with

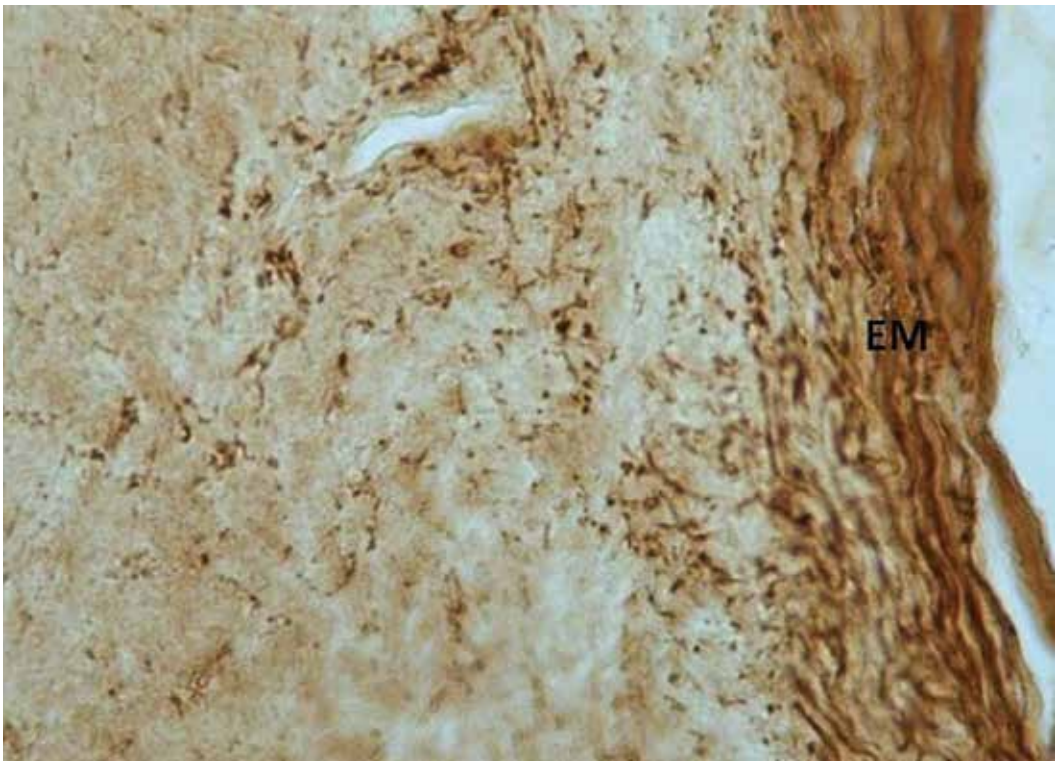

Fig. 1. Capsule and underlying tissue of the prostate. The capsule revealed elastic thick membranes (EM). Magn. × 450

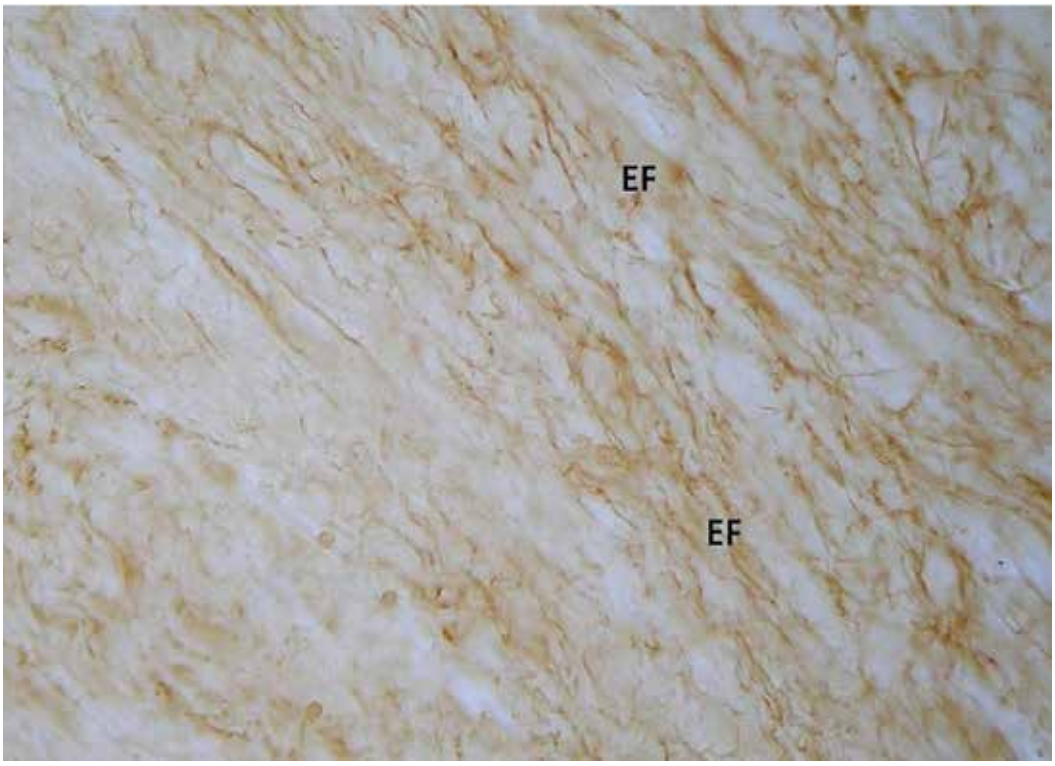

Fig. 2. Section of the large trabeculae. Elastic fibres (EF) form a dense meshwork with a prevalence in the longitudinal direction. Magn. × 450

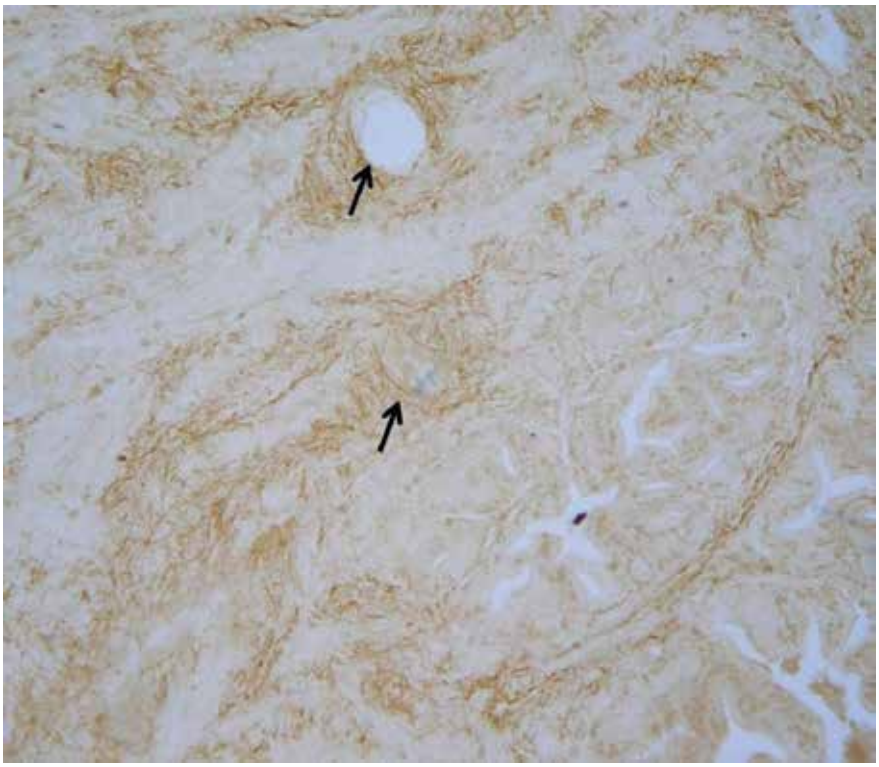

Fig. 3. Section of prostatic lobule. Elastic fibres in different amount are increased around blood vessels (arrows). Magn. × 200

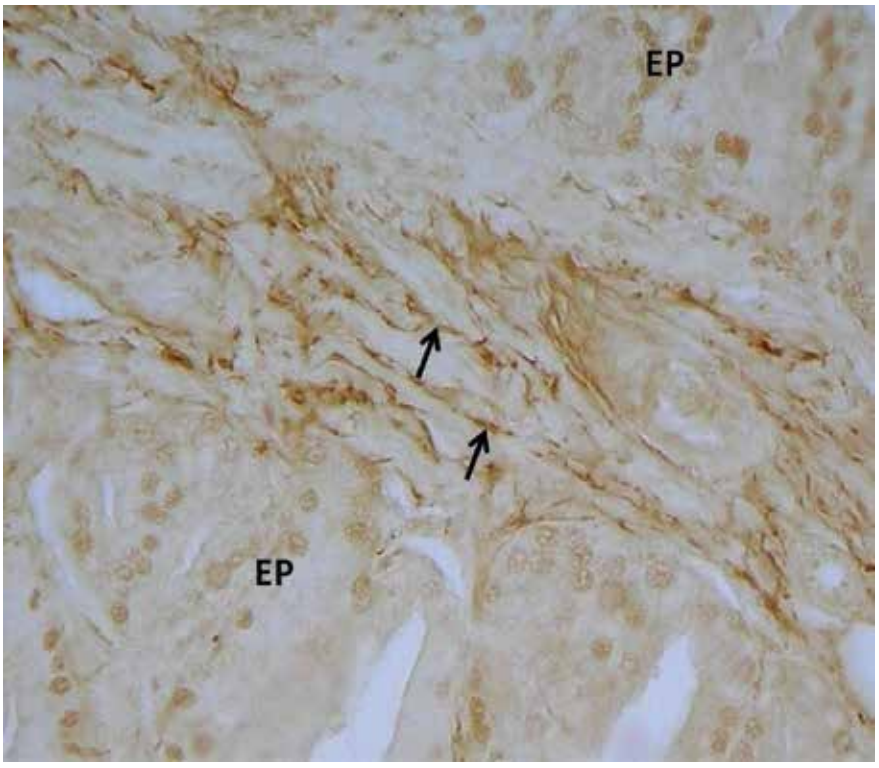

Fig. 4. Section of prostatic lobule. An accumulation of elastic fibres and membranes (arrows) among the secretory alveoli with secretory epithelium (EP). Magn. × 1000

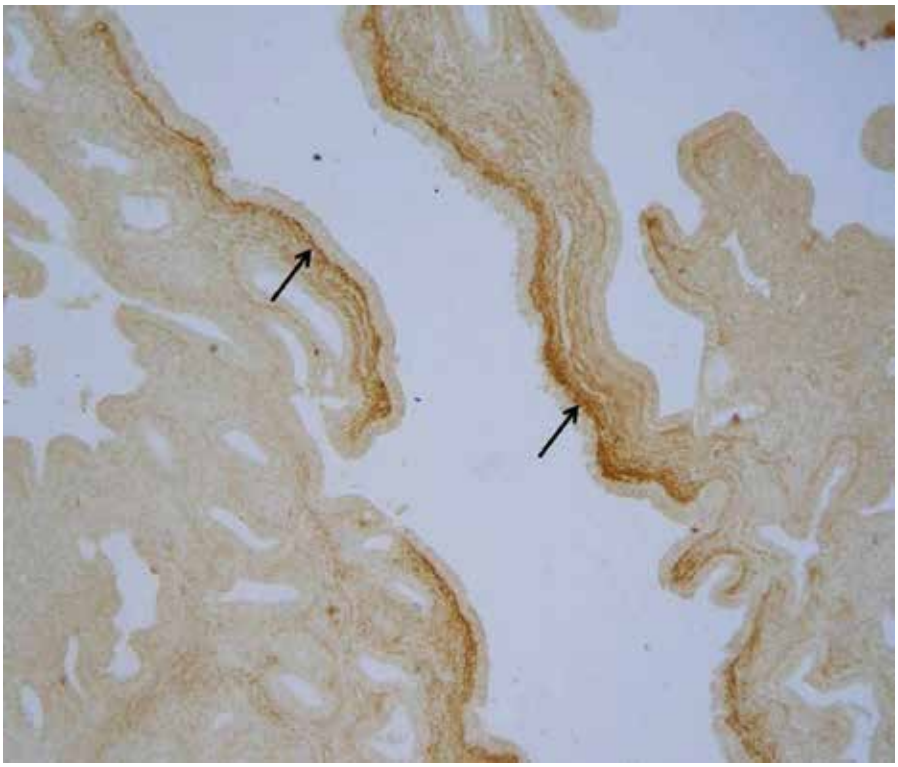

Fig. 5. Prostatic duct opening into prostatic portion of the urethra.
An accumulation of elastic fibres below the lining epithelium (arrows). Magn. × 200

smooth muscle cells. Among the secretory alveoli, elastic fibres are involved in a concentric fibrous extracellular matrix surrounding the normal acini. The stroma undergirds the acinar epithelium through its fibromuscular substance [5]. In the rat prostate, the elastic fibres in this area were thin and inconspicuous as was described [3].

An accumulation of elastic fibres was observed in the connective tissue in the area of the urethra. A similar increase of elastic fibres was reported in the vesicourethral junction and urethra of the guinea pig [2] and also in the cat [4]. An abundance of elastic fibres amongst the smooth muscle bundles associated with the human male membranous urethral sphincter has been reported by Ho et al. [7]. The fibres were seen to be associated with the longitudinal smooth muscle bundles of the urethra and urinary bladder of the guinea pig [10, 11]. Dass et al. [2] considered the elastic fibres associated with the smooth muscle bundles of the urethra suitable for both uniting the contractile force of the smooth muscle bundles to shorten the urethra, as well as augmenting passive recoil of the longitudinal smooth muscle bundles, hence restoring the urethral in length.

The number and organization of elastic tissue change in the case of benign prostatic hyperplasia where the larger proportion of elastin in the area of the urethra and at the base of prostate was noted [12]. In benign prostatic hyperplasia the elastic components were of variable thickness and formed a three-dimensional network at the base of the epithelium [15]. An increased variability in the elastic fibre distribution was observed in adenocarcinomas, depending on the tumour grade. In adenocarcinomas with little differentiation, in some hyperplasic acini, and in the stroma adjacent to tumour masses, ruptured and residual elastic fibres indicative of matrix degradation or remodelling were seen. In more undifferentiated tumours, a pre-elastic network, perhaps indicative of a new extracellular matrix microenvironment was seen [15]. These results indicate that prostate cancer cell invasion involves extensive remodelling of the fibres of the elastic system. According to Delella and Felisbino [3], the deposition of collagen and elastic system fibres in the prostatic stroma may counterbalance the absence of smooth muscle tone during α-blockers treatment.

Although it is supposed that elastic fibres in the prostatic connective tissue are predominantly mechanical, they may also have a specific function. The elastic fibres may play a role in the resistance of these components in the prostate during secretory activity and in restoration after expulsion of prostate secretion following ejaculation. An accumulation of elastic fibres present around the external layer of blood vessels may be involved in this action. According to Weinberg et al. [16], the ability of elastin-rich structures to deform and subsequently recover depends on the interactions of elastin with other fibrillary components and with proteoglycans and non-collagenous glycoproteins.

CONCLUSIONS

The results of this study revealed that the capsule and stroma of the dog prostate was rich in elastic tissue. The capsule of the dog prostate contained a large number of elastic membranes of different thicknesses forming one thick layer and partially integrated in the muscle layer of the capsule. In the stroma of the canine prostate, the elastic fibres were observed with almost equal density in the different parts of the glandular interstices. Among the secretory acini, elastic fibres were involved in concentric fibrous extracellular matrix.

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BREED-SPECIFIC HAEMATOLOGIC REFERENCE VALUES IN ADULT TURKEYS (*Meleagris gallopavo*) IN THE HUMID TROPICS OF NIGERIA

Daniel-Igwe, G.[1], Okwara, N.[2]

[1]Department of Veterinary Pathology
[2]Department of Veterinary Medicine
College of Veterinary Medicine, Michael Okpara University of Agriculture
Umudike, Abia State
Nigeria

gloria.danieligwe@gmail.com

ABSTRACT

One hundred (50 males and 50 females) B-not strain indigenous turkeys, *Meleagris gallopavo*, were used to determine the reference values for their haematological parameters. The turkeys were housed in the poultry unit and jugular venepunctures were used to collect their blood. The haematological parameters were determined using standard procedures. The mean values of: the packed cell volume (PCV; 37.29 ± 0.37 %), red blood cell (RBC) counts ($2.50 \pm 0.44 \times 10^6$.$\mu l^{-1}$), haemoglobin concentration (Hbc; 10.89 ± 0.34 g.dl^{-1}), mean corpuscular volume (MCV; 150.63 ± 0.73 fl), mean corpuscular haemoglobin (MCH; 44.29 ± 1.78 pg), mean corpuscular haemoglobin concentration (MCHC; 29.10 ± 0.73 g.dl^{-1}), and white blood cell (WBC) counts ($12.41 \pm 0.83 \times 10^3$ μl^{-1}) were determined. No significant differences were found between the male and female B-not strain turkeys in this study. The results will help in the interpretation of cases of disease when there are variations in the values and serve as baseline data for B-not strain of turkeys in the humid tropics.

Key words: breed-specific; haematology; indigenous; Nigeria; turkey

INTRODUCTION

The turkey is an important species for the poultry industry in Nigeria, which is usually raised for economic benefits [2, 10]. Haematological values are an important tool in monitoring the health of poultry species [1]. These values have also been shown to be markers of ongoing events in the body [23, 25]. It can be used as a diagnostic tool to assess the health status of an individual and/or a flock [5]. Haematological parameters also provide valuable information on the immune status of animals [14]. Reference values for the erythrocytic indices are vital for the diagnosis of anaemia and polycythaemia and also for the assessment of the efficacy of therapy instituted to correct these abnormalities [9]. Establishing an accurate set of reference values is critical in the interpretations of the results of clinical pathology [15, 22].

This study was carried out to determine the normal haematological parameters of indigenous B-not strain of

turkeys raised in the humid tropics under intensive management system. The result of this study would help to improve diagnostic evaluation of sick turkeys and serve as baseline information for the interpretation of laboratory data in research.

MATERIALS AND METHODS

Animals and sampling

The College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike ethical committee approved this study. A total of 100 adult B-not strains of apparently healthy indigenous turkeys (50 males and 50 females) were used for this study. They were housed in the poultry unit of Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. They were fed a commercially prepared growers mash by Top feeds Nigeria Limited®. Feed and water were provided *ad libitum* to the turkeys. The turkeys were vaccinated against known infectious diseases in the area and preventive medications were given to them as endorsed by the National Veterinary Research Institute (NVRI), Vom, Nigeria, and they were cared for in accordance with the principles of humane laboratory animal care throughout the study.

Blood samples (1 ml) were collected from the right jugular vein into sample bottles containing potassium ethylenediamine tetra-acetic acid (EDTA). The blood samples were collected in the morning and analyzed immediately.

Methods of blood analysis

The packed cell volume (PCV) was determined by the microhaematocrit method [24] using a microhaematocrit centrifuge and reader (Hasksley, England); the haemoglobin concentration (Hbc) was determined by the cyanomethaemoglobin concentration method [11, 13] using digital colorimeter (Lab Tech, India); the red blood cell count (RBC) and white blood cell count (WBC) were carried out following the haemocytometer method [9, 24] using an improved Neubaur counting chamber (Marienfeld, Germany). The mean corpuscular values were calculated using a standard formula [9, 24].

Statistical analyses

The results were presented as the mean ± standard error of the mean (SEM) with minimum and maximum values. The results were subjected to descriptive statistics and the differences between the sexes (males and females) were analyzed using the Student's *t*-test. All statistics were carried out using the SPSS 15 Statistical Package (SPSS 15.0 for windows, SPSS Inc., Chicago, IL, USA). Significant differences were accepted at $P < 0.05$.

RESULTS

The overall means and ranges for the PCVs, Hbc, RBC counts and basic indices (MCV, MCH, MCHC) and WBC counts of the turkeys are shown in Table 1. There were no significant differences ($P > 0.05$) between the male and female turkeys used for this study (Table 2).

DISCUSSION

Haematologic reference ranges were established in this study for live clinically healthy B-not strain of indigenous turkeys. The PCV, Hbc, RBC values obtained in the present study were within the range as reported for other domestic

TABLE 1. The haematological variables of apparently healthy B-not strain turkeys (*Meleagris gallopavo*)

Parameter	Mean ± SEM [n = 100]	Range
PCV [%]	37.29 ± 0.37	35.00—39.00
Hbc [g.dl^{-1}]	10.89 ± 0.34	8.60—12.17
RBC count [10^6.µl^{-1}]	2.50 ± 0.44	2.28—2.81
MCV [fl]	150.63 ± 2.78	133.08—163.79
MCH [pg]	44.29 ± 1.78	34.34—51.77
MCHC [g.dl^{-1}]	29.10 ± 0.73	23.89—31.61
WBC count [10^3.µl^{-1}]	12.41 ± 0.83	7.70—17.40

PCV — packed cell volume; Hbc — haemoglobin concentration; RBC — red blood cell; MCV — mean corpuscular volume; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration; WBC — white blood cell; SEM — standard error of the mean

Table 2. Sex differences in the haematological variables of apparently healthy B-not strain turkeys (*Meleagris gallopavo*)

Haematological parameter	Males		Females	
	Mean ± SEM (n = 50)	Range	Mean ± SEM (n = 50)	Range
PCV [%]	37.43 ± 0.65	35.00—39.00	37.29 ± 0.42	36.00—38.50
Hbc [g.dl^{-1}]	11.20 ± 0.56	9.03—12.17	10.57 ± 0.40	8.60—11.98
RBC count [10^6.µl^{-1}]	2.53 ± 0.55	2.32—2.63	2.46 ± 0.71	2.28—2.81
MCV [fl]	149.35 ± 4.76	133.08—163.79	151.91 ± 3.21	137.01—161.70
MCH [pg]	45.42 ± 2.97	34.34—51.77	43.15 ± 2.11	37.26—50.98
MCHC [g.dl^{-1}]	29.84 ± 1.04	25.80—31.61	28.36 ± 1.03	23.89—31.53
WBC count [10^3.µl^{-1}]	13.91 ± 0.95	10.30—16.05	10 91 ± 1.16	7.70—17.40

PCV — packed cell volume; Hbc — haemoglobin concentration; RBC — red blood cell; MCV — mean corpuscular volume; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration; WBC — white blood cell; SEM — standard error of the mean

[3, 4, 12, 16], foreign [3, 12] and crossbred [12] turkeys. In contrast to earlier findings [3, 16], the total WBC count of the current study was higher which agreed with the findings of Bounous et al. [7] and Azeez et al. [6] who also observed higher value for the WBC counts. Isidahomen et al. [12] also observed higher WBC count in local turkeys. Other avian species had been reported to have higher WBC counts as observed in the Nigerian ducks [19] and chestnut-breasted mannikins [17].

In this study, the PCV and Hbc values were slightly but not significantly higher in males than in females. The observed slight decrease in PCV and Hbc values as well as RBC counts in females could be attributed to the presence of oestrogens in female turkeys [21]. Oestrogens have been reported to decrease Hbc and RBC counts by inhibiting erythropoiesis [8, 18, 21], thus the males have higher values of RBC [3, 20, 21]. The erythrocytic indices of MCV, MCH and MCHC showed no significant sex variations in this study. However, the MCV of the current study do not support the previous reports of Azeez et al. [6] who reported higher MCV value in males.

CONCLUSIONS

This study has demonstrated that there were no significant differences in the haematologic variables studied between males and females of apparently healthy B-not strain indigenous turkeys raised in humid tropics under intensive system of management. The reference intervals established in this study provide a useful baseline for the assessment of haematologic data in B-not strain indigenous turkeys in Nigeria.

CONFLICT OF INTERESTS

The authors declare that there was no conflict of interests concerning the publication of this paper.

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ACQUISITION AND EXPANSION OF ADULT RAT BONE MARROW MULTIPOTENT MESENCHYMAL STROMAL CELLS

Šulla, I.[1], Balik, V.[2], Šarišský, M.[3]

[1]Department of Anatomy, Histology and Physiology, University of Veterinary Medicine and Pharmacy Komenského 73, 041 81 Košice
[2]Department of Neurosurgery
[3]Department of Pharmacology, P. J. Šafárik University, Faculty of Medicine, Tr. SNP 1, 040 11 Košice
Slovakia

igor.sulla@uvlf.sk

ABSTRACT

This study was initiated in order to test a mini-invasive method of mesenchymal stem/progenitor cells (MS/PCs) isolation from a rat bone marrow (BM), and subsequently their expansion, differentiation, and evaluation of their immunophenotypic characteristics; and later their preservation as donor cells in an optimal condition for potential autotransplantation. The study group comprised of 6 adult male Sprague-Dawley (S-D) rats, weighing 480—690 g. The rats were anaesthetised by isoflurane with room air in a Plexiglas box and maintained by inhalation of a mixture of isoflurane and O_2. Their femurs were surgically exposed and their diaphyses double-trephined. Then BM cells were flushed out by saline with heparin and aspirated into a syringe with a solution of DMEM (Dulbecco's modified eagle's medium) and heparin. The mononuclear cells from the BM were isolated by centrifugation and expanded in a standard culture medium supplemented with ES-FBS (es-cell-qualified foetal bovine serum), L-glutamine and rh LIF (recombinant human leukemia inhibitory factor). Following 14 days of passaging cultures, the cells were split into 2 equal parts. The first culture continued with the original medium. The second culture received additional supplementation with a human FGFβ (fibroblast growth factor beta) and EGF (epidermal growth factor). The populations of these cells were analysed by light-microscopy, then the mean fluorescence intensities (MFIs) of CD90 and Nestin were evaluated by a tricolour flow cytometry using monoclonal antibodies. The type of general anaesthesia used proved to be appropriate for the surgical phase of the experiments. All rats survived the harvesting of the BM without complications. The total number of mononuclear cells was 1.5—4.0 × 10^6 per sample and the proportion of CD90/Nestin expressing cells was < 1 %. Following 14 days of expansion, the cells became larger, adherent, with fibrillary morphology; the proportion of cells expressing CD90/Nestin increased to almost 25 %, i. e. they earned basic phenotypic characteristics of MSCs. Throughout the further cultivation a gradual decrease of the CD90/Nestin expression occurred. This suggested that the suitability of rat bone marrow derived MS/PCs for replacement therapy would probably be the highest between days 12—15 of cultivation and then would diminish.

Key words: adult rat; bone marrow; expansion; isolation; multipotent cells

INTRODUCTION

Bone marrow multipotent mesenchymal stromal cells (MSCs), also known as mesenchymal stem/progenitor cells (MS/PCs), represent a heterogeneous population of the non-blood producing fraction of bone marrow [2, 3, 4, 14]. Their ability to differentiate into tissues of mesenchymal origin such as bone, tendon, and cartilage or, under specific conditions, into neural, muscle, myocardial or liver cells makes them a valuable source of different types of progenitors for transplantation [6, 13, 21, 22]. Being members of a team participating in a study of new approaches in the treatment of spinal cord injuries, including replacement therapy of damaged neurocytes, astrocytes and oligodendrocytes by autologous stem cells, the authors developed, and with positive results, tested a modified method of harvesting and expansion of adult rat bone marrow MSCs enabling an easy autotransplantation to donors, thus avoiding possible adverse immune reaction [2, 3, 4, 10, 19, 20].

MATERIALS AND METHODS

The experimental protocols were prepared with respect to the rules of the Animal Protection Act of Slovakia No. 15/1995 [1], approved by the Ethical Commission of the P. J. Šafárik University, Faculty of Medicine in Košice, as well as the State Veterinary and Food Administration of the Slovak Republic in Bratislava by the Decision No. SK P 10552/03-220. The experimental procedures in animals were performed under general anaesthesia and the postoperative painful sensations were suppressed by the intramuscular administration of anodynes. Six adult male Sprague-Dawley (S-D) rats weighing 480-690 g were used in this study.

Harvesting of rat bone marrow stromal cells

The general anaesthesia was induced by inhalation of a mixture of isoflurane (1-chloro-2,2,2-trifluoroethyldifluoro-methylether) with room air in a Plexiglas box. Following the 2—3 min inhalation of anaesthetics, when the surgical phase of narcosis was achieved, animals were removed from the box and placed on a heated platform which kept their rectal temperature between 36.6 and 37.2 °C. The general anaesthesia was further maintained by administration of 1.5 % isoflurane with medical oxygen (O_2) via a special mask designated for rodents. The lateral parts of both femoral regions were shaved and the exposed area of skin was disinfected by an iodine solution (Iodopolyvidonum). The disinfected skin and femoral fascia were incised, adjacent parts of *musculus rectus femoris* and *musculus vastus lateralis* were dissected and between them the ventrolateral surface of femur was approached. After removal of the periosteum, two burr-holes (one at the proximal, the second at the distal end of *diaphysis ossis femoris*) were performed using a tooth-drill. The bone marrow from the femoral medullary cavity was flushed by 2 ml of saline with 0.1 ml of heparin (*solutio heparini natrici sterilisata et titrata* 5 000 u. i.) instilled into one of the burr-holes and aspirated by an injection needle from the other into a syringe with 2 ml of collecting medium "Invitrogen", i. e. Dulbecco's modified eagle's medium (DMEM) with heparin under aseptic conditions (Fig. 1). After aspiration of the bone marrow (BM), the dissected thigh muscles were fixed in their original position by several non-absorbable stitches, then the femoral fascia and skin were sutured in anatomic layers. The position of the rat was changed, the skin of the contralateral thigh was disinfected, incised, and the femur was approached, double-trephined and the bone marrow was harvested from the opposite side, also. Before finishing the experimental operation, antibiotics (*amoxicillinum natricum* with *kalii clavulans*) were administered intramuscularly in a total dose of 30 mg and 2 ml of anodyne tramadol [2-dimethylamino-methyl-1-1-(3-methoxyphenyl)-cyclohexanol] was injected intramuscularly at two different places. After suture of the second surgical wound, the rat was transferred to a disinfected cage, where it was provided with drinking water *ad libitum* and food granules. Two randomly chosen rats were chosen as shame controls. Blood samples (0.5 ml) aspirated by a thin needle from the right heart ventricle were collected into a syringe containing 0.2 ml of heparin solution just before finishing the administration of general anaesthesia.

Cultivation of multipotent stromal cells

Immediately after aspiration of the BM from both femurs, the material in the collecting medium was removed to a special cell culture laboratory at the Department of

Pharmacology, P. J. Šafárik University, Faculty of Medicine. The medium was dissected into small pieces, homogenized and filtered through a 70 µm filter (to remove bone fragments), diluted 1:1 with Hank's balanced salt solution (HBSS) and mononuclear cells were isolated by Ficoll-Urographin density gradient centrifugation (DCG) at 1 600 rpm for 30 minutes. After centrifugation, the mononuclear cells were collected from the interface, washed two times in DMEM, suspended at 10^6 cells . ml^{-1} in culture media and plated in 12 well culture dishes. The cells were cultured in alpha-MEM (minimum essential medium) supplemented with 10 % ES-FBS (embryonic stem-cell-qualified foetal bovine serum), 2 mM L-glutamine, 10 ng.ml^{-1} human rh LIF (recombinant human leukemia inhibitory factor), 100 u. i./ml^{-1} PNC (benzylpenicillinum kalicum) and 10 µg.ml^{-1} STM (streptomycinum sulphate), half volume of the culture solution being replaced with a fresh medium every three days.

At passage 4 (after 14 days of cultivation), the cell cultures were divided into two equal parts. In the first subgroup of cultures, we continued to use the same medium, whereas in the second subgroup, the medium was additionally supplemented with rh FGF-β (recombinant human basic fibroblast growth factor beta) and rh EGF (recombinant human epidermal growth factor). At the 14th, 21st, 36th and 45th day of cultivation (passages P4, P7, P12, P15) the samples of cells were stained with a combination of FITC/PE/PE-Cy5, i. e. monoclonal antibodies (mouse IgG1) against rat CD45 (thyrosine phosphatase expressed by haemopoietic cells), CD90 (protein expressed by mesenchymal cells) and cy-Nestin (type VI intermediate filament-associated protein expressed mostly by nerve cells) and the mean fluorescence intensities (MFIs) were evaluated by the three-colour flow cytometry [3, 12].

RESULTS

The general anaesthesia by vapours of isoflurane with O_2 met our expectations. Its induction phase was short (2—3 min.), the course of the narcosis was smooth, its depth sufficient for performing the surgical phase of the procedure, and subsequent awakening of the animals was uneventful and quick. All six rats survived the aspiration of the bone marrow without complications, incisions of skin, fascia, and dissections of thigh muscles; the trephinations of the femurs healed by first intention (Fig. 2).

The light-microscopic analyses of the cells isolated from the fresh adult rat bone marrow showed that a total number of mononuclear cells was 1.5—4.0 million per sample and the proportion of CD90/Nestin expressing multipotent cells was less than 1 %. After 14 days of *in vitro* expansion, the proportion of CD90 and cy-Nestin expressing cells increased to 19.2—24.9 % in individual samples. The cells were large, adherent with fibrillary morphology (Fig. 3). They expressed inconsistently for CD45 (a phenotype characteristic for haemopoietic lineage), but the cells were strongly positive for CD90 and cy-Nestin, compatible with the basic phenotypic characteristics of MSCs (Fig. 4). P2 — primary data space region 2. In the P2 area are located stem/progenitor cells strongly expressing CD90 marker. In flow cytometric images their colour is green. The MFIs for CD90 cells were 9 552, 9 356 and 9 250; for nestin 3 620, 2 681 and 1 628. Throughout the further cultivation the gradual decrease of expression of CD90, as well as cy-Nestin was observed. At passages 7, 12 and 15, i. e. after 21, 36 and 45 days of expansion, the MFIs of CD90 decreased to 9 320, 8772, 6 671 and of cy-Nestin to 3 605, 1 757, 541.

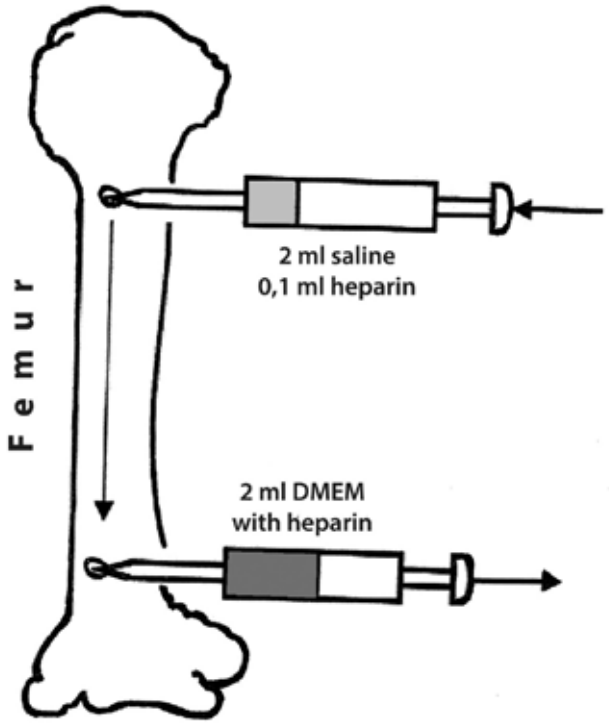

Fig. 1. Pattern of double-trephination perfusion technique of harvesting rat bone marrow cells

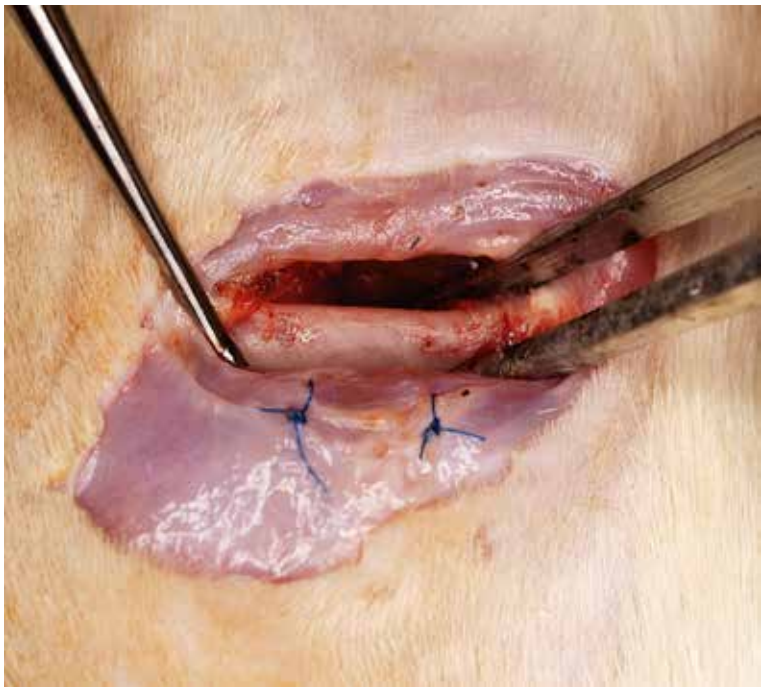

Fig. 2. Situation two months following aspiration of bone marrow. Trephinations in a rat femoral bone are almost completely healed

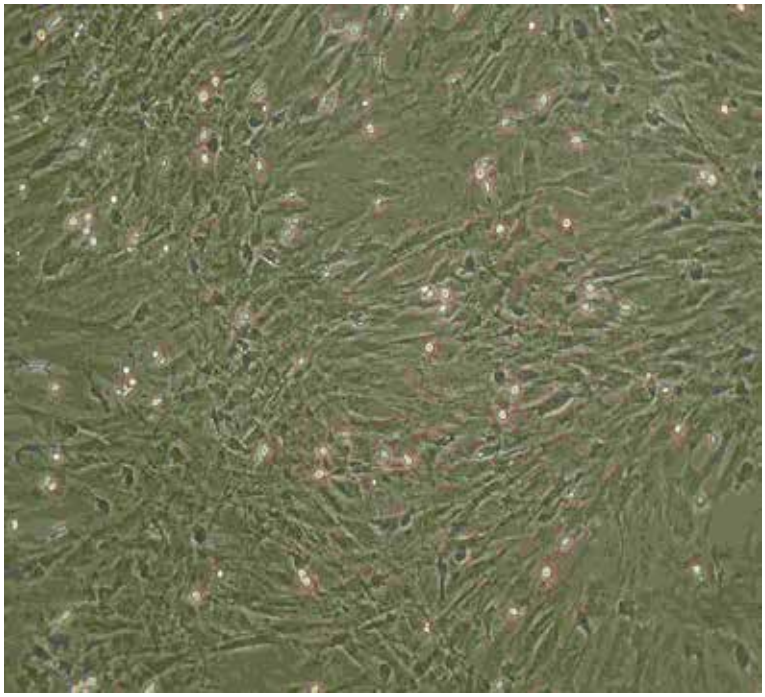

Fig. 3. A heterogeneous culture of spindle-shaped fibroblastic morphology adherent cells obtained after the initial 14 days of cultivation of bone marrow cells (passage 0). Original magnification Magn. ×100

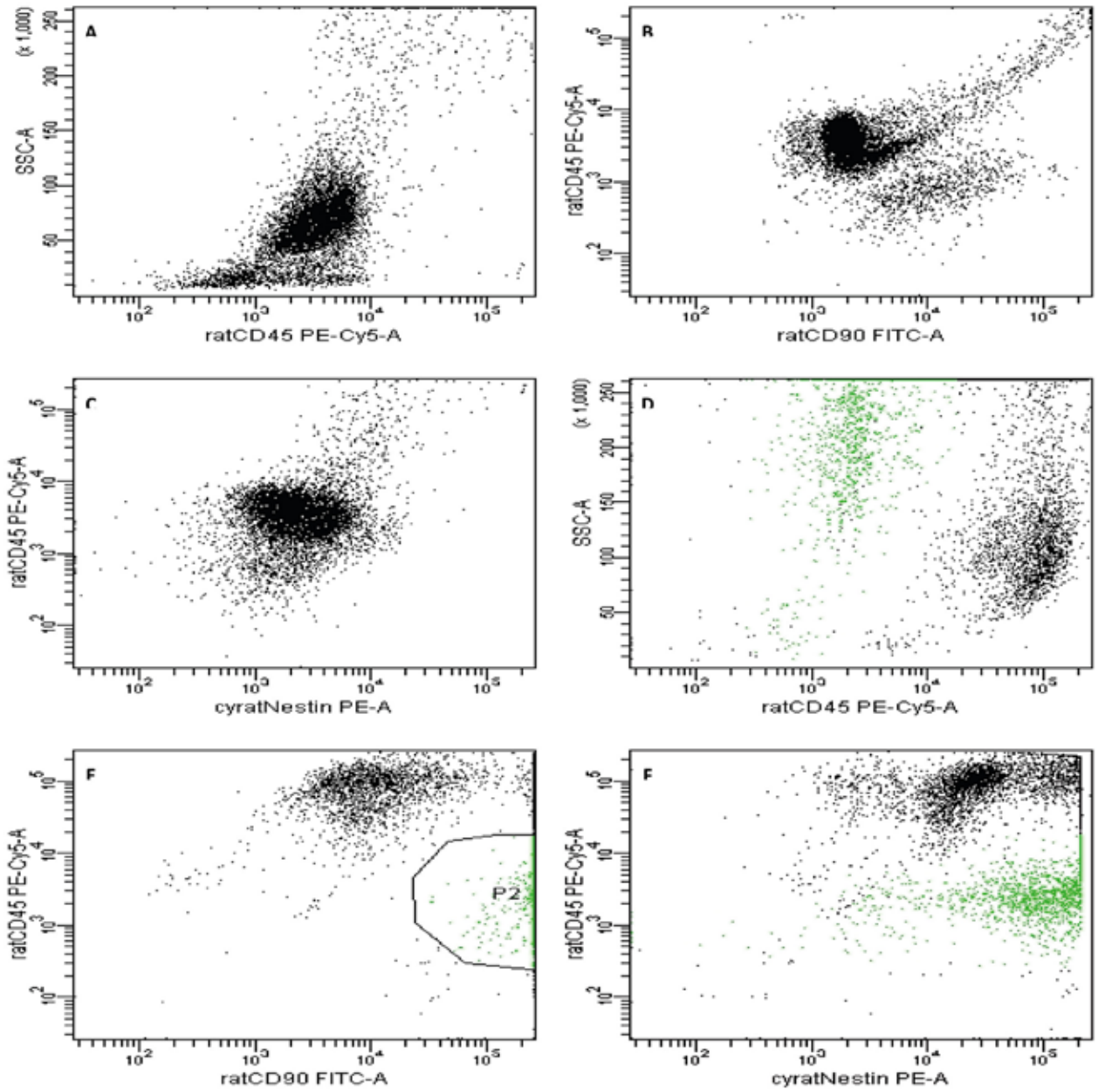

Fig. 4. Results of flow cytometry immunophenotyping analysis of rat bone marrow derived stem cells. Illustrative bivariate dot plots show expression of CD45, CD90 and nestin by the cells before (A, B, C) and after initial 14 days – passage 3 of cultivation (D, E, F). P2 - primary data space region 2

DISCUSSION

Spinal cord and brain injuries, ischemic or haemorrhagic strokes, neurodegenerative disorders (e.g. Parkinson's and Alzheimer's disease, and amyothrophic lateral sclerosis), muscular dystrophy, myocardiopathies, and myocardial infarctions, belong to serious pathological conditions connected with high morbidity and mortality [6, 10, 13, 21, 22]. The treatment modalities of these diseases are limited and attempts to renew impaired or lost functions are unsuccessful in the majority of cases, so far [3, 6, 17]. The well-known fact, that the viability of tissues during the whole lifespan of an animal, is maintained by replacement of damaged cells through a process provided by special cells, called stem/precursor cells (SC/PCs), inspired scientists to use SC/PC transplants to treat different defects of organs with restricted regenerative capacity [3, 4, 5, 6, 13, 15].

In general, there are five distinct groups of stem cells (SCs): i. e.

1. Totipotent SCs (able to differentiate into all embryonic as well as extraembryonic structures),

2. Pluripotent SCs (able to differentiate into cells of all three basal germ layers, i.e. ectoderm, mesoderm and endoderm),

3. Multipotent SCs (able to give rise to a limited SC types, e. g. skin, cartilage, bone marrow, and neurocytes),

4. Oligopotent SCs (able to differentiate only into a few SC types, such as lymphoid or myeloid SCs), and

5. Unipotent SCs, (which are able to produce only one cell type) [3]. Embryonal stem cells (ESCs) are the best source of omnipotent stem cells as they have an unlimited potential to produce any specialised cell type of an organism [3, 16].

Experimental results suggest the almost unrestricted capacity for their utilisation in replacement therapy and regenerative medicine [16, 17]. On the other hand, the fact that a blastocyst developing from a fertilized egg has to be destroyed to obtain ESCs, their use is highly controversial and considered an act of violating human rights in many countries. Later on, experimental studies confirmed the possibility to acquire MS/PCs from several tissues of adult mammals. MS/PCs occur, e. g. in bone marrow, brain subventricular zone, olfactory bulb, adipose tissue, skin, and even malignant tumours [3, 4, 5, 11, 12, 13]. They are not omnipotent or pluripotent as embryonal or foetal stem cells, however their capacity to expand, transdifferentiate into multipotent progenitors and eventually replace damaged cells, produce growth factors and trophic mediators is not substantially reduced [13, 14, 15, 21]. Since the utilisation of adult MS/PCs avoids, on one hand emotional and legal problems, but on the other hand, it permits successful autotransplantation; this practice has become standard in animal experiments, as well as in clinical trials [2, 3, 4, 5, 6, 10, 13, 14, 15, 22].

There is a well established cell laboratory at the Department of Pharmacology, P. J. Šafárik University, Faculty of Medicine in Košice, specialized in the diagnostics of different types of leukemias and bone marrow diseases. This laboratory has also been able to perform phenotypic analyses of cancer cells obtained from patients after surgical resection of their tumours. The sufficient experience with the bone marrow research and high-class equipment at their disposal inspired the authors to concentrate on various problems accompanying harvesting, cultivation, expansion and transdifferentiation of multipotent mesenchymal stem cells derived from bone marrow of adult organisms. Several scientific teams dealing with SCs/PCs research have published reports recommending experimental models using different mammals and their tissues in basic spinal cord injury and transplantation research — from mice, rats and rabbits to cats, dogs, minipigs, exceptionally apes or human embryonal, foetal and adult tissues [2, 3, 4, 5, 6, 7, 8, 9, 11, 14, 15, 16, 17, 18, 21]. The authors decided to use S-D rats in the presented study. Their decision was influenced by several facts: namely, rats are much bigger than mice; S-D rats are more robust than Wistar rats, so they tolerate better the stress associated with complex and repeated surgical procedures; the volume of bone marrow (BM) in their femurs are greater; and the aspiration of BM from both femurs does not endanger the life or health of experimental animals.

Various techniques of bone marrow MSCs acquisition have been tested recently [4, 6, 15, 18]. The majority of them require longitudinal splitting of femurs and excochleation of the cellular contents of the medullary cavity, or resection of both ends of a femur and flushing out the bone marrow from the diaphysis. In consequence, the donors are severely mutilated and perish, which sometimes is emotionally distressing and it excludes autotransplantation of cultivated MSCs. With an aim to avoid these disadvantages, the authors developed a less invasive surgical procedure. The operative technique developed proved simple and straight- forward; the amount of obtained bone marrow cells was sufficient; and the procedure did not increase the morbidity or mortality of the experimental animals. The authors believe their technique could be recommended as a method of choice for experimental acquisition of rat BM derived MSCs (BMDMSCs).

For the expansion of BMDMSCs, commercially available standard culture media and supplements were used which were acquired from well-established firms, such as: BD Biosciences, USA, GIBCO Products International Inc., USA, Chemicon International Inc., USA and Stemcell Technologies Inc., Canada. Their products are expensive, but of high quality, and provided reliable results. The preparation of culture media and all manipulations with cell cultures were performed in a special box with laminar air circulation at the stable temperature of 37.0 °C, in a room air containing approximately 20 % of O_2 (considering the

altitude of Košice at 208 metres above the sea level). Some authors point out that hypoxic environments (oxygen concentrations 2—3 %) markedly increase the expansion of MSCs, without negative influence to the multilineage differentiation capabilities [8]. The construction of the cell culture box that the authors had at their disposal did not allow to work in such an atmosphere. In addition, it was not necessary — the amount of BM derived MSCs increased substantially during the first four passages. The acquired cells were viable and expressed essential markers characteristic for mesenchymal and neural SCs. The laboratory equipment at the Department of Pharmacology, P. J. Šafárik University, Faculty of Medicine allowed us to test only CD45, CD90 and cy-Nestin expressions. The more detailed identification of cells made ready for actual transplantation is recommended [7]. The authors plan to adopt this protocol in future transplantation experiments.

Since cy-Nestin is predominantly expressed by NS/PCs, the expression of this marker is considered to be the first step in the progression of BMDSCs along the neural lineage [19]. Following 14 days *in vitro* cultivation of BMDSCs in alpha-MEM solution supplemented with 10 % ES-FBS, 2 mM l-glutamine and 10 ng.ml^{-1} human rh LIF, the proportion of CD45 (marker characteristic for haemopoietic lineages at all stages of leukocyte's development) was negative or ±, but SC/PCs expressing CD90 (a cell surface marker expressed mainly by mesenchymal stem cells — MSCs) and cy-Nestin expressing cells raised from less than 1 % to 19.2—24.9 %.

As a next step in their research, the authors decided to find out, if BMDMSC/PCs would be able to maintain their original properties, i. e. the strong (+++) expression of cy-Nestin and CD90 markers, and none or weak (±) expression of CD45. The gradual decrease of both, CD90 as well as cy-Nestin markers expression, confirmed by a decline of the mean fluorescence intensities of these markers during medium-term cultivation, was detected at the 21st, 36th and 45th day of passage. This observation indicates, that BMDMSC/PCs are most suitable for replacement therapy between the 12th to the 15th day of cultivation.

The results of this study are promising, however further *in vitro* and *in vivo* experiments, aimed to detect the potential of harvested NS/PCs to differentiate into mature cells of the CNS and to determine whether they gained functional properties of neurons, astrocytes or Schwann cells, eventually cells producing growth factors or trophic mediators, will be necessary. Several studies suggest that BMDMSC/PCs have none or only limited ability to provoke an adverse immunologic reaction [4, 21]. The authors do not share this opinion. That is why they decided to use a minimally invasive bone marrow harvesting surgical procedure offering the opportunity to proceed with MSC/PCs autotransplantation in the same experimental animals.

CONCLUSIONS

This study demonstrated that the use of the BM-harvesting method was simple and reliable. The standard culture media supplemented with ES-FBS, L-glutamine and human rh LIF can be utilized for the expansion of BMDMS/PCs. The experimental procedure did not increase morbidity or mortality. The healing of the surgical wounds was uneventful, thus easily allowing for the autotransplantation of the expanded MSCs.

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INFECTIOUS BRONCHITIS VIRUS IN CAPTURED FREE-LIVING, FREE-RANGE AND INTENSIVELY REARED BIRDS IN SOUTHWEST NIGERIA

Adebiyi, A. I.[1], Fagbohun, A. F.[2]

[1]Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan
[2]Federal College of Animal Health and Production Technology, Moor Plantation, Ibadan
Nigeria

adebiyiade@gmail.com

ABSTRACT

Infectious bronchitis (IB) is an acute infectious viral disease causing severe economic losses in poultry production. In Nigeria, there has only been monitoring of the disease in chickens with little attention given to other bird species. For this study, blood samples were collected from 184 apparently healthy, unvaccinated birds which comprised of 61 captured free-living pigeons, 60 free range indigenous chickens and 63 intensively reared Japanese quails. Sera from these birds were screened for IB virus antibodies (IBV) using a commercial ELISA kit. The birds were from Oyo and Osun States, in southwest Nigeria. Overall, 63 (34.2%) sera were positive for IBV with 3.3% (2/61), 95.0% (57/60) and 6.3% (4/63) from pigeons, indigenous chickens and Japanese quails, respectively. These findings suggest that they were subclinically infected with either field or vaccine virus and could thus serve as possible reservoirs of this virus to domestic poultry. Thus, there is need for continuous surveillance of the disease in different bird species and their possible role in the spread of IBV in Nigeria.

Key words: antibodies; indigenous chickens; infectious bronchitis virus; pigeons; quails

INTRODUCTION

Infectious bronchitis (IB) disease is distributed worldwide. It is an important highly contagious upper-respiratory tract viral disease of avian of all ages and is considered one of the main causes of economic losses in poultry production [5], causing: reduced weight gain, poor feed conversion efficiency, and high condemnation rates in meat-type birds [7]. The causative agent, infectious bronchitis virus (IBV), is a coronavirus which belongs to the family *Coronaviridae* and subfamily *Coronavirinae* within the genus of *Gammacoronavirus* [16]. This enveloped, positive-strand RNA virus has been reported not only to cause respiratory diseases but has been isolated from kidneys, different parts of the oviduct and alimentary tract of chickens [3, 4, 12] with adverse effects on egg quality, egg production and marked depression of growth especially in the laying birds [7]. The most notable signs of the disease are those which affect the respiratory tract, hence the term 'infectious bronchitis'. These signs include: rales, gasping and sneezing, sometimes accompanied by lacrimation and facial swelling [18]. Infectious bronchitis virus is present in respiratory discharges, faeces and on contaminated eggshells and can survive for a considerable time in faeces and is suspected

to represent a continuing source of re-infection in the recovery phase of the disease [14]. This virus is transmitted by air droplets, ingestion of contaminated feed and water, and contact with infected chickens. Also, it can be spread through contaminated equipment and clothing of caretakers [5]. For surveillance purposes, an antibody-detecting enzyme-linked immunosorbent assay (ELISA) is the most appropriate test which will detect seroconversion to IBV regardless of the type or origin of the virus [14].

Poultry coronaviruses are antigenically and phylogenetically related [6]. Thus, avian coronavirus from one species has been shown to replicate in other avian species without observation of clinical signs in most instances [13, 15]. Chickens are the main natural hosts of IBV [5]. Although other species such as pheasants, geese, ducks, quails and pigeons have been indicated to play a role in the spread of IBV strains worldwide, to date, the virus only tends to cause disease in chickens [8, 11, 14]. Despite the availability of effective vaccines and efforts to control IB disease by routine use of live and inactivated vaccines in commercial poultry production, IBV being a single stranded RNA virus, has a high propensity to change by both spontaneous mutation and genetic recombination [5]. This tendency continues to cause poor health, high production loss and welfare concerns through recurrent outbreaks due to the emergence of variants especially in the areas of intensive poultry farming [5, 23].

In Nigeria, although the southwest region plays a leading role in poultry production [1], there is limited information on infectious bronchitis disease in the region. Most of these studies were on chickens from commercial and backyard farms [10, 21]. Thus, this study was designed to investigate the presence of IBV antibodies in captured free-living pigeons, intensively reared Japanese quails and free-range indigenous chickens in Oyo and Osun states, in southwest Nigeria.

MATERIALS AND METHODS

Sample animals

A total of 184 unvaccinated apparently healthy birds were used in this cross sectional study. The birds comprised of 61 (33 males and 28 females) pigeons from a bird market in Oyo state, 63 (60 females and 3 males) Japanese quails from three flocks in Osun state and 60 (44 females and 16 males) indigenous chickens from five rural areas in Oyo state. While the ages of the pigeons were deemed to be over 6 months by the traders, the quails and indigenous chickens were estimated to be about 12 months old.

Sample collection

About 2 ml of blood was aseptically collected from the brachial vein of each bird. The blood was allowed to clot at room temperature while sera were separated and stored at –20 °C until tested.

Detection of IBV antibodies

The sera were screened using a commercially available Green Spring® indirect enzyme-linked immunosorbent assay (ELISA) kit (Shenzhen Lvshiyuan Biotechnology, China) that detects infectious bronchitis virus IgG antibodies. The ELISA procedure was carried out according to the manufacturer's protocol and the optical density (OD) was read using an ELISA reader at double wavelengths of 450 and 630 nm. For each sample, the result was expressed as sample/positive (S/P) value using:

$$S/P = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$$

Samples that presented $S/P \geq 0.2$ and less than 0.2 were considered positive and negative, respectively.

Statistical analysis

Results obtained were analyzed using the statistical package GraphPad Prism version 5.01 (San Diego, USA). Data were subjected to One-way ANOVA and subsequently to Tukey's post test to perform multiple comparisons in order to assess the statistical significance of differences between all possible pairs of groups. The level of statistical significance was $P < 0.05$.

RESULTS

Out of the 184 birds sampled, 3.3 % (2/61), 95.0 % (57/60) and 6.3 % (4/63) were positive for IBV antibodies in pigeons, indigenous chickens and Japanese quails respectively (Table 1).

TABLE 1. Seroprevalence of infectious bronchitis virus in species of birds

Species	Number sampled	Positive [%]
PIGEONS		
Female	28	0 (0.0)
Male	33	2 (6.1)
	61	**2 (3.3)**
INDIGENOUS CHICKEN		
Female	44	41 (93.2)
Male	16	16 (100.0)
	60	**57 (95.0)**
QUAILS		
Female	60	4 (6.7)
Male	3	0 (0.0)
	63	**4 (6.3)**

The mean S/P value (0.63 ± 0.3) was highest in the indigenous chickens, followed by Japanese quails (0.45 ± 0.4) and pigeons (0.20 ± 0.0). The overall seroprevalence of IBV in all species of birds in this study was 34.2 %. The seroprevalence of IBV based on sex of the species of birds varied but was not statistically significant ($P > 0.05$).

DISCUSSION

Infectious bronchitis is a major viral disease of birds that cause significant economic loss in poultry production but whose impact on African poultry is still poorly known [9, 19). This study investigated the presence of infectious bronchitis antibodies in captured free-living pigeons, free range indigenous chickens and intensively reared Japanese quails in Oyo and Osun states, in southwest Nigeria. Additionally, IBV antibody prevalence of 3.3 %, 95.0 % and 6.3 % were obtained respectively for pigeons, indigenous chickens and Japanese quails, an indication that the infection was more prevalent in indigenous chickens than in quails and pigeons. This finding is similar to previous reports of high levels of circulation of IBV in chickens in some West African countries with seroprevalence rates above 70% [10, 19, 20] and supports the possible carrier status of indigenous chickens in the transmission of the virus particularly to commercial poultry [2] and perhaps, to other birds. Furthermore, since these birds were not vaccinated against IB and were apparently healthy, the detection of antibodies is an indication that they had been naturally exposed to the virus. In addition, it has been reported that IB virus replicates in the gut for longer periods than in the respiratory tract and the infection of enteric tissues usually does not manifest itself clinically, but persists for long periods and results in faecal virus excretion [17]. Hence, these birds could serve as reservoirs shedding the virus into the environment, thus playing a key role in the epidemiology of the disease.

Avian coronavirus from one species has been shown to replicate in other avian species without observation of clinical signs in most instances [13, 15]. Consequently, the detection of IBV antibodies in pigeons and Japanese quails in this study indicate seroconversion following natural exposure to the virus. Infectious bronchitis virus can survive for a considerable time in faeces and is suspected to represent a continuing source of re-infection of the disease (14], hence, the detection of IBV antibodies in pigeons and Japanese quails in this study may be due to contact with contaminated feed and water, and may be further supported by the observation of pigeons around commercial poultry houses in southwestern Nigeria scavenging for feed and housing of quails in close proximity to chickens. Similarly, the detected antibodies could be as a result of contact with contaminated equipment and clothing of farm handlers [5] which have also been implicated in the spread of IBV. This fact is further supported by studies elsewhere that revealed the detection of IBV in ducks, turkeys, pigeons, quails and geese and their possible role in the spread of IBV [8, 11, 22].

CONCLUSIONS

The findings of this study revealed that infectious bronchitis virus presently circulate in pigeons, indigenous chickens and Japanese quails in Oyo and Osun states, in Southwest Nigeria; indicating that these species of birds, mainly indigenous chickens, serve as reservoirs for infectious bronchitis virus or related viruses. Thus, this finding underscores the importance of the routine surveillance for IBV in different avian species. There is a need for further studies to determine the genotype and serotype of IBV strains circulating in southwestern Nigeria.

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THE STUDY OF THE PROBIOTIC POTENTIAL OF THE BENEFICIAL BACTERIA ISOLATED FROM KEFIR GRAINS

Englerová, K., Nemcová, R., Mudroňová, D.

Department of Microbiology and Immunology
University of Veterinary Medicine and Pharmacy, Komenského 73, 04181 Košice
Slovakia

radomira.nemcova@uvlf.sk

ABSTRACT

The aim of this study was to identify beneficial bacteria with probiotic potential from kefir grains. The lactobacilli isolated from kefir grains were characterised as: *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus paracasei*, and *Lactobacillus kefiri*. The strains *Lb. plantarum* 1Ž, *Lb. paraplantarum* S10, and *Lb. paracasei* 2Ž tolerated better the test gastric juice at pH 2 and 2.6 during 120 min of incubation in comparison with the strains *Lb. kefiri*. On the other hand, the strains *Lb. kefiri* were resistant to 0.3 % bile acid salts. The *Lb. paracasei* 2Ž showed the significantly highest survival ($P < 0.001$) at pH 2 in comparison with all other strains tested and was also able to tolerate 0.3 % concentration of the bile salts. All strains produced medium to strong biofilms on abiotic surfaces and inhibited the growth of selected potential pathogens with varying intensity. All kefir isolates were susceptible to the antibiotics tested and exhibited positive β-galactosidase activity with the exception of *Lb. paracasei* 2Ž which did not show any activity of undesirable enzymes, such as β-glucosidase and β-glucuronidase. Additional testing and validation of the biological properties and safety of the strain *Lb. paracasei* 2Ž under *in vivo* conditions are needed to confirm the prospective use of this strain in practice.

Key words: biofilm; inhibitory activity; lactobacilli; probiotics; safety; tolerance of GIT conditions

INTRODUCTION

Kefir is a popular drink originating in the Caucasian mountains in Central Asia where it has been consumed since the middle Ages. At the present it is an important consumer commodity in many areas of the world including Africa and the Middle East. Kefir is traditionally made using kefir grains as a starter culture. The grain matrix is composed of a complex of proteins and polysaccharides and consists of densely populated lactic fermentation bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*), acetic fermentation bacteria and yeasts, the proportion of which is affected by geographic regions [29].

Although some of the health benefits of kefir have not yet been validated by exact scientific and clinical investigations, several *in vitro* studies conducted on animals have confirmed the positive effects of kefir on: intolerance of lactose [20], immunomodulation [21], antimicrobial activity against pathogenic micro-organisms [9] and harmonisation of intestinal microflora [44]. The functional properties of kefir have been traditionally ascribed to its biologically active proteins and bacterial exopolysaccharide — kefiran [40]. However, the potential beneficial effects could also be mediated by the microbial composition of this fermented milk or by secondary metabolites [37].

Although a considerable number of commercial well-characterised probiotic strains are available today, there is still interest in screening new productive strains [5, 36]. Strains exhibiting unique and specific properties important for health can be selected during characterisation of natural fermented dairy products such as kefir [46]. This traditional product may be an interesting source of potential probiotic bacteria with specific functional properties. Despite the fact that many authors have advocated the importance of the human origin of a strain and a selective criterion for its use by humans, the professional group at FAO/WHO [17] stressed more the probiotic activity of the strain than the source of the relevant micro-organism.

The functionality of probiotics can be determined by two groups of tests [34]. The first group of tests focuses on the physiological properties and safety, for example: the tolerance of gastric juice and bile acids; ability to adhere to intestinal mucosa and colonise the intestine; production of inhibitory substances and inhibitory activity against pathogenic bacteria; susceptibility of the strain to antibiotics and enzymatic activity; and immunomodulatory properties. The second group of tests focuses on the technological properties of probiotic micro-organisms, such as the viability during the production process and stability of the strain during storage.

The aim of this study was to identify lactobacilli isolated from kefir grains and characterise their selected properties according to instructions recommended by FAO/WHO with respect to their biological effects and safety under *in vitro* conditions.

MATERIALS AND METHODS

Isolation of lactobacilli from kefir grains and their identification

Homogenised samples of kefir grains were applied to de Man-Rogosa-Sharpe agar (MRS; Carl Roth GmbH, Germany) with addition of 200 ppm antimycotic cycloheximide (Glentham Life Sciences Ltd., UK) to suppress the growth of yeasts. The plates were cultivated in an anaerobic environment (Gas Pak Plus, BBL, Microbiology Systems, Cockeysville, USA) at 30 °C, 37 °C and 40 °C for 48—72 hours. Individual morphologically different colonies were stained according to Gram. According to microbiological features (shape, colour, size, arrangement) selected isolates were deposited in the cryosystem MicrobankTM (Pro-Lab Diagnostics, Canada) for the purpose of their preservation for additional analyses.

The isolates were identified by means of MALDI-TOF mass spectrometry (Microflex LT instrument Bruker Daltonik GmbH, Leipzig, Germany) using the method of Bessede et al. [3]. On the basis of the obtained spectra (BioTyper software, version 2.0 Bruker Daltonik), the probability of identification was evaluated as follows: score ≥ 2.30 — high probability of identification at the level of species; 2.30 ≥ score ≥ 2.00 — high probability of identification at the level of the genus; and 2.00 ≥ score ≥ 1.70 — probable identification at the level of the genus.

Testing of viability in gastric juice

Simulated gastric juice (pH 2 and 2.6) was prepared according to the method by Kos et al. [26]. A fresh suspension of a respective strain (approximately 1×10^8 CFU.ml^{-1}) obtained by multiplication in de Man Rogosa and Sharpe (MRS) broth (Carl Roth GmbH) and subsequent washing in saline solution was added to the sterile gastric juice. The incubation took place in a water bath at 37 °C with shaking. The viability of the strains was determined after 0, 60, 90 and 120 min of incubation by means of a flow cytometer BD FACSCantoTM (Becton Dickinson Biosciences, USA) using BD FACS DivaTM software. For the measurement, we used a mixture of 50 µl of the strain sample in gastric juice, 5 µl of carboxyfluorescein diacetate stain and 445 µl of phosphate buffer saline (PBS) containing 1 mM dithiotreitol (Sigma-Aldrich, USA). This mixture was incubated for 30 min at 37 °C. Blue laser of wavelength 488 nm was used for excitation and the subsequent emission was measured

at wavelength 530/30 nm (FL1). The percentage proportion of live and dead bacteria was evaluated by means of a histogram with FL1 fluorescence setting against the Count (cell numbers). The results were presented as the arithmetic mean of three measured values (three cultivations) ± standard deviation (SD).

Testing of toxicity of bile salts

The testing of toxicity of the bile salts to the lactobacilli was carried out on MRS agar containing 0.3 % bile acid salts (sodium taurocholate and glycocholate, Sigma-Aldrich, USA). Discs impregnated with suspensions of lactobacilli strains were placed onto agar and the plates were incubated under anaerobic conditions (Gas Pak Plus, BBL) at 30 °C and 37 °C for 72 hours. The ability of the lactobacilli to grow in the presence of the above salts was evaluated qualitatively.

Monitoring of the biofilm production

The production of the biofilm was observed in 96-well microtitration plates using the method of O'Toole et al. (38). The capacity of biofilm production was assessed using crystal violet, a standard stain used for the determination of biofilm production. Crystal violet bound to adhered cells (biofilm) was extracted with 200 µl of 95 % ethanol and the optic density of the solution was measured spectrophotometrically (Synergy Reader 4, BioTek, Merck, SRN) at a wavelength of 570 nm. The medium without bacterial culture was used as a control. The produced biofilm was evaluated as thick ($OD570 \geq 1$), medium ($0.1 \leq OD570 < 1$) or negative ($OD570 < 0.1$) (6). The strains were tested by three independent experiments, each repeated 8 times. The results are presented as the arithmetic means ± SD. The biofilm produced and fixed to slides according to Kubota et al. [27] was detected by a scanning electron microscopy (SEM). The SEM images were obtained by a scanning electron microscope JEOL JSM-7000F (magnification: ×200, ×2500, ×15000; high vacuum; voltage 15.0 kV; working distance 11.3 mm).

Testing of the inhibitory activity

The disc-diffusion method was used to determine the inhibitory activity of selected lactobacilli against potentially pathogenic micro-organisms. The following indicator strains were used: Escherichia coli 0149 F4 (Research Institute of Veterinary Medicine in Brno, CR); *Salmonella* Typhimurium CCM 7205 (Czech Collection of Micro-organisms in Brno, CR); Staphylococcus aureus and Bacillus cereus (isolates obtained at Laboratory of gnotobiology, UVMP in Košice, SR). Sterile disks of diameter 6 mm (BBL, Cockeysville, USA) were placed on the surface of 20 ml of peptone-yeast extract-glucose (PYG) agar in Petri dishes. The composition of PYG agar was as follows: peptone for bacteriology 5 g; enzymatic casein hydrolysate 5 g; yeast extract 10 g; glucose 10 g; and agar 18 g.1000 ml^{-1} distilled water (pH 6.9). The discs were inoculated with 5 µl of the night cultures of lactobacilli (1×10^8 CFU. ml^{-1}l) and the plates were then cultivated anaerobically (Gas Pak Plus, BBL) at 37 °C or 30 °C for 48 hours. After incubation, the plates were overlayed with 3 ml of 0.7 % PYG agar, inoculated with 0.3 ml of the night culture of a respective indicator strain and incubated aerobically at 37 °C for 24 hours. The results are presented as the arithmetic means of 3 measurements ± SD.

Evaluation of safety

The minimum inhibitory concentration (MIC) of an antibiotic against the lactobacilli strains were tested by MIC Test Strip test (Liofilchem, Italy). The test is based on the use of antibiotic strips impregnated with the following concentration gradients of antibiotics: 0.016—256 $mg.l^{-1}$ for ampicillin, vancomycin, kanamycin, erythromycin, clindamycin, tetracycline, chloramphenicol and 0.064—1024 $mg.l^{-1}$ for gentamycin and streptomycin. The strips were placed onto solid nutrient medium Mueller-Hinton agar (Oxoid Unipath, Ltd., Basingstoke, UK) with 10 % addition of MRS agar (Carl Roth) which was inoculated with 100 µl of the lactobacilli suspension (McFarland Standard No.1). The plates were incubated anaerobically (Gas Pak Plus, BBL) at 30 °C, or 37 °C for 48 hours and the value of MIC was obtained after the incubation. The results obtained were compared with the critical values of MIC ($mg.l^{-1}$) recommended by EFSA [14].

The enzymatic activity of the lactobacilli was determined by means of a commercial semi-quantitative API ZYM test (BioMérieux, France) according to the producer's instructions. The suspension of the lactobacilli (65 µl) of turbidity, equal to McFarland Standard No. 5, was inoculated into each well of the API ZYM strips. The enzymatic activity was evaluated after a 4-hour incubation under anaerobic conditions (Gas Pak Plus, BBL) at 37 °C or 30 °C

for 4 hours. The intensity of colouring ranging from 0 (no activity) up to 5 (≥ 40 nmol of hydrolysed substrate during 4-hour incubation) was recorded according to the API-ZYM colour reaction diagram.

The haemolytic activity was tested on Trypticase soy agar (TSA; Carl Roth GmbH, Germany) with 5 % ram blood. The presence of α- or β-haemolysis was evaluated on the basis of the production of bright or greenish zones around the colonies.

Statistical evaluation

The results of the individual analyses were evaluated by one way variance analysis (ANOVA) supplemented with Tukey test using the software GraphPad Prism version 3.00.

RESULTS AND DISCUSSION

Lactobacilli are an important part of the microflora of kefir grains. A number of authors have described the isolation of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* from kefir grains originating from various regions[2, 7, 19, 46]. With a high probability at the level of the genus and probable identification at the level of the species, we identified isolate R7 as *Lactobacillus plantarum* (score 2.091), isolate 1Ž as *Lactobacillus plantarum* (score 2.105), isolate 2Ž as *Lactobacillus paracasei* (score 2.078), isolates 4/30 and 13/30 as *Lactobacillus kefiri* (scores 2.058 and 2.117, resp.). With probable identification at the level of the genus, we identified isolate S10 as *Lactobacillus paraplantarum* (score 1.725). We were not able to identify the isolate marked as 6/30; however its growth properties and morphology were almost identical with those of strains 4/30 and 13/30. In order to confirm the results obtained by MALDI-TOF mass spectrometry, it is necessary in future studies to subject the isolates also to genotype identification, such as the sequencing of 16S RNA amplificate obtained by PRC methods.

During the testing of the individual properties of isolates obtained from kefir grains, we used for comparison animal strains *Lactobacillus reuteri* 2/6 and *Lactobacillus reuteri* L26 isolated from the digestive tract of the animals in our laboratory.

The viability and survival of probiotic bacteria under unfavourable conditions in the digestive tract are the most important parameters for achieving their therapeutic effects [18]. In order to survive in the digestive tract these bacteria must be able to resist the extreme conditions due to the presence of hydrochloric acid or bile acids. It was confirmed that the viability of bacteria exposed to such conditions are species and strain specific [28]. The first barrier these bacteria must overcome is the very low pH in the stomach (values in the range of 1—3) to which they are exposed for 90 min on average. Our observations of the incubation in simulated gastric juice at pH 2.6 showed 98—88 % survival of strains *Lb. plantarum* 1Ž, *Lb. paraplantarum* S10, *Lb. paracasei* 2Ž and strains *Lb. reuteri*. These strains exhibited significantly higher tolerance ($P < 0.001$) of the gastric juice at pH 2.6 (Fig. 1a) in comparison to the strains *Lb. kefiri*. A similar trend was also observed at pH 2 (Fig. 1b). During 120 min incubation we recorded higher than 60 % survival of strains *Lb. plantarum* 1Ž and *Lb. paraplantarum* S10 and higher than 80 % survival of strain *Lb. paracasei* 2Ž, which was significantly higher ($P < 0.001$) in comparison with the survival of strains *Lb. kefiri* and animal strains *Lb. reuteri*. The *Lb. kefiri* strains showed only 10 % survival at 90 and 120 min incubation. A significantly highest percentage of survival ($P < 0.001$) at pH 2 compared to all tested strains was observed for *Lb. paracasei* 2Ž.

Probiotic bacteria should be able to grow in the presence of 0.15—0.3 % bile acids [11]. *Lb. kefiri* strains were resistant to 0.3 % natrium taurocholate and glycocholate, while the growth of *Lb. plantarum* R7, *Lb. paraplantarum* S10 and *Lb. plantarum* 1Ž was inhibited by the presence of these bile salts. The exception was the strain *Lb. paracasei* 2Ž which was able to tolerate 0.3 % concentration of both bile salts and, as reported earlier, exhibited a high tolerance also to simulated gastric juice with pH 2. It is well known that exposure of bacterial cells to one type of stress can induce a response protecting the cells against multiple stresses [13]. Some previously published data indicated that intestinal isolates of lactobacilli were usually more resistant to bile salts than isolates from other sources [25]. However, our results did not confirm this observation, as intestinal isolates of animal strains of *Lb. reuteri* were susceptible to the salts of bile acids.

One of the properties most frequently observed during the selection of probiotic candidates is their ability to adhere to the mucus and epithelial cells, or their congregational ability. Despite the important role of biofilm in commensal bacteria which affects immunomodulation, the exclusion of pathogens and increased contact with the in-

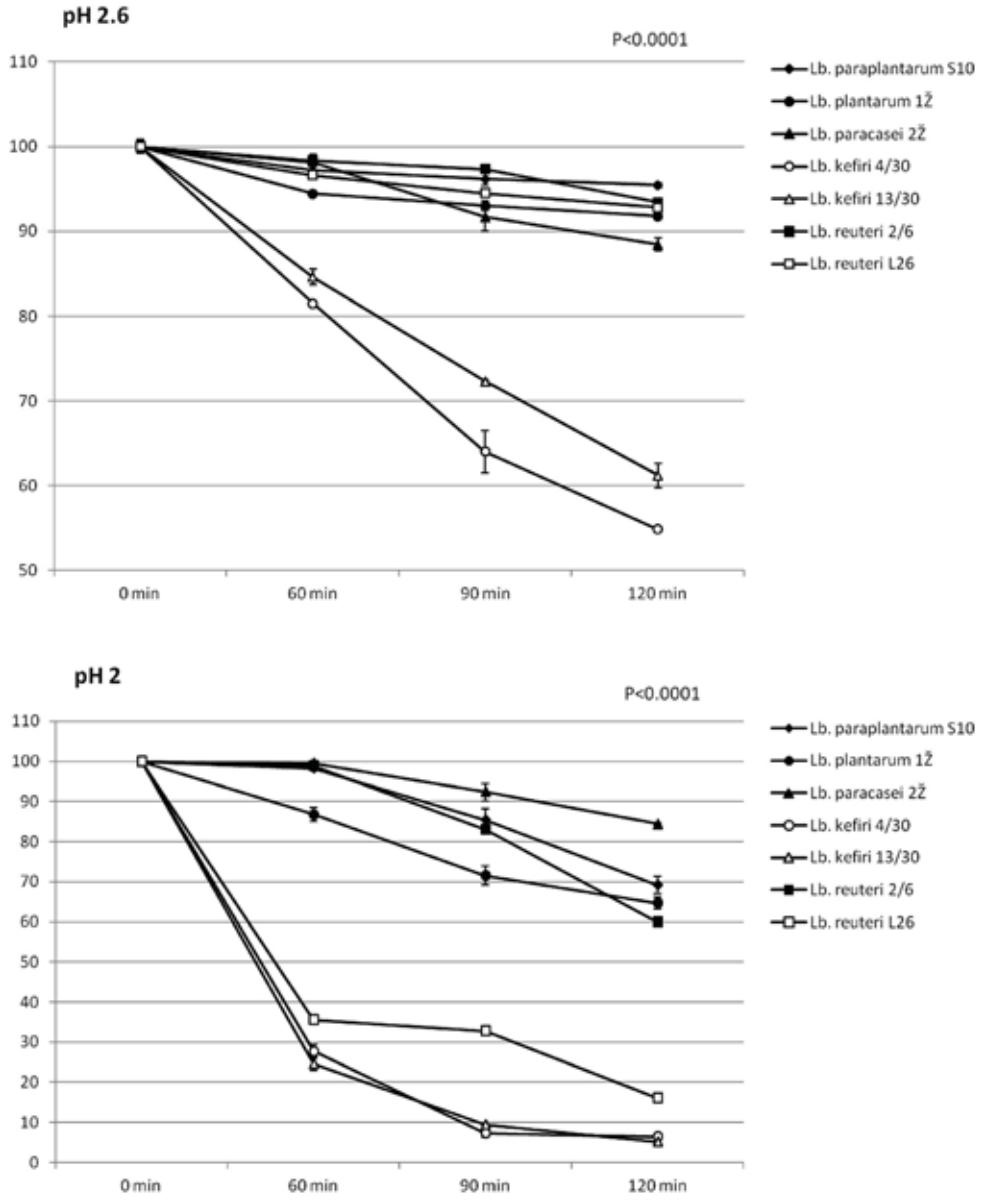

Fig. 1. Time dependence of survival (in per cent) of lactobacilli in simulated gastric juice at different pH. The values presented are means of 3 measurements ± SD

testinal mucosa [30], the potential probiotic strains are not routinely tested for their ability to produce complex biofilm structures on various surfaces. Biofilms can protect probiotic bacteria against unfavourable conditions in the gastrointestinal tract (GIT), allow them to communicate directly with the host and support survival of these bacteria in GIT at higher beneficial metabolic effectiveness [24]. Moreover, production of biofilms would increase their stability in the process of production and storage [8]. The strains in our study produced biofilms on the abiotic surfaces in MRS medium free of Tween. These biofilms were either medium thick (*Lb. plantarum* R7, *Lb. paraplantarum* S10, *Lb. paracasei* 2Ž, *Lb. kefiri* 13/30) or thick (*Lb. plantarum* 1Ž, *Lb. kefiri* 4/30, *Lactobacillus* sp. 6/30, *Lb. reuteri* 2/6, *Lb. reuteri* L26) (Fig. 2). The highest production of biofilm (OD570 = 4.5) was recorded and also confirmed by SEM with the strain *Lb. plantarum* 1Ž (Fig. 3). A similar production of biofilms by lactobacilli from various sources was observed by Terraf et al. [43] and Slížová et al. [42]. However, these authors pointed out that there are significant differences in the production of biofilms related to the composition of the medium and cultivation conditions. Although observation

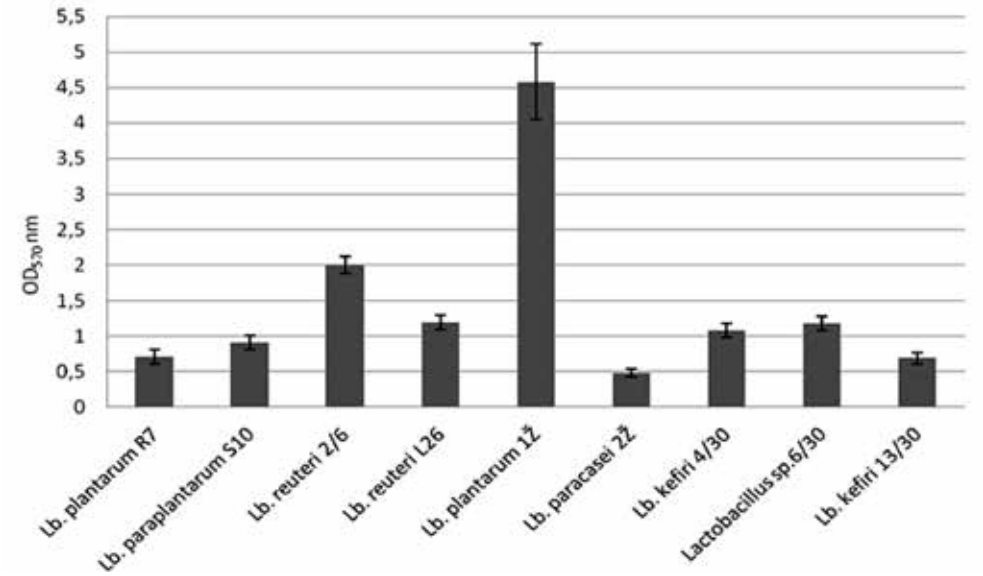

Fig. 2. Production of biofilms by lactobacilli (MRS medium free of Tween)
Results are presented as arithmetic means ± SD

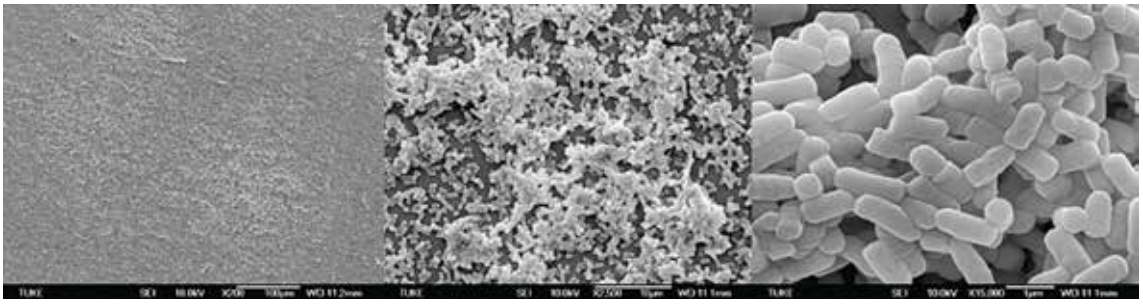

Fig. 3. SEM of biofilm produced by strain Lb. plantarum 1Ž.
Magn. ×200; ×2 500; ×15 000.

of the production of biofilms on abiotic surfaces cannot replace the testing of the ability to adhere to biotic surfaces, several authors who tested the potential of probiotic bacteria to produce biofilms reported significant positive correlation between adherence to biotic surfaces and their ability to produce biofilms on abiotic surfaces [32, 39].

Lactobacilli are known for their ability to produce antimicrobial substances against pathogenic bacteria. The antimicrobial activity of lactobacilli is based mostly on organic acids, hydrogen peroxide and bacteriocins [23]. Our examination showed that the strains of lactobacilli inhibited the growth of indicator strains *E. coli* 0149 F4, *S.* Typhimurium CCM 7205, *S. aureus* and *B. cereus*, with the size of the inhibition zones dependent on the individual strains (Table 1). The highest inhibitory properties were exhibited by the strains *Lb. plantarum* 1Ž and *Lb. paracasei* 2Ž. The susceptibility of *E. coli* 0149 F4 and *S.* Typhimurium CCM 7205 to the lactobacilli was approximately the same; however the Gram positive bacteria *B. cereus* were more susceptible than *S. aureus*. Similarly, Bilková et al. [4] reported that *S. aureus* was the strain with the highest resistance to the strains *Lb. murinus*, *Lb. mucosae* and *Lb. reuteri*, isolated from the GIT of lambs. We assume that the inhibitory effect observed in our study could be mediated by the production of organic acids. In our previous studies we observed the production of lactic acid and acidic acid by the strains tested. The antimicrobial effect of these acids may be related to the inhibition of the various metabolic functions of bacterial cells with the highest inhibitory effect ascribed to the non-dissociated form of organic acids [41].

The system of assessment of safety of probiotic micro-organisms strictly specifies criteria such as resistance

Table 1. Antimicrobial activity of the lactobacilli strains

Strains	Diameter of inhibition zones (mm)			
	***S.* Typhimurium CCM 7205**	***E. coli* O149 F4**	***S. aureus***	***B. cereus***
***Lb. plantarum* R7**	24.67 ± 0.47	29.00 ± 0.82	14.33 ± 1.25	34.00 ± 4.32
***Lb. paraplantarum* S10**	28.00 ± 1.63	29.33 ± 0.94	18.67 ± 0.94	39.00 ± 0.82
***Lb. plantarum* 1Ž**	30.33 ± 0.47	34.67 ± 0.47	26.67 ± 1.25	44.67 ± 2.49
***Lb. paracasei* 2Ž**	29.33 ± 0.94	30.00 ± 0.82	20.00 ± 1.63	39.33 ± 0.94
***Lb. kefiri* 4/30**	16.50 ± 0.50	21.00 ± 1.00	12.00 ± 2.00	25.00 ± 1.00
***Lactobacillus* spp. 6/30**	23.00 ± 1.00	22.00 ± 0.00	11.00 ± 1.00	23.00 ± 1.00
***Lb. kefiri* 13/30**	25.00 ± 0.82	24.33 ± 0.47	11.33 ± 0.94	21.67 ± 0.47
***Lb. reuteri* 2/6**	23.00 ± 2.16	28.33 ± 0.47	11.33 ± 0.94	33.00 ± 2.16
***Lb. reuteri* L26**	34.67 ± 3.68	28.00 ± 1.93	20.33 ± 0.47	40.33 ± 0.47

Results are presented as arithmetic means of 3 measurements± SD

Table 2. Minimum inhibitory concentration (MIC) of antibiotics against the lactobacilli strains

Strains	MIC mg.l^{-1}							
	AMP	**GEN**	**CAN**	**STR**	**ERY**	**CLI**	**TTC**	**CMP**
***Lb. kefiri* 4/30**	1	0.5	16	16	0.38	0.38	6	1.5
***Lactobacillus* spp. 6/30**	1.5	0.75	12	12	0.25	0.38	6	1.5
***Lb. kefiri* 13/30**	1.5	1	16	12	0.19	0.19	4	3
***Lb. paracasei* 2Ž**	3	8	48	64	0.75	0.75	0.19	3
***Lb. plantarum* R7**	0.19	2	32	24	0.50	0.016	16	8
***Lb. plantarum* 1Ž**	0.19	4	96*	16	0.50	0.38	8	3
***Lb. paraplantarum* S10**	0.5	2	32	24	0.75	0.032	24	8
***Lb. reuteri* 2/6**	0.38	3	64	32	1	1	24*	8*
***Lb. reuteri* L26**	0.25	0.75	32	24	2*	0.047	64*	4

AMP — ampicillin; GEN —gentamycin; CAN — canamycin; STR — streptomycin; ERY — erythromycin; CLI — clindamycin; TTC — tetracycline; CMP — chloramphenicol; * — values MIC exceeding critical values defined for the respective species of lactobacilli by EFSA commission [14]

to antibiotics and transfer of resistance genes. Probiotic micro-organisms should not increase the existing risk associated with normal microflora in the intestine or foods. In relation to the evaluation of resistance to antibiotics of probiotic strains, one should distinguish the type of resistance involved. Natural resistance is not transferred horizontally and raises no risk of transfer to other, particularly potentially pathogenic bacteria. On the other hand, the acquired resistance present in some strains within one species normally susceptible to the assessed antibiotics can spread horizontally among bacteria. With regard to the therapeutic use of antibiotics, this resistance presents a serious problem [35]. The MIC values of antibiotics against strains of lactobacilli isolated from kefir grains correlated with the critical values of MIC recommended for the respective species or groups of lactobacilli by EFSA commission (Table 2) with the exception of strain *Lb. plantarum* 1Ž in the case of kanamycin. Danielsen and Wind [10] re-

Table 3. Enzymatic activity of lactobacilli by API ZYM system

Enzymes[a]	Strains								
	Lb. plantarum R7	*Lb. paraplantarum* S10	*Lb. plantarum* 1Ž	*Lb. paracasei* 2Ž	*Lb. kefiri* 4/30	*Lb.* sp. 6/30	*Lb. kefiri* 13/30	*Lb. reuteri* 2/6	*Lb. reuteri* L26
Naphthol-AS-BI-phospho-hydrolase	20	10	10	20	5	5	5	20	5
Acid phosphatase	5	5	5	10	20	20	20	30	20
Esterase (C4)	5	5	5	5	0	0	0	10	10
Esterase lipase (C8)	5	5	5	0	5	5	5	10	5
Leucine arylamidase	20	20	20	30	20	10	10	10	10
Valine arylamidase	20	20	20	30	10	5	10	0	0
Cystine arylamidase	0	0	5	0	0	0	5	0	0
α-galactosidase	0	0	5	0	20	20	20	20	20
α-fucosidase	0	0	0	0	0	0	0	0	0
β-galactosidase	5	5	5	10	30	30	30	30	30
β-glucuronidase[b]	0	0	5	0	10	20	20	0	0
α-glucosidase	5	10	10	20	10	20	10	20	20
β-glucosidaseb	10	10	20	0	0	0	0	0	0

[a] — enzyme activity measured as approximate nmol of hydrolysed substrate during 4-hour incubation; [b] — unwanted enzymatic activity; strain *Lb. paracasei* 2Ž — isolate from kefir grains; strains *Lb. reuteri* 2/6 and *Lb. reuteri* L26 — isolates from animal intestines without unwanted enzymatic activity

ported that some lactobacilli strains are naturally resistant to aminoglycosides which include also kanamycin. All kefir isolates were susceptible to ampicillin with MIC values ranging from 0.19 to 3 mg.l^{-1}, to gentamycin 0.5—8 mg.l^{-1}, to kanamycin 12—64 mg.l^{-1}, to streptomycin 12—64 mg.l^{-1}, to erythromycin 0.19—0.75 mg.l^{-1}, to clindamycin 0.016—0.75 mg.l^{-1}, to tetracycline 0.19—24 mg.l^{-1} and to chloramphenicol 1.5—8 mg.l^{-1}. On the other hand, strains of *Lb. reuteri* of animal origin exhibited higher values of MIC, namely with *Lb. reuteri* 2/6 for tetracycline and chloramphenicol and with strain *Lb. reuteri* L26 for erythromycin and tetracycline. The phenotype results obtained for these strains should be supplemented by observation of the presence of transferrable resistance genes at the molecular level. Lactobacilli are generally susceptible to these antibiotics [16]. Plasmids encoding resistance to tetracycline, erythromycin and chloramphenicol were detected in *Lb. reuteri*, *Lb. fermentum*, *Lb. acidophilus* and *Lb. plantarum* isolated from raw meat, silage and excrements. Many of these resistance genes may have been acquired by horizontal transfer [33].

The safe use of probiotic strains is related to the activity of their enzymes. The strains showed different enzymatic profiles (Table 3). From that point of safety, the absence of activity of β-glucosidase and β-glucuronidase is desirable. The activity of β-glucosidase is associated with undesirable effects in the large intestines; β-glucuronidase may release aglycons and deconjugate carcinogens conjugated with glucuronic acid [12]. The *Lb. plantarum* (R7 and 1Ž) and *Lb. paraplantarum* S10 strains exhibited medium activity (10—20 nmol of hydrolysed substrate) of β-glucosidase; *Lb. plantarum* 1Ž showed weak activity (5 nmol of hydrolysed substrate); and *Lb. kefiri* (4/30, 13/30) and *Lactobacillus* spp. 6/30 medium activity of β-glucuronidase (10—

20 nmol of hydrolysed substrate). On the other hand, the activity of β-galactosidase affects favourably the human metabolism. This enzyme hydrolyses lactose to glucose and galactose and in this way alleviates the unpleasant manifestations of lactose digestion disorders. Moreover, oligomerisation of products and substrate by β-galactosidase stimulates the cytotoxic and humoral immunity through the activation of macrophages and T-cells [22]. All kefir isolates exhibited activity of β-galactosidase with the highest activity (30 nmol of hydrolysed substrate) observed with *Lb. kefiri* (4/30, 13/30) and *Lactobacillus* spp. 6/30.

Haemolysis is a common factor of virulence of pathogenic bacteria which makes iron available to bacteria as a co-factor important for the action of some bacterial enzymes, and causes anaemia and oedemas in hosts [45]. Lactobacilli are able to grow in the absence of iron which is considered an ecological advantage in the environment where they have to compete with pathogenic bacteria. However, some observations indicated the presence of haemolytic activity from lactobacilli. According to Elli et al. [15] this haemolytic activity may be associated with the requirements of lactobacilli on iron in the metabolism of pyrimidine and purines in the environment with limited sources of specific nucleotides. The lactobacilli strains in our study showed no haemolysis of blood agar which is in agreement with the results of a number of authors who observed no haemolytic activity in lactobacilli isolated from clinical samples, faecal samples from children and adults and samples of dairy products [1, 31].

CONCLUSIONS

The results obtained in this study have allowed us to conclude that lactobacilli strains isolated from kefir grains could fulfil the parameters set for the required properties of probiotics. The strain *Lb. paracasei* 2Ž provided the best preliminary results as it: showed a high resistance to simulated gastric juice and bile salts; produced biofilm; exhibited strong inhibitory activity against potential pathogens; was susceptible to the test antibiotics; and showed no harmful enzymatic or haemolytic activity. In this study, we did not perform all the tests that must be used for testing of potential probiotic strains which opens additional space for further investigations focused particularly on the validation of biological properties and safety under *in vivo* conditions. After complex characterisation and relevant *in vivo* studies, the selected strain appears prospective for the use as probiotics in functional food or clinical practice.

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EFFICIENCY OF ENZYMATIC DEBRIDEMENT IN THE HEALING PROCESS OF CHRONIC WOUNDS IN SMALL ANIMAL PRACTICE

Hamilton, L., Kožár, M.

University of Veterinary Medicine and Pharmacy, Komenského 73, 04181 Košice
Slovakia

martin.kozar@uvlf.sk

ABSTRACT

Skin wounds are a common presentation in small animal practice. These wounds may be acute or chronic with a complicated healing process. An important aspect of the healing of wounds is debridement which may be carried out by surgical, autolytic, mechanical or enzymatic methods. The debridement method is chosen according to the individual skin defect and influenced by factors such as wound size and location, the age of the wound, and the presence of infection or exudate. Enzymatic debridement is a method that is not commonly used in veterinary practice, and involves the use of enzyme preparations to remove necrotic tissue from a wound. The aim of this study was to investigate the effects of the enzymatic ointment collagenase as a method of debridement, and its effect on the macroscopic appearance of chronic skin wounds in cats and dogs. We observed that the application of Iruxol Mono directly to the wound changes the progress of the healing process, with no obvious adverse effects. The time of healing of chronic wounds was decreased and healthy granulation tissue was developed within a couple of days after application of the ointment. Enzymatic debridement appears to be a promising method of debridement for use in chronic wounds, and should be considered in cases where more conventional methods of debridement are ineffective or unsuitable.

Key words: collagenase; chronic wounds; debridement; enzymes

INTRODUCTION

Wounds are commonly encountered in small animal practice. In the year 2015, the Small Animal Clinic, section of surgery, orthopaedics, roentgenology and reproduction of the University of Veterinary Medicine and Pharmacy in Košice examined 2172 patients, of which there were 182 cases of wounds. In order to heal successfully, wounds must pass through three phases: inflammation, proliferation and maturation [9]. If this process is interrupted or unsuccessful, a wound may become chronic. Chronic, non-healing wounds require more intervention than acute wounds in

order to heal successfully. Chronic wounds often contain a large amount of necrotic tissue, decreased blood supply and substantial exudate which impedes healing.

An important aspect in the healing of chronic wounds, and the focus of this study, is debridement. The purpose of debridement is to remove any bacteria and damaged or necrotic tissue in order to improve healing of the remaining tissue [9]. There are four common methods of debridement that may be used alone or in combination. These methods are surgical, autolytic, mechanical and enzymatic. The choice of the method is based on the characteristics of the individual wound such as its size, location, aetiology, age, the presence of infection or exudate, and the overall condition of the patient [2].

Surgical debridement is the most common and rapid method of debridement. It involves the use of a scalpel or other blade to scrape or cut away unhealthy tissue. The disadvantages of surgical debridement are that it can be painful, causes bleeding, and may result in the accidental removal of viable tissue. Autolytic debridement is a slow but safe method that relies on the patient's own healing processes and the presence of phagocytic cells and proteolytic enzymes produced naturally in the wound. These natural processes are enhanced by the application of dressings which maintain a moist wound bed. Mechanical debridement involves removal of unhealthy tissue by force, using methods such as wet-to-dry dressings, lavage or ultrasound. This method may cause further damage and pain to a wounded area, for example the use of wet-to-dry dressings may remove viable tissue along with necrotic tissue.

Enzymatic debridement is not commonly used in veterinary medicine compared to other methods. It involves the direct application of proteolytic enzymes to a wound, which breaks down necrotic tissue. There have been only a few studies on the efficacy of enzymatic debridement in animal patients, however it has been used very successfully in human patients [5, 6]. Collagenase-based preparations are the most common type of enzymatic debriding agents. Collagenase is a water-soluble proteinase derived from bacteria such as Clostridium histolyticum and Vibrio spp. It has specific activity against collagen in non-viable cells which means it does not break down healthy tissue, making it safe for use in wound management [1]. Studies in human medicine have shown that the use of collagenase increases the speed of healing and reduction in wound size, as well as reducing inflammation [6, 8].

There are many advantages of enzymatic debridement over other more commonly used methods. Enzymatic debridement is minimally invasive, and its application can be carried out while the animal is conscious, minimising the necessity for general anaesthesia and its associated risks. Potential disadvantages of enzymatic debridement are that it may not be suitable as the sole treatment in patients with large, deep, burn wounds [4]. It has also been proposed that enzymatic debridement may not be safe in wounds with a high bacterial burden [3]. Payne et al. [7] were able to demonstrate the safety of both collagenase and papain-urea agents in wounds with high bacterial burdens via the use of a rodent model.

Many researchers have attempted to evaluate the efficacy of enzymatic debridement on the healing of wounds in humans, and in animal models under experimental conditions. However, there have been few studies carried out on animal patients. The aim of this study was to assess the healing process of wounds in cats and dogs that have been treated with enzymatic debridement.

MATERIALS AND METHODS

The enzymatic debriding agent used in this study was Iruxol Mono, a collagenase preparation produced by Smith & Nephew Ltd. (London, Great Britain). Iruxol contains the enzyme clostridiopeptidase A and associated proteases, contained in excipients liquid paraffin and white soft paraffin. It is indicated for the debridement of necrotising wounds, such as decubital ulcers in human patients. The recommended method of application is a 2 mm thickness of ointment once per day, applied directly to the wound or wound dressing.

The animals used in this clinical study were 5 dogs and 2 cats which were patients of the Small Animal Clinic, section of surgery, orthopaedics, roentgenology and reproduction of the University of Veterinary Medicine and Pharmacy in Košice. They were presented with chronic, non-healing wounds during the period between November 2015 and March 2016. Before starting treatment with Iruxol, cultures were taken from the wounds to ensure they were free from bacterial contamination.

The Iruxol ointment was applied once per day directly to the wound and the surrounding skin in a layer of at least 2 mm thick. The volume of Iruxol used per day in each case

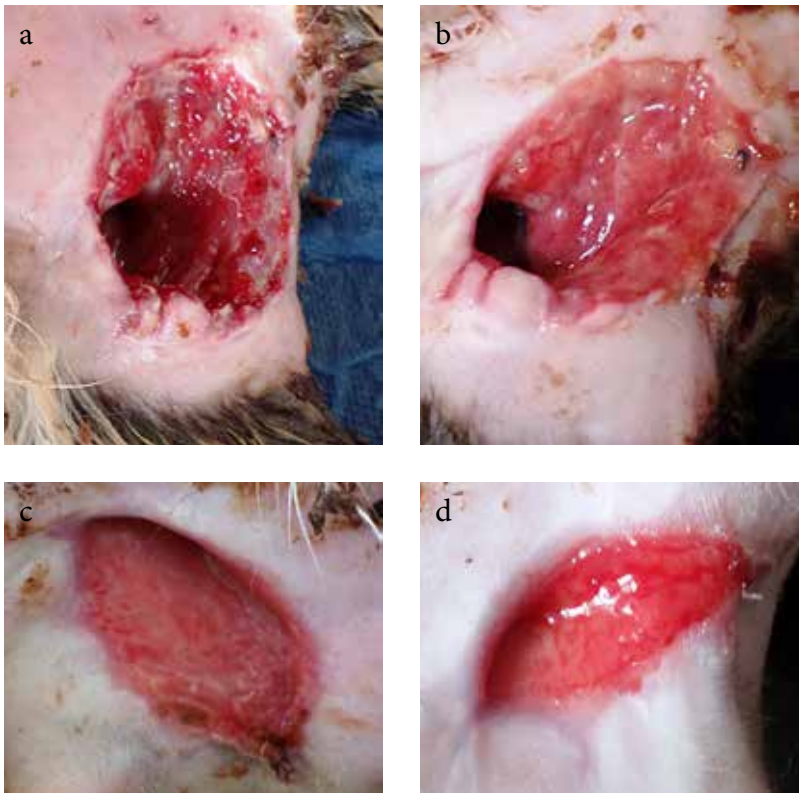

Fig. 1. Wound of the right thigh in a cat
a — before treatment; b — after 1 day of treatment; c — after 8 days of treatment; d — after 14 days of treatment
(Source: Authors)

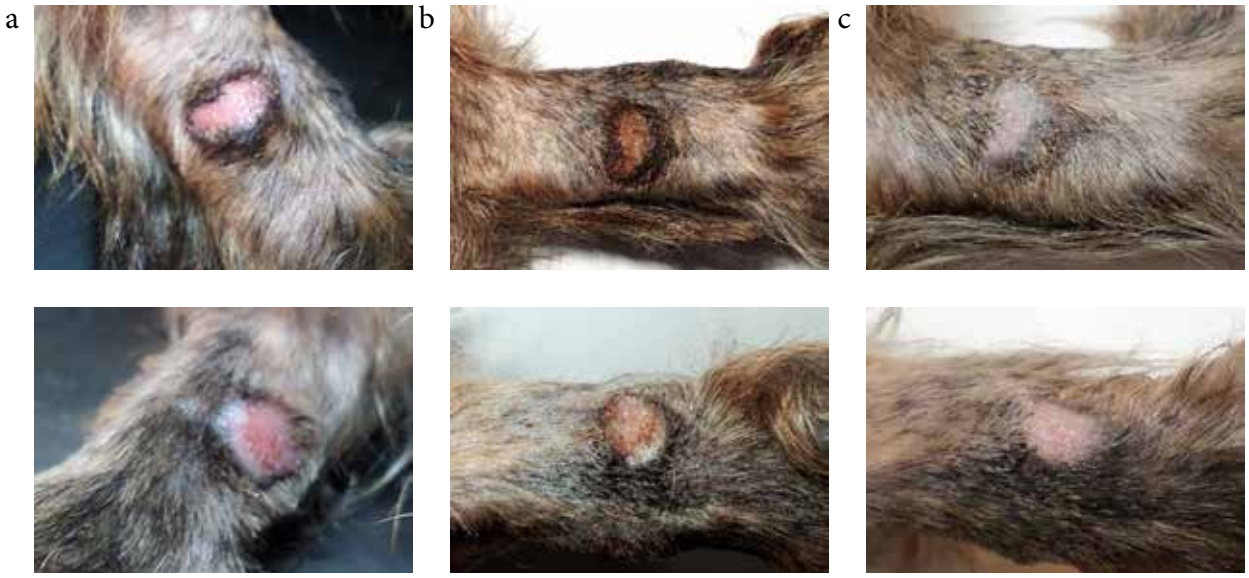

Fig. 2. Wounds of the right tarsus in a dog (top = lateral side, bottom = medial side)
a — before treatment; b — after 1 day of treatment; c — after 7 days of treatment
(Source: Authors)

was based on the size and depth of the wound. The wound was examined each day to assess the healing process and size of the wound. During the wound healing process, we also examined colour changes, desiccation of wound edges, the production of granulation tissue in the wound bed and production of exudate. Iruxol treatment was stopped when the wound was completely healed, and the time of healing was recorded.

RESULTS AND DISCUSSION

Enzymatic debridement was carried out in 7 animals; 5 dogs and 2 cats. In 6 out of 7 cases the wounds healed well following enzymatic debridement, even in patients in which other methods of debridement and surgical treatment had previously been unsuccessful. In the remaining patient, the wound reduced in size during the first week of application of Iruxol, however it became contaminated with Staphylococci bacteria and underwent dehiscence so the Iruxol treatment was halted.

In all patients, the production of granulation tissue was observed within 2 days of beginning Iruxol treatment. In a few cases, a small amount of serous wound exudate was observed, which was cleaned from the wound bed before the daily application of Iruxol. The wound edges remained well-defined throughout the treatment and reduction in the wound size progressed rapidly. In some cases, such as the case pictured in Figure 1, there was a visible reduction in the size within 2 days of Iruxol treatment.

The patient in Figure 1 was a kitten which presented with a large, deep wound of unknown aetiology on the medial right thigh. The patient also had desiccated toes on the right hind foot which were amputated during the course of the Iruxol treatment. 0.5 ml of Iruxol was applied directly to the wound once per day and left uncovered, the patient wore an Elizabethan-collar to prevent licking of the wound.

Within 24 hours there was a visual improvement to the surface of the wound. The edges of the wound were cleaner and more defined than prior to the application of Iruxol, and healthy granulation tissue was visible. The wound decreased rapidly in size, beginning on the third day after initiation of Iruxol treatment. Iruxol treatment was continued for 2.5 weeks until the wound was completely healed.

The patient in Figure 2 was a 4-year old, castrated male mixed-breed dog with a history of atopic dermatitis. The patient had self-inflicted wounds to the lateral and medial right tarsus which had been present for approximately 3 months. 0.2 ml Iruxol was applied once per day to each wound and covered with a bandage to prevent licking.

Within 2 days of beginning Iruxol treatment, the wound reduced in size. Within 1 week, the wound began epithelialization. Iruxol treatment was continued by the owner for approximately 2 weeks until the wounds were completely healed.

In all but one of the cases studied so far, the wounds healed successfully when treatment with Iruxol ointment was used as the sole method of debridement. No negative effects of the treatment were observed. Following treatment with Iruxol, granulation tissue was formed within 2 days, which indicated that the Iruxol treatment improved the growth of granulation tissue.

Studies of enzymatic debridement in human patients have yielded similar results. For example, retrospective evaluations of the use of collagenase for enzymatic debridement in human patients have been published by Marazzi et al [5], and Ramundo and Gray [8]. Both studies found the treatment to be well-tolerated by patients and even to reduce pain at the wound site. Another human study by Onesti et al [6] compared the efficacy of enzymatic and mechanical debridement and showed that cases treated with enzymatic debridement had more rapid reduction in wound size and caused less pain than debridement using wet-to-dry dressings.

Further research into the efficacy of enzymatic debridement in small animal practice should include a comparison with different methods of debridement used in similar wounds, as carried out in human patients by Onesti et al [6]. Further research should also include histological analysis of the wound before and after enzymatic debridement. However this will be difficult to accomplish in animal patients due to reluctance of the owner and the ethical implications of taking tissue samples for histological analysis.

CONCLUSIONS

The results observed in this study support the use of enzymatic debriding agents such as Iruxol in the healing process of chronic wounds. The advantages of this method compared to other methods of debridement are that it is non-invasive, painless and can be carried out without an-

aesthesia. It is therefore worth considering enzymatic debridement for the treatment of chronic wounds in cases where other methods of debridement may be unsuitable.

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THYROID HORMONES, INSULIN, BODY FAT, AND BLOOD BIOCHEMISTRY INDICES IN DAIRY COWS DURING THE REPRODUCTION/PRODUCTION CYCLE

Paulíková, I.[1], Seidel, H.[2], Nagy, O.[1], Tóthová, Cs.[1] Konvičná, J.[1], Kadaši, M.[1], Kováč, G.[1]

[1]Clinic for Ruminants
[2]Clinic for Swine, University of Veterinary Medicine and Pharmacy, Košice
Slovakia

iveta.paulikova@uvlf.sk

ABSTRACT

This study investigated the changes in: thyroid hormones, amount of subcutaneous fat, and selected indices of blood biochemistry in dairy cows in relation to the reproduction/production cycle. The blood samples were collected both *ante-* and *post-partum* every two weeks. When evaluating the mean values of the investigated indices, the major changes were recorded in dairy cows 3 to 14 days after calving. During this period, we observed a significant decrease in the mean serum levels of T_3 ($P < 0.05$), T_4 ($P < 0.01$), and triglycerides ($P < 0.01$). An opposite trend was observed with a significant increase after calving in the: mean serum levels of β-hydroxybutyrate ($P < 0.05$), urea ($P < 0.01$), and mean AST activities ($P < 0.05$). A significant increase over the normal range was recorded in the average levels of non-esterified fatty acids ($P < 0.01$) and total bilirubin ($P < 0.01$). From the next sampling (28 days after calving) onwards we recorded a significant increase in the blood serum levels of cholesterol ($P < 0.01$), total lipids ($P < 0.01$), total protein ($P < 0.01$), as well as a significant decrease in the insulin levels ($P < 0.05$) and a reduced layer of subcutaneous fat ($P < 0.01$). The blood serum iodine concentration showed only slight significant changes ($P < 0.05$) during the observation. Blood serum levels of glucose did not show any significant changes during the whole observation period. Within the whole observation period we found a negative correlation between T_3 levels and the layer of subcutaneous fat ($r = -0.2606$; $P < 0.05$). This correlation was much more marked in cows 3 to 14 days after calving ($r = -0.5077$; $P < 0.05$), which may indicate a possible relationships between the thyroid status, body condition, and *post partum* negative energy balance.

Key words: body fat volume; dairy cows; insulin; negative energy balance; thyroid

INTRODUCTION

The transitional period in dairy cows includes 3 weeks before and 3 weeks after calving, when the metabolic pro-

cesses are adapting to provide energy and nutrients for the synthesis of milk compounds [15, 39].

During this period, the dairy cows undergo dramatic changes in lipid metabolism during the transition from gestation to lactation [8, 14]. Even during ongoing lactation, homeostatic control of the metabolism varies markedly depending on the stage of lactation [8]. The *post partum* feeding pattern of nutrient intake does not keep up with the requirements. The peak milk production, at about 8 to 10 weeks *post partum*, occurs earlier than the maximum energy intake. Therefore, a negative energy balance (NEB) develops, most severely after parturition [14]. As a consequence, the body fat reserves are mobilized, resulting in elevated non-esterified fatty acids (NEFA) concentrations in the plasma [8]. These are taken up by the liver where they are either processed by the β-oxidation pathway or re-esterified to triglycerides (TG) and exported as very low-density lipoproteins (VLDL) [16]. If the TG synthesis exceeds the TG export capabilities as VLDL, a fatty liver develops [16]. A fatty liver usually provokes other metabolic diseases and reproductive disorders that are initially derived from NEB during early lactation.

A negative energy balance in the transition period is the key factor determining the adaptation of a dairy cow's metabolism [17], including adaptation of the endocrine system, which is crucial to maintaining the metabolic balance [2]. The changes in the endocrine system affects predominately the glucose and lipid metabolism, to ensure the homeorhetic nutrient partitioning towards the prioritized mammary gland despite a catabolic state [14]. This selectivity in directing nutrients coincides with the reduced responsiveness and sensitivity of extrahepatic tissues to insulin, i. e. insulin resistance is thought to be markedly involved in developing ketosis and hepatic lipidosis [19].

Hormonal changes during the transition period are characterized by an increase in growth hormone and a decrease in insulin, thyroid hormones and insulin like growth factor (IGF-I) [30]. A positive correlation between circulating thyroid hormones and energy balance is well known in many species including cattle [3, 4, 23, 33, 34, 36].

The thyroid gland function and hormonal changes in the different reproductive periods in cows have been investigated by many authors [1, 37, 48]. The concentrations of thyroid hormones change significantly during the reproduction cycle. In accordance with the nutritional and metabolic processes during advanced pregnancy, dried cows showed high concentrations of thyroid hormones followed by a significant decrease in the *peri partum* period. Blood levels of thyroid hormones in *peri partum* cows decrease, particularly in early lactation, when the body reserves are mobilized for high milk production [21, 22, 25, 28, 44, 47, 48].

During *post partum* negative energy balance (NEB), dairy cows respond by lowering T_3 and T_4 and increasing rT_3 concentrations [35, 42]. Dairy cows in the first third of lactation showed low concentration of T_3 and T_4 [40], despite recovery of ß-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) [12]. The concentrations of T_3 and T_4 correlate negatively with milk yield [48].

This study was aimed at the evaluation of energy metabolism, thyroid hormones, insulin, and body fat thickness in *pre partum* and *post partum* dairy cows.

MATERIALS AND METHODS

The experiments were carried out in accordance with the established standards for animal care and use on a farm near Košice. Dairy cows (n = 21) were in certain phases of *ante partum* (a. p.) and *post partum* (p. p.). The mean production age was 2.5 lactations (3—5 years of age). The milk yield during the previous lactation was 6 668.5 kg milk during a 305-day lactation. The animals were fed a total mix ration (TMR) twice daily, nutrient composition of the TMR varied with the stage of pregnancy and lactation (Table 1). The dairy cows had free access to drinking water.

Blood samples were collected by direct puncture of *vena jugularis*, 3 h after feeding, every two weeks (from the 6th week before expected calving until the 12th week after calving). In the blood serum we analysed the concentrations of: triiodothyronine (T_3), thyroxin (T_4), insulin, iodine (I), aspartate aminotransferase (AST), glucose (Glu), total protein (TP), urea (U), cholesterol (Chol), triglycerides (TG), total lipids (TL), non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), and total bilirubin (TBil). The hormones T_4 and T_3 were determined by the ELISA method with the use of commercial ELISA kits (Human, Germany) and microtitration plates. The readings of absorbancies and calculations of the concentrations were done by automatic photometer Opsys MR (Dynex Technologies). The insulin ($IU.ml^{-1}$) was determined by an ELISA method using a commercial assay (Cusabio, China) according to the manufacturer's instructions. The iodine

Table 1. Components of *pre partum* (a. p.) and *post partum* (p. p.) diets [kg.head^{-1}day^{-1}]

	Weeks 6–1 a. p.	Week 1 p. p.	Week 3 p. p.	Week 6 p. p.	Week 9 p. p.
Meadow hay	5.5	1.5	1.5	1.5	1.5
R-24*	0.3	0.25	0.3	0.25	0.25
Haylage	4	4	6	6	6
Alfalfa silage	13	24	22	22	22
Green fodder		25	25	25	25
Soybean meal		0.8	0.8		
Rape meal		2.5	2.5	2.5	2.5
Wheat meal		3	4	2.5	
Limestone		0.2	0.2	0.2	0.2
Flaxseed meal			0.5	1	1
Maize meal				1	
Triticale					3.5

*R-24 — mineral supplement (10.4% Ca; 9% P; 11% Na; 4% Mg; 7000 mg Cu; 3000 mg inorganic Mn; 6000 mg inorganic Zn; 40 mg Se; 100 mg I; 20 mg Co; 1 000 000 IU vitamin A; 100 000 IU vitamin D3; 2 000 IU vitamin E)

concentrations were estimated by a photometric method using a catalytic reaction NO_2^-/SCN^- [49]. The concentrations of: glucose, AST, TP, urea, Chol, TG, and BHB were determined by using commercial diagnostic kits (Randox, UK) on an automatic biochemical analyser Alizé (Lisabio, France). The concentrations of NEFA and TL (Randox, UK) were assessed by the spectrophotometric method Specord 210 Plus (Analytic Jena, Germany). The concentrations of total bilirubin were determined by a classic photometric method according to Jendrassik and Grof [24]. The backfat thickness (BFT) measurements were obtained by using a 3.5 MHz linear transducer and were assessed according to Staufenbiel [45]. The examination site was located in the sacral region between the caudal one-quarter and one-fifth connection line going from the dorsal part of the tuber ischia (pins) to the tuber coxae (hooks). This site corresponds to the area between the end of the crista sacralis and the end of the os sacrum (i. e. beginning of the first coccygeal vertebra). Animals were scored for BFT on the day of their blood collection.

The evaluation of the results was performed by the assessment of the mean values (x) and standard deviations (SD) in each group of dairy cows. The significance of differences in the mean values in relation to the several monitored periods were evaluated by a one-way analysis of variance (ANOVA). The significance of differences in the mean values between groups was evaluated by Tukey's multiple comparisons test. The statistical analyses were done with the GraphPad Prism 3.0 software. The level of significance was set to $P < 0.05$, respectively.

RESULTS

The results of our investigation are presented in tables 2—16. When evaluating the mean values of investigated indices, the major changes were recorded in dairy cows 3 to 14 days after calving. During this period, we observed a significant decrease in the mean serum levels of T_3 ($P < 0.05$) and T_4 ($P < 0.01$) (Tables 2, 3), a decrease in triglycerides ($P < 0.01$) below the normal range (Table 4), and a slight insignificant decrease in the mean serum cholesterol concentrations (Table 5).

An opposite trend, i. e. a significant increase after calving was found in the mean serum levels of β-hydroxybutyrate ($P < 0.05$), urea ($P < 0.01$), and mean AST activities ($P < 0.05$) (Tables 6, 7, 8). A significant increase over the normal range was recorded in the average levels of non-esterified fatty acids ($P < 0.01$) and total bilirubin ($P < 0.01$) (Tables 9, 10).

From the next sampling (28 days after calving) onwards we recorded a significant increase in the blood serum levels of cholesterol ($P < 0.01$) (Table 5), total lipids ($P < 0.01$) and total protein ($P < 0.01$) (Tables 11, 12), as well as a significant decrease in the insulin levels ($P < 0.05$) and reduced layer of subcutaneous fat ($P < 0.01$) (Table 13, 14).

The blood serum iodine concentration showed only a slight significant change ($P < 0.05$) during the observation (Table 15). The blood serum levels of glucose did not show any significant change during whole observation period (Table 16).

Within the whole observation period we found a significant negative correlation between T_3 levels and layer of subcutaneous fat ($r = -0.2606$; $P < 0.05$). This correlation was much more marked in cows 3 to 14 days after calving ($r = -0.5077$; $P < 0.05$).

Table 2. Mean blood serum T_3 concentrations [ng.ml^{-1}] in dairy cows *ante partum* (a. p.) and *post partum* (p. p.)

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p.p.								
x	2.00	1.88	2.03[a]	1.55[b, c]	1.98	2.00	2.11d	1.92	1.82
± SD	0.232	0.377	0.218	0.352	0.391	0.48	0.536	0.398	0.572
ANOVA	**0.0051**								

[a, b; c, d] — values with the superscripts differ at P < 0.05

Table 3. Mean blood serum T4 concentrations [µg.dl^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	6.82[e]	7.01[a, g]	5.78	4.75[c, f, h]	5.13[b]	5.41	5.86	6.26[d]	5.77
± SD	0.496	1.019	0.885	1.381	1.117	0.63	1.293	1.216	1.46
ANOVA	**0.0001**								

[a, b; c, d] — values with the superscripts differ at P < 0.05; [e, f; g, h] — values with the superscripts differ at P < 0.01

Table 4. Mean blood serum triglycerides concentrations [mmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	0.217	0.273[a]	0.265[e]	0.135[b, f, g]	0.281[h]	0.345[c, h]	0.285[h]	0.231[d]	0.206[d]
± SD	0.074	0.085	0.084	0.055	0.169	0.109	0.091	0.031	0.037
ANOVA	**0.0001**								

[a, b; c, d] — values with the superscripts differ at P < 0.05; [e, f; g, h] — values with the superscripts differ at P < 0.01

Table 5. Mean blood serum cholesterol concentrations [mmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	2.6[e]	2.8[g]	2.2[i]	2.2[d, k]	3.3[b, c, m]	4.7[f, h, j, l, n]	4.8[f, h, j, l, n]	5.6[f, h, j, l, n]	4.7[d, f, h, j, l, n]
± SD	0.35	0.59	0.30	0.51	0.86	0.98	1.37	1.54	0.78
ANOVA	**0.0001**								

[a, b; c, d] — values with the superscripts differ at P < 0.05; [e, f; g, h; i, j; k, l; m, n] — values with the superscripts differ at P < 0.01

Table 6. Mean blood serum BHB concentrations [mmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	0.355	0.429	0.429	0.762	0.794	0.554	0.514	0.418	0.506
± SD	0.044	0.15	0.094	0.285	0.927	0.292	0.257	0.196	0.134
ANOVA	**0.035**								

Table 7. Mean blood serum urea concentrations [mmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	2.2a	2.3c	2.7e	4.6b,d,f	4.9b,d,f	5.7b,d,f	5.2b,d,f	4.9b,d,f	4.2b,d,f
± SD	0.73	0.63	0.84	1.87	1.27	1.24	1.19	0.97	0.90
ANOVA	**0.0011**								

[a, b; c, d; e, f] — values with the superscripts differ at P < 0.01

Table 8. Mean blood serum AST activities [µkat.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	1.35	1.19a	1.19c	1.74b,d	1.54	1.54	1.58	1.41	1.40
± SD	0.221	0.135	0.205	0.477	0.358	0.509	0.466	0.197	0.166
ANOVA	**0.002**								

[a, b] — values with the superscripts differ at P < 0.05; [c, d] — values with the superscripts differ at P < 0.01

Table 9. Mean blood serum NEFA concentrations [mmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	0.347[a]	0.513[c]	0.595[e]	1.566[b, d, f, g]	0.941[h]	0.815	0.702[h]	0.632[h]	0.406[h]
± SD	0.102	0.22	0.264	0.63	0.397	0.394	0.282	0.393	0.111
ANOVA	**0.0001**								

[a, b; c, d; e, f; g, h] — values with the superscripts differ at P < 0.01

Table 10. Mean blood serum total bilirubin concentrations [µmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	4.67[a]	5.10[c]	5.06[e]	9.85[b, d, f, g]	7.36	5.98[h]	5.94[h]	5.49[h]	4.86[h]
± SD	0.542	1.036	1.107	4.484	4.621	1.26	1.355	1.618	1.187
ANOVA	**0.0001**								

[a, b; c, d; e, f; g, h] — values with the superscripts differ at P < 0.01

Table 11. Mean blood serum total lipid concentrations [g.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	3.95$^{a, g}$	3.9$^{c, e, i}$	3.7^{k}	4.1^{m}	5.4$^{d, o}$	7.1$^{h, j, l, n, p}$	6.9$^{h, j, l, n, p}$	6.5$^{h, j, l, n, p}$	5.7$^{b, f, l, n}$
± SD	0.301	0.24	0.21	0.722	1.449	1.04	1.4	0.624	0.732
ANOVA	**0.0001**								

$^{a, b; c, d; e, f}$ — values with the superscripts differ at P < 0.05; $^{g, h; i, j; k, l; m, n; o, p}$ — values with the superscripts differ at P < 0.01

Table 12. Mean blood serum total protein concentrations [g.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	72.0^{a}	72.6^{e}	71.3$^{c, g}$	75.1^{i}	79.3	79.8^{d}	81.6^{h}	84.1$^{b, f, h, j}$	81.8^{d}
± SD	11.96	5.69	4.77	6.15	6.40	5.558	6.96	7.76	7.50
ANOVA	**0.001**								

$^{a, b; c, d}$ — values with the superscripts differ at P < 0.05; $^{e, f; g, h; i, j}$ — values with the superscripts differ at P < 0.01

Table 13. Mean blood serum insulin concentrations [IU.ml^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	589.8	577.6	616.0	624.4	654.3	353.9	394.6	374.9	392.9
± SD	94.1	37.8	181.5	553.7	575.1	115.5	155.9	157.8	128.5
ANOVA	**0.0434**								

Table 14. Mean thickness of subcutaneous fat [cm] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	3.30^{a}	3.39^{e}	3.45	3.02$^{c, g}$	2.50^{f}	2.35$^{b, d, f}$	2.35$^{b, f, h}$	2.39	2.41
± SD	1.025	0.555	0.092	0.491	0.513	0.404	0.318	0.226	0.253
ANOVA	**0.0001**								

$^{a, b; c, d}$ — values with the superscripts differ at P < 0.05; $^{e, f; g, h}$ — values with the superscripts differ at P < 0.01

Table 15. Mean blood serum iodine concentrations [μg.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	55.30	55.55	56.21	56.43	53.57	51.65	52.18	51.82	52.30
± SD	3.83	6.191	4.224	10.013	3.287	1.622	2.011	1.611	0.526
ANOVA	**0.0401**								

Table 16. Mean blood serum glucose concentrations [mmol.l^{-1}] in dairy cows a. p. and p. p.

Sampling	1	2	3	4	5	6	7	8	9
	Days 3—14 p. p.								
x	3.98	3.92	3.85	3.64	3.77	3.95	3.82	3.85	3.56
± SD	0.128	0.195	0.245	0.966	0.413	0.425	0.344	0.301	0.213
ANOVA	**n. s.**								

n. s. — non-significant

DISCUSSION

Within our observations, the indices of energy and lipid metabolism corresponded to the well-known findings reported in fresh cows [2, 8, 9, 13, 14, 16].

The blood serum levels of glucose, which are considered to be a direct indicator of energy balance [38], did not show any significant changes during the entire observation period. However, a more reliable indicator of the cow's energy status is the concentration of β-hydroxybutyrate [41, 46]. Within our observations, a decrease in triglycerides ($P < 0.01$) below the normal range, as well as an increase in the mean serum levels of β-hydroxybutyrate ($P < 0.05$), and non-esterified fatty acids ($P < 0.01$) in dairy cows 3 to 14 days after calving indicated some degree of negative energy balance.

The negative energy balance in the transition period is the key factor determining adaptation of a dairy cow's metabolism [17], including adaptation of the endocrine system, which is crucial in order to maintain the metabolic balance [2]. Hormonal changes in the transition period are characterized by an increase in growth hormone and a decrease in insulin, thyroid hormones and insulin like growth factor (IGF-I) [31]. A similar decrease in the mean serum insulin and thyroid hormones was observed also in our observation. The level of insulin in bovine serum strongly correlates with the body weight increase rate [11, 18, 20] and limited feed intake by the animals results in a decrease in the content of insulin in blood serum [53]. A decrease in insulin after calving is probably related to inappetence, which is typical during the periparturient period [9]. The plasma insulin is additionally known to suppress lipolysis from adipose tissues [19]. However, the plasma insulin concentration in dairy cows is decreased after parturition and enables, along with concomitant insulin resistance and the associated loss of inhibitory effects on lipolysis, the high degree of metabolic priority [14].

Limited food energy content decreases also the serum levels of triiodothyronine (T_3) and thyroxin (T_4) [11]. The thyroid hormones are of importance in adapting the endocrine system during lactation, since their very low blood levels in peri-partal cows leads to a decrease in energy metabolism, mobilization of body fat reserves and their partitioning towards high milk production [21, 29, 48].

Periparturient hormonal changes including thyroid hormones and their relation to lipid metabolism and body condition has been studied by many authors [5, 6, 7, 10, 17, 26, 27, 31, 32, 50].

Kapp et al. [26] suggested an important role of an endocrine disorder, particularly thyroid, in the pathogenesis of liver steatosis in high-yielding Holstein-Friesian dairy

cows. The authors consider "fatty liver" syndrome as a consequence of hypothyreoidosis, when insufficient thyroid function (low serum levels of T_4 and T_3) leads to endocrine dysfunctions, liver disorders and frequent puerperal complications.

A decrease in circulating T_4 and T_3 hormones and functionless thyroid hypertrophy was observed in dairy cows suffering from adipose-hepatic fat syndrome. Presumably, there is protein-energy (or another) deficiency accompanied by obesity [27]. This syndrome is associated with hormonal imbalance and metabolic disorders followed by reproduction disorders (stillbirths, retained placenta, metritis, low fertility).

Durdevič et al. [10] compared T_4 and T_3 levels in the blood serum of cows with and without ketosis. In dairy cows with ketosis they found significantly lower hormone levels: T_4 0.7 ± 0.4 vs. 3.6 ± 1.1 μg.dl^{-1}; T_3 0.83 ± 0.22 vs. 1.22 ± 0.23 ng.ml^{-1}. Similarly, Djokovič et al. [7] studied the blood concentrations of thyroid hormones, lipids, glucose, and liver lipid content in dairy cows during the transitional period. In ketotic dairy cows, they suggested established a hypothyroidal status. Kostopanagiotou et al. [32] reported during acute liver failure markedly decreased serum thyroxin (T_4) and triiodothyronine (T_3) levels, whereas free-triiodothyronine and thyroxin-stimulating hormone levels did not change. T_4 and T_3 levels correlated with the degree of liver failure. Gvozdič et al. [17] reported significantly lower thyroid hormone levels in obese cows. Over conditioned dry dairy cows showed also decreased insulin sensitivity and decreased insulin responsiveness of the glucose metabolism [6], which may contribute to lipomobilisation level.

These data indicate an association between thyroid status, level of lipomobilisation and body condition, respectively. Within all of our observation period, we found a significant negative correlation between T_3 levels and the layer of subcutaneous fat ($r = -0.2606$; $P < 0.05$). This correlation was much more marked in cows 3 to 14 days after calving ($r = -0.5077$; $P < 0.05$). However, there is a question of what is primary and what is secondary — low thyroid status or over conditioning. It is well known that hypothyroidism causes a weight increase together with a decrease in basal metabolic rate [43, 52]. On the other side, obesity also contributes to thyroid dysfunction in a form of mildly elevated TSH levels [51].

CONCLUSIONS

Within our observations, the indices of energy and lipid metabolism indicate some degree of negative energy balance and corresponds to well-known findings reported in fresh cows. The analyses of thyroid hormones and back fat thickness indicate an association between thyroid status, body fat volume, degree of negative energy balance, and the level of lipomobilisation. However, the cause of lower thyroid status in cows with subcutaneous fat needs to be clarified.

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List of abbreviations

ANOVA	analysis of variance
a. p.	*ante-partum* (*pre-partum*)
AST	aspartate aminotransferase
BFT	backfat thickness
BHB	β-hydroxybutyrate
Glu	glucose
I	iodine
IGF-I	insulin like growth factor
p. p.	*post-partum*
T_3	triiodothyronine
T_4	thyroxin
TBil	total bilirubin
TG	triglycerides
TL	total lipids
TMR	total mix ration
TP	total protein
NEB	negative energy balance
NEFA	non-esterified fatty acids
SD	standard deviation
U	urea
VLDL	very low-density lipoproteins
x	mean value

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MALASSEZIA VERSUS *CANDIDA* IN HEALTHY DOGS

Sihelská, Z., Pangrácová Piterová, M., Čonková, E. Harčárová, M., Böhmová, E.

Department of Pharmacology and Toxicology,
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
Slovakia

zuzana.sihelska@uvlf.sk

ABSTRACT

The genera *Malassezia* and *Candida* include yeasts which are members of the normal mycobiota of the skin and mucosal sites of humans and other warm-blooded animals. These yeasts are associated with a variety of dermatological disorders and also systemic diseases in humans and other animals. This study confirms the occurrence of *Malassezia* and *Candida* species in healthy dogs. Samples were collected from different body sites: external ear canal, interdigital area, skin of the axilla and of the neck, and the oral and rectal mucosae. The isolates were identified using phenotypic methods (biochemical-physiological and morphological characteristics). The presence of yeasts were investigated in the specimens from 70 healthy dogs. *Malassezia* species were isolated in 44 dogs from which 84 *Malassezia* isolates were obtained. Only one *Candida* isolate was obtained from the dogs examined. It was found that *Candida* does not occur in dogs normally and *Malassezia* was the main colonizing yeast in healthy dogs.

Key words: *Candida*; dog; incidence; *Malassezia*; mycoses; skin

INTRODUCTION

The most frequent fungal infections in animals are dermatophytosis and dermatomycosis. Dermatophytes are keratinophilic and keratinolytic fungi, characterized by a high affinity to keratin-containing tissues. In dogs, dermatophytosis are most often caused by genera of *Microsporum* and *Trichophyton* [1]. Dermatomycosis are also very frequently diagnosed in veterinary medicine, especially those caused by yeast of the genera of *Malassezia* and *Candida*. The yeasts of these genera are part of an animal's normal flora. However, despite being saprobes, there have been many reports of infections caused by these microorganisms, which present different clinical manifestations.

In dogs, *Malassezia* spp. have been associated with *otitis externa* and *dermatitis* [27, 29, 31] and as a possible aggravating factor in the physiopathology of corneal ulcers [33]. The predisposing factors that determine the formation of *Malassezia* mycoses include: the alteration of the host´s

immune system, greasy skin, high temperature, humidity, corticosteroid treatment and others [2]. Currently, the genus *Malassezia* includes 16 species: *M. globosa, M. restricta, M. slooffiae, M. obtusa, M. furfur, M. sympodialis, M. japonica, M. yamatoensis, M. dermatis, M. pachydermatis, M. caprae, M. equina, M. nana, M. cuniculi, M. brasiliensis and M. psittaci* [7, 8].

Several reports have shown that *Candida* spp. are also important pathogens in dogs, being related to: urinary infections [32], endophthalmitis [26], cutaneous lesions [28], and systemic infections [6]. The group with the most relevant clinical species includes: *C. albicans, C. parapsilosis, C. glabrata, C. orthopsilosis, C. metapsilosis, C. tropicalis, C. viswanathii C. tropicalis, C. krusei, C. guilliermondii, C. lusitaniae, C. dubliniensis, C. pelliculosa, C. kefyr, C. lipolytica, C. famata, C. inconspicua,* and *C. rugosa* [13, 37]. The transition to pathogenicity in *Candida* species, mainly in *C. albicans*, occurs generally due to the host's immunological condition, leading to a microbiota unbalance, which is associated with important virulence factors, such as biofilm formation and hydrolytic enzymes production, and promotes fungal dissemination to other sites and organs. The clinical manifestations vary from a localized mucosal or skin infection to disseminated disease [23].

The purpose of this study was to compare the occurrence of *Malassezia* and *Candida* yeasts on the skin and the mucous membranes in healthy dogs.

MATERIALS AND METHODS

Animals and samples

The survey was carried out on 70 healthy dogs in good general health with no history of skin or ear diseases and with no antimicrobial treatments in the past 12 months. The ages of animals ranged from 4 months to 10 years. From each dog, four samples originating from four different anatomical sites of the body (external ear canal, interdigital area, skin of the axilla and of the neck) were collected by a standard swab method. A sterile cotton swab soaked in sterile saline was used to rub against the skin surface, with continuous rotation of the swab over at least a 10 seconds period. In addition, 20 dogs had samples taken also from oral and rectal mucosae.

Cultivation and microscopy

The samples were inoculated on specific media for the culturing of *Malassezia* and *Candida*: Sabouraud dextrose agar with chloramphenicol (SCH) (HiMedia Laboratories Pvt. Ltd., Mumbai, India), Modified Leeming & Notman agar medium (MLNA) [25], and Modified *Candida*-Chrom agar (HIT) with Tween 40 [21]. The plates were incubated at 32 °C for 7 days. All yeast cultures were identified to the genus level by using conventional mycological methods, including the examination of both macroscopic appearance of the colonies (colonies shape, texture and colour) and the microscopic cell morphology (cell size, shape and budding characteristics). In order to assess the lipid dependency, the colonies were subcultured on Sabouraud dextrose agar. Each isolate was stained by Gram and examined by microscopy for the presence of the typical *Malassezia* and *Candida* yeast cells. More detailed identification of *Malassezia* was performed according to Kaneko et al. [20]. Reference strains *M. pachydermatis* (CBS 1879, Utrecht, Holandsko) (Fig. 1) and *C. albicans* (CCM 8512, Brno, CR) (Fig. 2) were used as positive controls.

RESULTS

Forty four out of 70 examined dogs were scored positive for *Malassezia* spp. (62.9 %). Out of all of the samples only one was identified as a *Candida* isolate which was obtained from the rectal mucosa and identified as *C. albicans*. No *Candida* yeasts were found on the skin. From the samples, a total of 84 *Malassezia* isolates were obtained. All 84 *Malassezia* isolates were identified as *M. pachydermatis* (Fig. 3). The information about the occurrence of yeasts on the skin in the dogs is presented in Table 1. Seventy three isolates were collected from the skin of the following body sites: 36 isolates — external ear canal (51.4 %); 20 isolates — interdigital area (28.6 %); 10 — axilla (14.3 %); and 7 — neck (10.0 %). Six *Malassezia* cultures (30.0 %) were obtained from the oral mucosa and five (25.0 %) from the rectal mucosa (Table 2).

DISCUSSION

In dogs, yeasts preferably colonize moist areas such as mucous membranes, mucocutaneous junctions, skinfolds, interdigital areas and ears. *Candida* and *Malassezia* spe-

Fig. 1. Cultivation of *M. pachydermatis* reference strain on HIT

Fig. 2. Cultivation of *C. albicans* reference strain on HIT

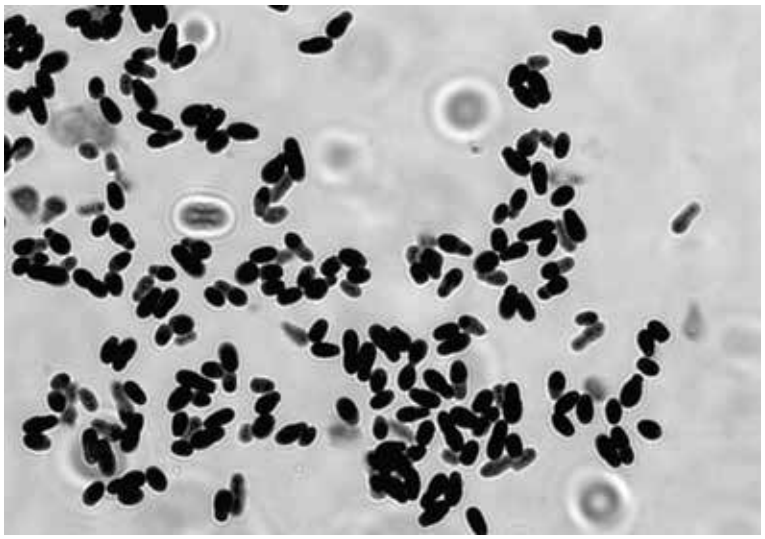

Fig. 3. Cells of *M. pachydermatis* isolate (Axio Observer Z.1, Magn. ×1000).

Table 1. Presence of *Malassezia* and *Candida* yeasts on the skin in dogs (70 animals)

Examined body sites	Number of *Malassezia* isolates	Number of *Candida* isolates
External ear canal	36 (51.4%)	0 (0%)
Interdigital area	20 (28.6%)	0 (0%)
Skin of the axilla	10 (14.3%)	0 (0%)
Skin of the neck	7 (10.0%)	0 (0%)
Total	**73**	**0**

Table 2. Presence of *Malassezia* and *Candida* yeasts on the mucous membrane in dogs (20 animals)

Examined body sites	Number of *Malassezia* isolates	Number of *Candida* isolates
Oral mucosa	6 (30.0%)	0 (0%)
Rectal mucosa	5 (25.0%)	1 (5.0%)
Total	**11**	**1**

cies are ubiquitous organisms existing almost exclusively as commensal organisms, which rarely become pathogenic [18]. A range of skin micro-environmental factors, such as the bacterial microbiota present, pH, salts, immune responses, biochemistry, and physiology, may play a role in the adherence and growth of yeasts, favouring distinct genotypes depending on the geographical area and/or the skin sites [17].

Currently, the results of scientific studies on *Candida* yeasts in animals vary. Brito et al. [5] and Cleff et al. [11] stated, that *Candida* genus is considered to be part of the microbiota of dogs. We did not identify *Candida* yeasts on the skin of our examined dogs. This give rise to the question about *Candida* presence/absence on the skin of dogs. Lee et al. [24] asserted that the presence of *Candida* in a dog is always the expression of a pathologic state and of its intrinsic pathogenicity. Our results indicate that *Candida* spp. is not a member of the normal skin flora. We identified one *Candida* isolate from the rectal mucosa. *Candida* yeasts colonize mostly mucous membrane [16, 34, 35] and their occurrence on the skin is not common and may be bound with outbreak of a disease.

The most *Malassezia* affected animal is the dog [31]. In general, *Malassezia* yeasts are associated with the skin and mucous membranes in healthy and diseased dogs [9, 10, 22, 30, 31]. Even 62.9 % of the dogs are positive for *Malassezia* yeasts. In our study, *M. pachydermatis* was isolated as the sole species in all dogs examined. Generally, lipophilic *M. pachydermatis* is the main colonizing yeast in healthy and also in diseased dogs [31]. Only a few times lipid-dependent *Malassezia* yeasts (*M. furfur, M. nana* and *M. obtusa*) were identified [11, 12, 14, 15, 36].

In our group of dogs, the external ear canal was the most frequently colonized (51.4 %). However, Nardoni et al. [30] appointed the frequency of *Malassezia* isolation: interdigital area (70.7 %), ears (63.4 %), axilla (23.8 %), and perineum and anus (19.0 %). Cafarchia et al. [9] stated that the perianal region is the most frequently colonized and in the ear canal is 12.1 % of all *Malassezia*. But Kumar et al. [22] detected *Malassezia* yeasts in 39.39 % of healthy ears and Campbell et al. [10] represented 17 % prevalence of *Malassezia* in healthy ears. The occurrence of *Malassezia* on the different body sites varies and depends on: the group of examined dogs, season, type of hair and ear and other factors.

Malassezia yeasts have been isolated from different mucosal sites from healthy dogs. In our group, *Malassezia* occurred on the oral mucosa in 30.0 % of the samples and on the rectal mucosa in 25.0 % of the samples. Hajsig et al. [19] isolated this yeast also from the anus, anal sacs, and vagina of healthy dogs. Bond et al. [4] recovered *M. pachydermatis* from the anus and mouth of 27.5% of healthy dogs of various breeds. However, Bond and Lloyd [3] suggested that in some cases, mucosal colonization may be secondary to skin proliferation of the lipophilic yeasts.

In conclusion, *Candida* spp. does not occur on the skin in healthy dog normally. The presence of this yeast is linked rather with the mucosae. On the skin and mucosa is the highest occurrence of *Malassezia* yeasts and *M. pachydermatis* remains the most prevalent species in the healthy dog.

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COLLECTION OF *LISTERIA MONOCYTOGENES* ISOLATES FROM MILK, DAIRY PRODUCTS AND FOOD PROCESSING ENVIRONMENTS IN SLOVAKIA FOR THE PURPOSES OF EUROPEAN MOLECULAR DATABASE

Kubicová, Z., Filipová, M., Jurovčíková, J., Cabanová, L.

State Veterinary and Food Institute, Veterinary and Food Institute in Dolný Kubín
Jánoškova 1611/58, 026 01 Dolný Kubín
Slovakia

miriam.filipova@svpu.sk

ABSTRACT

The molecular typing of *Listeria monocytogenes* isolates is an important tool for monitoring the spread of the strains in food chains, providing evidence for epidemiological investigations and for the detection of outbreaks. The demand of European typing data centralization, collection and sharing stimulated the generation of "EURL *L. monocytogenes* Database (EURL Lm DB)" in 2012 led by the European Union Reference Laboratory (EURL) for *L. monocytogenes* (ANSES Maisons-Alfort Laboratory for Food Safety, France) in close collaboration with Applied Maths. This database includes the typing results and epidemiological information on strains isolated from food, environmental or animal samples and it is in connection with human strains database TESSy (The European Surveillance System) led by the ECDC (European Centre for Disease Prevention and Control). In total 147 *L. monocytogenes* isolates were examined by PFGE (pulsed field gel electrophoresis) in 2014—2015 in VFI Dolny Kubin from different sources. Nearly half (68) of the 147 isolates in the national Slovak database came from milk or dairy products samples and the related manufacturing environment. In this work, 68 isolates associated with milk were selected and divided into 27 clusters (95 % similarity level) after combined comparison analysis (AscI and ApaI) by BioNumerics 6.6 software. Eight clusters included three or more similar PFGE profiles.

Key words: database; *Listeria monocytogenes*; molecular typing; PFGE

INTRODUCTION

The molecular typing of bacterial DNA by different methods (pulsed field gel electrophoresis — PFGE, multi locus variable-number tandem repeat analysis — MLVA, whole genome sequencing — WGS) has become a standard process of pathogenic foodborne bacteria characterization. The PFGE typing of foodborne microorganisms is very important for surveillance purposes, especially monitoring the spread of strains in food chains, ensuring evidence for

epidemiological investigations and revelation of national or international outbreaks. PFGE is regarded as the "gold standard" among typing methods [6].

A surveillance network based on PFGE method including food and clinical isolates (PulseNet) has been used in USA and Canada for many years and it helped to detect numerous outbreaks [1, 2, 3]. PulseNet Europe was created with the same aim in 2003 [8], but it was cancelled in 2006 due to lack of funding [9]. In 2012 the European Centre for Disease Prevention and Control — ECDC developed a pilot Molecular Surveillance System (MSS) as a component of The European Surveillance System — TESSy. The scope of this databasing system is to share epidemiological information and molecular typing data on *L. monocytogenes* strains isolated in cases of human disease [10]. However, there was a constant need to collect and share information on European *L. monocytogenes* isolates sourced from food, animal and environmental samples. This need was satisfied in 2012 by the creation of "EURL *L. monocytogenes* Database (EURL Lm DB)" led by European Union Reference Laboratory (EURL) for *Listeria monocytogenes* (ANSES Maisons-Alfort Laboratory for Food Safety, France).The National Reference Laboratories (NRLs) across Europe can store and also share their molecular and epidemiological data on *L. monocytogenes* strains isolated from food, animal and environmental samples. The principle of data sharing is based on internet communication using BioNumerics software (Applied Maths Saint-Martens-Latem, Belgium) [5].

MATERIALS AND METHODS

In sum, 147 *L. monocytogenes* isolates from different sources were typed by PFGE in NRL for *L. monocytogenes* at the Veterinary and Food Institute (VFI) in Dolný Kubín during 2014—2015. However, this study was focused on 68 isolates from milk, dairy products and related food processing environments. The isolates originated in official control samples or from samples of producer's self-control. All *L. monocytogenes* isolates were analysed according to the EURL protocol [7]. Agarose plugs with bacterial DNA were prepared and restriction enzymes AscI and ApaI (Thermo Scientific, USA) were used for PFGE profiles production. Electrophoresis was performed in 1 % agarose (SeaKem Gold Agarose, Lonza, USA) on a CHEF Mapper® XA (Bio-Rad, USA). *Salmonella* Braenderup H9812 DNA cleaved with XbaI enzyme (Thermo Scientific, USA) was used as a reference system. The molecular profiles obtained by the PFGE procedures were analysed by BioNumerics v6.6 software (Applied Maths, Belgium) using the Dice coefficient and unweighted pair group method (UPGMA) with arithmetic mean analysis, with "optimization" and "tolerance" settings of 1 %. The inclusion of PFGE profiles into one group — cluster — was done according to 95 % similarities among ApaI/AscI compared profiles.

RESULTS

From 2014 to 2015, a total of 147 *L. monocytogenes* from different sources were examined by PFGE in NRL for *L. monocytogenes* at VFI Dolný Kubín. These milk-associated isolates (milk, dairy products and related manufacture environments) formed nearly half (46.26 %) of all investigated isolates. The combined clustering analysis of 68 ApaI/AscI profiles resulted in 27 clusters with 95 % similarity.

Only 8 of the 27 clusters contained 3 or more identical, similar or nearly similar PFGE profiles (Fig. 1, Table 1). All 8 clusters were comprised of 44 isolates in total. The biggest cluster IV was composed of *L. monocytogenes* isolates from one specific source — sheep (sheep milk — 2 samples, products from sheep milk — 8 samples and specific sheep milk processing environments — 2 samples), and from one manufacturer. Conventional serotyping classified all 12 isolates into 1/2a serogroup. The epidemiological data of the other 7 clusters (I.—II., V.—VIII.) were also investigated in detail, but there was no confirmation of either the presence of isolates from the same source and time, or the connection with one manufacturer.

The remaining 19 clusters were formed only by one or two isolates per cluster and were comprised of 24 isolates in total (data not shown).

DISCUSSION

A surveillance of the *L. monocytogenes* spread in food chains is an essential part in preventing disease and monitoring public health issues. The surveillance may include sampling of food or environments for the presence or absence of the organisms which may contribute to the identification of the risky food batches or identify colonization of

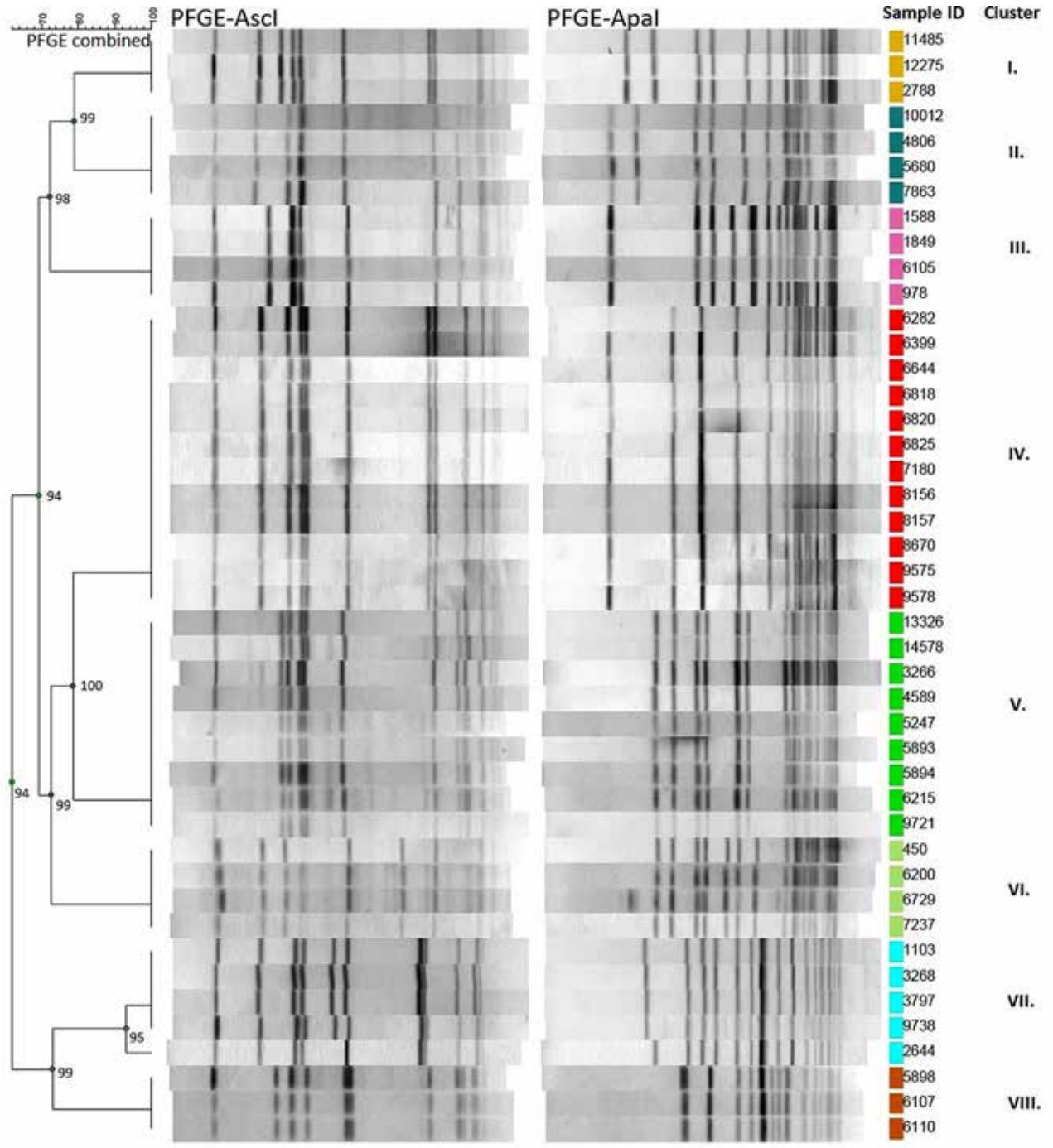

Fig 1. Similarity dendrogram of 8 PFGE clusters identified in the positive samples of milk, dairy products and related food processing environments

Combined analysis of AscI and ApaI profiles of 44 *L. monocytogenes* strains resulted in 8 clusters (I.—VIII.). Cluster IV was composed of 12 *L. monocyogenes* samples isolated from a specific source and one manufacturer. Despite the profiles similarity, all of the other clusters did not show a connection either with the source and time of sampling or with one producer

Table 1. Detailed epidemiological data concerning the samples of 8 PFGE clusters

Cluster	Sample ID	Conventional serotype	Source	Sample category	Sampling date
I.	11485	1/2a	Sheep	Cheese category not specified	2014-09-30
	12275	1/2a	Sheep	Soft cheese	2014-10-17
	2788	1/2a	Sheep	Cheese category not specified	2014-03-28
II.	10012	1/2a	–	Food processing environment	2014-08-26
	4806	1/2a	Sheep	Cheese category not specified	2014-05-13
	5680	1/2a	Sheep	Cheese category not specified	2024-05-26
III.	7863	1/2a	Sheep	Cheese category not specified	2015-07-16
	1588	1/2a	Bovine	Semi soft cheese	2015-03-05
	1849	1/2a	Sheep	Cheese category not specified	2014-03-07
	6105	1/2a	–	Food processing environment	2014-06-03
IV.	978	1/2a	Bovine	Cheese category not specified	2015-02-17
	6282	1/2a	Sheep	Soft cheese	2015-06-16
	6399	1/2a	Sheep	Cheese category not specified	2015-06-17
	6644	1/2a	Sheep	Soft cheese	2015-06-23
	6818	1/2a	–	Food processing environment	2015-06-24
	6820	1/2a	–	Food processing environment	2015-06-24
	6825	1/2a	Sheep	Milk	2015-06-24
	7180	1/2a	Sheep	Semi soft cheese	2015-07-01
	8156	1/2a	Sheep	Semi soft cheese	2015-07-23
	8157	1/2a	Sheep	Soft cheese	2015-07-23
	8670	1/2a	Sheep	Cheese category not specified	2015-07-29
	9575	1/2a	Sheep	Soft cheese	2015-08-21
	9578	1/2a	Sheep	Milk	2015-08-21
V.	13326	1/2a	Sheep	Soft cheese	2014-11-10
	14578	1/2a	Sheep	Soft cheese	2014-12-11
	3266	1/2a	Sheep	Cheese category not specified	2015-04-16
	4589	1/2a	Sheep	Soft cheese	2014-05-07
	5247	1/2a	Sheep	Soft cheese	2014-05-20
	5893	1/2a	Bovine	Semi soft cheese	2011-05-28
	5894	1/2a	Sheep	Milk	2014-05-26
	6215	1/2a	Sheep	Soft cheese	2014-06-05
VI.	9721	1/2a	Bovine	Other dairy product	2014-08-25
	450	1/2a	Bovine	Semi soft cheese	2014-01-28
	6200	1/2a	Bovine	Semi soft cheese	2014-06-05
	6729	1/2a	Bovine	Semi soft cheese	2014-06-17
	7237	1/2a	Bovine	Semi soft cheese	2014-06-25
VII.	1103	4b	Bovine	Semi soft cheese	2014-02-11
	3268	4b	Bovine	Semi soft cheese	2015-04-16
	3797	4b	Bovine	Semi soft cheese	2015-04-28
	9738	4b	Sheep	Cheese category not specified	2015-08-26
	2644	4b	Sheep	Cheese category not specified	2015-03-30
VIII.	5898	1/2b	Bovine	Semi soft cheese	2014-05-28
	6107	1/2b	–	Food processing environment	2014-06-03
	6110	1/2b	–	Food processing environment	2014-06-03

environments (farms) with organism and potential cross-contamination of the food. Using more advanced tools (strains serotyping and molecular typing by PFGE), better epidemiologic understanding can be achieved. 46.26 % occurrence of milk associated isolates in a total 147 investigated *L. monocytogenes* isolates is consistent with the fact that milk and dairy product are one of the most frequent source of listeriosis [4]. The combined analysis of AscI and ApaI patterns of 68 *L. monocytogenes* strains isolated from dairy product and related environments resulted in strain discrimination into 27 clusters (95 % similarity level). After the selection of clusters with 3 or more profiles, 44 samples were grouped into 8 clusters and one of them (cluster IV) showed the close relationship between samples. All 12 samples from cluster IV were identified to be from one source and one producer. In this case, the subtyping data of *L. monocytogenes* isolates facilitated finding the source of *L. monocytogenes* presence in final dairy products and, of course, allowed the producer to optimize the technical and sanitation measures to be taken to ensure hygiene of the food production.

Moreover, all PFGE profiles were sent for curation processing in EURL Lm DB. The majority of them were accepted and involved in the transnational database for sharing with other European countries.

In Slovakia, there have been only a few similar studies focused on solving the secondary contamination problems in food producers using modern typing methods. In one of them, Veghova et al. [11] examined 20 *L. monocytogenes* strains isolated from sheep milk products and related food processing environments. In contrast with our results, the dominated serogroup in the collection was IIa. The authors identified 14 clusters at a similarity level of 100 % without confirmation of epidemiological clone ECI-ECIII. This high strain similarity suggested the external environment as an origin of contamination [11]. Using molecular tools on national and international levels one can better understand the potential route of disease transmission through the food chains. Databasing system based on PFGE in NRL for *Listeria monocytogenes* in Slovakia is connected with European databasing system and enables as to compare profiles from all participating countries, not only in the case of disease outbreak, but also for surveillance purposes and epidemiological investigations.

CONCLUSIONS

L. monocytogenes is a serious foodborne pathogen in human health, and also has important economic influence due to its persistence in food processing environments and potential food product contaminations. In the case of its source and route of contamination detection, suitable analytical method, such as molecular typing, needs to be used. In our surveillance, we referred the potential source and route of milk and dairy products contamination by the identification a cluster (cluster IV) of *L. monocytogenes* isolates from a specific source (sheep milk, products from sheep milk and related food processing environments) and from one producer. However, another 7 clusters mentioned in Fig. 1 did not show a connection in the context of source and season of contamination or coherence with a specific manufacturer. This example points out the importance and advantages of molecular typing and creating a national database of molecular profiles. Using national databasing systems we were able to search and compare molecular profiles collected through the years. Joining the European databasing system allows the comparison of molecular data with all participating countries and brings benefits during disease outbreak detection, epidemiological investigation and it is also helpful in the case of monitoring and surveillance programmes.

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POTENTIAL ROLE OF GUTTURAL POUCHES IN GAS EXCHANGE

Kovalyova, I. M.[1], Melnyk, O. P.[2], Kresakova, L.[3], Vdoviakova, K.[3]

[1]Department of Evolutionary Morphology, Schmalhausen Institute of Zoology
National Academy of Sciences of Ukraine, Kyiv
[2]Department of Animal Anatomy, Faculty of Veterinary Medicine, National University
of Life and Environmental Sciences of Ukraine, Kyiv
Ukraine
[3]Department of Anatomy, Histology and Physiology
University of Veterinary Medicine and Pharmacy in Košice
Slovakia

lenkakresak@gmail.com

ABSTRACT

Despite the obvious existence of guttural pouches in *Equidae*, the question of their function has not yet been adequately answered. We suggest a working hypothesis that the guttural pouches of horses may be an additional organ of gas exchange. Research on the topographical location of the guttural pouches of horses and on the micro-morphological structures of their walls were carried out. It appears possible that: the dense arrangement of the vascular system of the wall of the guttural pouches near the main cerebral vessels; the constant moistening of the inner surface of the wall of the guttural pouches; and the air circulation in it; strongly suggests that the guttural pouches of horses may serve as an additional organ of gas exchange. The guttural pouches becomes very useful, particularly during prolonged periods of intense physical activity.

Key words: gas exchange; guttural pouch; histological structure; horse; vascularization

INTRODUCTION

Unlike many other mammals, one-hoofed (*Equidae, Mammalia*) animals have guttural pouches (diverticulum tubae auditivae), which are ventro-lateral outgrowth of the mucosa of the auditory tubes (tuba auditiva, s. Eustachii). Despite the acknowledgement of the existence of such well defined anatomical structures, the question of their physiology has not yet been definitively answered. Akaevsky [1] believe that the guttural pouches have developed due to an increase in the mobility of the occipito-atlantic joint in one-hoofed animals. Another author noted that such structures are necessary for the balancing of the pressure during more intensive locomotion activity (unlike other animals) [11]. Others believe that the guttural pouches serve to cool the blood that goes to the brain during prolonged physical activity [6, 7]. However, the last statement was rejected folowing the conduction of experimental studies [10]. Thus, until the present day, the function and origin of the guttural pouches of horses remains unknown. The conduction of our own research has demonstrated that the wall of the guttural pouches of the horse has a special

structure — dense vascularization with a large surface area and thin blood-gas barriers [4]. Such a structural arrangement in the organ's wall which is filled with atmospheric air, give it an opportunity to participate in gas exchange in conditions of constant air circulation. In this regard, we suggested a working hypothesis that the guttural pouches of horses may be an additional organ of gas exchange. We conducted a biomechanical study in order to seek support of this hypothesis and also to trace the possible evolutionary pathway of the development of this organ in the horse.

MATERIALS AND METHODS

The materials for this study (15 heads of horses Equus caballus) was received from the funds of the Department of Animal Anatomy named after academician V. G. Kasyanenko of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine. The research was conducted on sagittal cuts of the horses' heads. The topography of the guttural pouches and their surrounding structures were studied. The air pathway in the guttural pouches and nasopharynx was analyzed. Samples of separate parts of the guttural pouch walls (size 1 × 1 cm) were fixed in 10 % formalin. They were embedded in paraffin blocks and were cut (thickness 20 μm) by a microtome-cryostat MK-25'Technologist' (Russia). Thin serial sections (up to 8 μm) of the paraffin blocks with the fixed material in the longitudinal and transverse directions were cut by using the microtome MC-2 (Ukraine). These sections were stained by hematoxylin-eosin by Ehrlich, hematoxylin by Van Gison, trichrome by Mallory and azure-2-eosin and analyzed by using the optical microscope Axio Imager M1 Karl Zeiss (Germany) with a built-in camera.

RESULTS

Topography

On the sagittal sections of the horse's heads, it was determined that the guttural pouches were located in the postero-jaw part of the parotid area. The anterior border of the pouch was situated 5 cm rostral from the posterior edge of the mandibular branch, and posterior border — on a line that connected the extremity of the jugular process of the occipital bone with the mandibular angle (Fig. 1).

The right and left guttural pouches touched each other with their medial walls, except at their apexes, where the pouches were divided by straight muscles of the head. The medial walls of the right and left pouches were tightly appressed to each other like forming a single structure that was gathered in folds in a free state.

The ventral wall of the guttural pouch was not attached to the bones of the skull. It was located near the dorsal wall of the pharynx. The bigger branch of the hyoid bone (stylohyoid) was pressed into the ventral wall of each pouch and divided it into a larger medial and smaller lateral parts that were connected with each other in the dorsal area.

The dorsal surface of each pouch was laying near the base of the skull, atlanto-occipital joint, atlas, straight muscles of the head, and the long flexor of the head. On its caudal surface, the main blood vessels with their branches that supply blood to the brain and brain tunic were located: occipital (*a. occipitalis*) and internal carotid (*a. carotis interna*) arteries. The ventral cerebral vein (*v. cerebralis ventralis*) also passed here and the glossopharyngeal, vagus, accessory and sympathetic nerves were located nearby.

Laterally, the guttural pouches were adjacent to the end of the common carotid artery (*a. carotis communis*) and its branches.

Thus, the dorsal walls of the right and left guttural pouches were attached to the bones of the skull, while the ventral and medial walls remained "free". The external surface of these areas of the pouches were located on the dorsal wall of the pharynx.

The slot-like foramina of the auditory tubes were located on both sides of the lateral walls of the oropharynx near the posterior nares. The medial edges of these foramina were fortified by chondral-like structures which prevent the closure of the foramina. On the ventral surface of the auditory tubes (immediately behind their foramina in the oropharynx) were openings that lead into the cavities of the guttural pouches. Thus, the air flowing out of the choanae, flows through the foramina of the auditory tubes into the guttural pouches (Fig. 1).

Micromorphology (histomorphology)

The general thickness of the wall of the guttural pouches was from 800 to 1000 μm. The wall was formed by an inner mucous membrane, an inner layer of connective tissue and an outer adventitial covering (Fig. 2a).

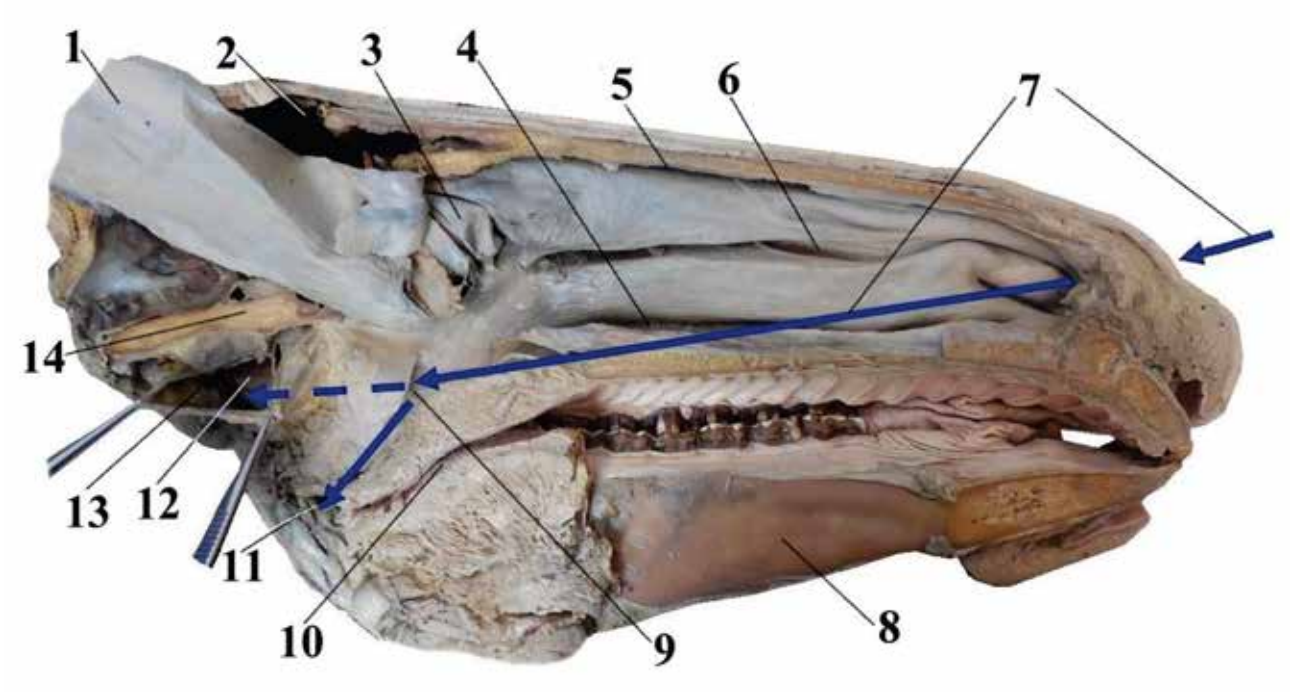

Fig. 1. Topographical location of the guttural pouch of horse and surrounding structures
1 — nasal septum (*septum nasi*), 2 — frontal sinus (*sinus frontalis*), 3 — ethmoid bone (*os ethmoidale*), 4 — ventral nasal meatus (*meatus nasi ventralis*), 5 — dorsal nasal meatus (*meatus nasi dorsalis*), 6 — middle nasal meatus (*meatus nasi medius*), 7 — path of the air flow in the guttural pouch, 8 — lower jaw (*mandibula*), 9 — foramen in the auditory (*Eustachian*) tube (*ostium tubae auditivae*), 10 — oropharynx (*pars oralis pharyngis*), 11 — path of the air flow in nasopharynx (*meatus nasopharyngeus*), 12 — stylohyoid (*stylohyoid*), 13 — cavity of the guttural pouch (*diverticulum tubae auditivae cavum*), 14 — base of the skull (*basis crania*)

The mucous membrane was covered with simple epithelia. The epithelial cells were cuboidal, columnar or squamous. The form of epithelial cells depended on their location in the mucosa, as well as on the area of the guttural pouches covered by the mucous membrane. Most of the general surfaces of the pouch was covered by elongated columnar epithelial cells appearing like cilia (Fig. 2a). The nuclei of these cells were located at the base of the cells. The flat squamous epithelial cells covered the mucous membranes of the guttural pouches in the places of their attachment to the bones of the skull (Fig. 2b). Sometimes, under the row of the superficial epithelial cells (columnar or squamous) cuboidal epithelial cells with centrally located nuclei were found.

Lymphoid formations were frequently found on the inner surface of the pouch — mature and immature lymph nodules without membranes in the form of islets which were formed by such accumulation of lymphoid cells (Fig. 2c). Their surface was covered by a number of ciliated epithelial cells (Fig. 2a).

The middle layer of the connective tissue was situated under the epithelial layer (Fig. 2a). There were numerous plexiform collagen and elastic fibers, large and small blood vessels, glands, among which the networks of lymphatic and blood capillaries with lymphocytes and blood cells were presented (Fig. 2b, d, e). The inner layer of the connective tissue formed numerous micro-folds that increased the total area of the inner surface of the guttural pouch and probably allowed the pouch to increase in its volume (Fig. 2f).

There were simple mucous and complex multicellular glands in the layer of the connective tissue. Branched glands of an alveolar type with ducts occurred among the multicellular glands (Fig. 2d, f). The ducts of the glands had thin walls that were covered with low cuboidal epithelium. These mucous glands secreted their products on to the inner surface of the pouch's wall and kept the inner surface of the pouch moist.

In the parts of the pouch attached to the skull bones, the internal epithelial layer of the mucosa, as well as the middle

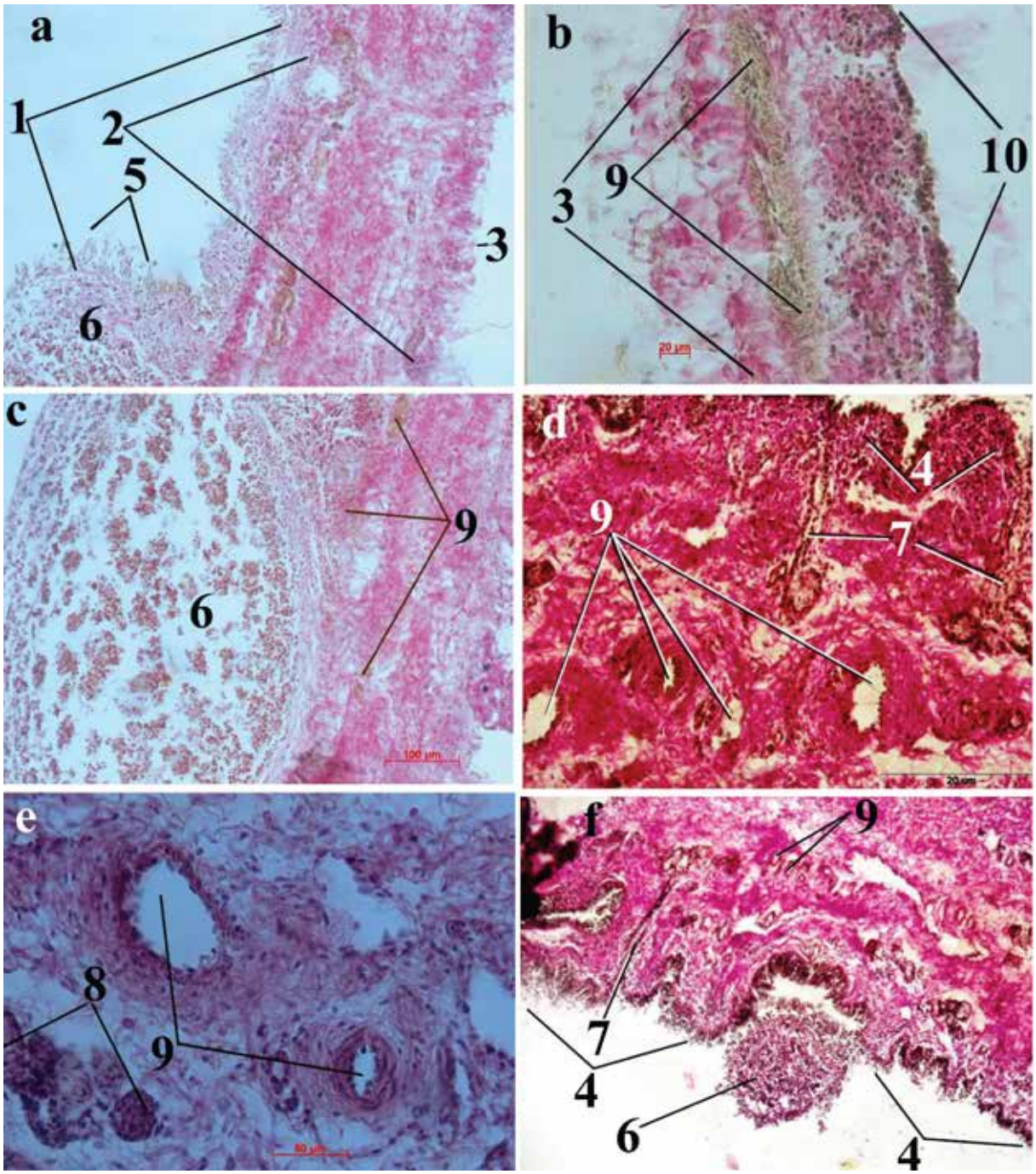

Fig. 2. Histological structure of the wall of the guttural pouch of the horse, × 400

Cross-section; **a.** Three layers of the "free" wall of the guttural pouch: inner epithelial, middle and external connective tissue. Dyeing by Van Gison; **b.** Squamous epithelial cells. Middle layer of the connective tissue of the pouch is much thicker than in the free areas of the pouch. Large and small diameter blood vessels on the external surface of the pouch. Dyeing by Van Gison; **c.** Lymphoid nodule filled with lymphocytes. Large and small diameter blood vessels on the external surface of the pouch. Dyeing by Van Gison; **d.** Blood vessels in the wall of the pouch. Secretory ducts of the glands have thin walls covered by simple low cuboidal epithelium. Dyeing by Van Gison; **e.** Blood vessels in the wall of the pouch. Dyeing with hematoxylin-eosin by Ehrlich; **f.** Middle layer of the connective tissue formed in the folds. Lymphoid nodule filled with lymphocytes. Secretory ducts of the glands have thin walls covered by simple low cuboidal epithelium. Dyeing by Van Gison. 1 — epithelial layer of the inner surface of the pouch (simple squamous epithelium), 2 — middle layer of the connective tissue, 3 — external layer of the pouch (adventitial covering), 4 — folds of epithelium, 5 — ciliated epithelium, 6 — lymphoid nodule with lymphocytes, 7 — secretory duct of the gland, 8 — complex gland of the alveolar type, 9 — blood vessels (arterioles, venules) and capillaries with erythrocytes, 10 — squamous epithelial cells

layer of the connective tissue, were much thinner than in the free parts of the pouch; also, the middle layer had no complex glands (Fig. 2b).

The external layer of the guttural pouch was covered by an adventitial tunica with blood vessels with large and small diameters (Fig. 2b, c). Unlike the inner and middle layers, the outer layer had no glands.

DISCUSSION

The micromorphological studies revealed the three-layered structure of the wall of the guttural pouches of the horse. The epithelial layer of the mucosa consisted mostly of single- or multi-layered epithelium. According to Sadovsky [5], the inner surface of the pouch was covered with simple squamous epithelium. We found such structure mainly in areas of the pouch attached to the bones of the skull or on the surface of lymphoid nodules. Sisson [11] indicated that the inner surface of the pouch was formed by columnar epithelial cells forming elongated papillae, and therefore they acted as cilia. Our study confirmed these observations: "that these papillae" are cytoplasmic elongations of epithelial cells of the external row of mucosa cells which spread to the inner surface of the pouch.

The basis of the middle layer of the connective tissue of the guttural pouch was composed of collagen and elastic fibers. Therefore, the pouch had a stable form, but at the same time it had the ability to stretch, especially in the «free» parts of the pouch (ventral and medial walls) that do no attach to the bones of skull. In these areas, a stratified (multi-layered) epithelium of the mucosa was collected in wrinkles — micro-folds. Such structure of the guttural pouch's wall would allow the pouch to change in volume. The changing of the volume could occur when the air flowed in and out of the guttural pouch and that an aeration of the internal environment of the pouch could be observed.

In the middle layer of the pouch, numerous blood vessels of different diameters and lymph vessels were found. The capillary network was located close to the epithelial cells of the inner surface of the pouch and could create conditions for gas exchange between the capillary blood and pouch's cavity air.

In our study, mucous glands of complex shape were located in the middle layer of the pouch. Their secretions flow via the ducts on to the inner surface of the pouch. It appears that the constant moisture of the inner surface of the pouch's wall could contribute to the process of gas exchange.

We found blood vessels entering the head running along the guttural pouch's wall in the adventitia. As was mentioned above, on the caudal surface of the guttural pouch, the occipital and internal carotid arteries were located and on the lateral surface of the guttural pouch, the final part of the common carotid artery and its branches were present.

In our opinion, the close location of the vascular network in the wall of the guttural pouch to the cerebral vessels and their branching may contribute to gas exchange between the blood of vessels of the guttural pouch and blood of the vessels that surround the brain. Due to this, as the blood passed through the capillaries of the guttural pouch's wall, it could probably become oxidized when a portion of fresh air entered the pouch and then enters the blood vessels supplying the brain. This pathway could be accepted as an additional way for oxygen to supply the brain.

Our hypothesis about the participation of the guttural pouch in gas exchange of animals differs from previous hypotheses of other researchers [2, 7, 11]. Our hypothesis is based not only on the materials of our macromorphological and histological examinations, but also in terms of biomechanics.

It is known that the wall of the guttural pouches of the horse arose from protrusions of the mucosa of the auditory tubes. Obviously, such protrusion was formed under the pressure of a substance (air or liquid). It is hard to imagine the origin of such an amount of liquid that would press the wall of the auditory tubes and form this protrusion. Meanwhile, air that enters the animal's nostrils and passes through the nasal passages may produce a considerable pressure during the animal's intensive breathing. It seems that while the animal is running, its breathing is very active and the air speed in the air-passages is significant. Moreover, the duration of the air pressure on the auditory tube's wall is very important. It is known that horses can run for several hours.

We believe that the air flow in the guttural pouches of the horse is determined by the topographic position of the pouches and their openings. A foramina that leads to the guttural pouches are located in the auditory tube in the lateral wall of the nasopharynx near the choanae. This means that in horses, these foramina are located directly along

the air passage way (Fig. 1). While leaving the choanae, air flows into the nasopharynx and, if the foramina to the auditory tube is open, the air can flow with a high pressure on the elastic wall of the auditory tube.

We have noted that in most animals, the foramina that leads into the auditory tubes opens only temporarily (e. g. while swallowing). Unlike other mammals, in horses the foramina of the auditory tubes (*ostium tubae auditivae*, *s. ostium pharyngeum tubae*) are always open, due to cartilage plates in the medial walls of the foramina [5]. Our research confirmed the presence of such structures. Indeed, this cartilage plate prevents the closure of the foramina that leads to the auditory tube. Thus, the air that freely leads into the auditory tubes, are also the paths to the guttural pouches through a special opening that leads from the auditory tubes into the guttural pouches.

In addition, a characteristic feature of horses is the presence of a long soft palate (*palatum molle*, *s. velum palatinum*); its arch touches the root of the tongue near the base of the epiglottis. The epiglottis (cartilage epiglottis), in turn, is also elongated and has a leaf shape. Thus, there are conditions under which air that passes from the choanae through the nasopharynx does not enter the oral cavity of the pharynx and flows directly into the larynx. Due to such structure of the soft palate and epiglottis, horses, unlike most other mammals, breathe only through the nose and cannot breathe through the mouth. In addition, this fact contributes to the powerful directed airflow.

It is interesting that a group of mammals from the family of horseshoe bats (*Rhinolophidae, Chiroptera*) that are supposed to be evolutionarily far from one-hoofed mammals has a similar structure of the larynx and soft palate that lead to the same result — horseshoe bats breathe only through the nose [1]. The peculiarity of horseshoe bats (unlike many other mammals) is that in the wall of the cranial part of their trachea, two paired and one unpaired protrusions are formed, whose origin is still unclear. In our opinion, they were formed under the influence of turbulent air flow that entered the larynx through a very short passage. It is known that in horseshoe bats, unlike, for example, vesper bats (*Vespertilionidae*), nasal passages are very short [3].

In our opinion, under the directional air flow (mentioned above), the structures of the respiratory tracts and adjacent organs were formed; particularly the structural peculiarities of the auditory tubes, whose walls formed saccular protrusions, i. e., guttural pouches. It is possible that such topography of the foramina of the auditory tubes and choanae were inherited by morphogenetic changes that occurred in the ancestors of one-hoofed animals.

We have noted that the possibility of free air passage in the guttural pouches of a horse does not exclude inflectional contamination and pollution in the form of dust with the air flow. The literature contains many references about horse diseases that have occurred due to inflammation in this area [8, 9]. This explains a large number of lymphoid formations and mucous glands in the wall of the guttural pouches. The fluid accumulating in the cavity of the guttural pouch, along with pollution, flows out freely through the opening of the pouch. Certainly, it helps to clean the internal environment of the pouch.

Each compression of the ventral surface of the guttural pouch helps to release air from it (e. g. during flexion of the head), followed by a new portion of air actively entering into the pouch.

Thus, based on: the general anatomy; topography of the guttural pouches and their surrounding structures; elasticity of their wall and the function of adjacent structures in horses; it can be assumed that the air circulation and renewal of gas composition in the guttural pouches (especially during the active locomotor loads of the animal) all of these factors, gas exchange could occur quite easily.

CONCLUSIONS

It seems that: the dense arrangement of the vascular system of the wall of the guttural pouches near the main cerebral vessels; the constant moistening of the inner surface of the guttural pouch's wall; and air circulation in it, strongly suggest that the guttural pouches of horses may serve as an additional organ of gas exchange. Therefore, the guttural pouches becomes very useful, particularly during the prolonged physical activity of animals.

Our hypothesis can be checked by conducting a new series of experiments based on the assumption of the possible utilization of sophisticated modern research methods. If independent researchers used animals in a static state to lend credence to our hypothesis, then in order to prove our hypothesis, observations on animals in dynamic locomotion at different speeds should be designed. Special remote micro detectors (previously attached to the inner wall of the guttural pouch of a horse) which record the passage

of air in two directions and analyze its gas composition by transmitting the data to a processor should be used for supporting our hypothesis.

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