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EFFECT OF SIMULATED MICROGRAVITY ON THE STRUCTURE OF THE VESTIBULAR APPARATUS IN JAPANESE QUAIL

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ABSTRACT

One of the model experiments of simulated microgravity on the Earth is experimental hypodynamy. We studied the effect of experimental hypodynamy on the morphological structure of the vestibular apparatus of Japanese quail. Morphological changes in the sensory epithelium of chicks reared from 1 to 14 days of age under hypodynamy conditions were evaluated. There were differences in shape and arrangement of the *macula utriculi* sensory epithelium hair cells of birds exposed to hypodynamy on day 14 in comparison with the controls.

Key words: hypodynamy; morphology; sensory epithelium

INTRODUCTION

The vestibular system detects head movements such as angular rotation, translation, and head position relative to gravity. It acts to stabilize the eyes and posture through subcortical reflexes. Its signals are also integrated at the cortical level to participate in the elaboration of a body scheme, used for different functions such as spatial orientation and motor control (11). The hair cells, in turn, transduce these stimuli into electrical signals which are transmitted to the brain (1).

One of the model experiments is hypodynamy. Hypodynamy is the most frequently used ground-based model to study the negative consequences of microgravity on an animal organism (9). Hypodynamy can be used to simulate weightlessness in laboratory conditions. (10). Macular synapses of the vestibular apparatus are sensitive to stress in space (4). Information about the status of balance that is provided by the sensory epithelium of the *utriculus and sacculus* and by proprioreceptors is getting disrupted under the influence of micro-gravitation (2). Some ultrastructural abnormalities were found in the inner ear of adult rats after 20 days of weightlessness (12). The mean number of synaptic ribbons in hair cells increased significantly in weightlessness (6).

In the study presented here, we investigated the effect of simulated microgravity on the morphological structure of the vestibular apparatus of Japanese quail reared under hypodynamy from hatching up to 14 days of age.

MATERIAL AND METHODS

Ten healthy 1-day-old male Japanese quail chicks (Laying Line 01 Ivanka pri Dunaji) were randomly assigned into experimental (n = 5) and control (n = 5) groups. Birds from the experimental group were exposed to hypodynamy and control birds were placed on the floor in a rearing box. The method by which hypodynamy simulates weightlessness is that birds are placed in special individual slings suspended in a flexible metal device so that their legs cannot touch the floor (8).

Japanese quails were exposed to the effects of hypodynamy from their 1st day of life, i.e. after their hatch/incubation, to their 14th day of age. At 5 and 14 days of age, one bird from each group

was decapitated and the inner ear tissue sample was taken for histological analysis of the vestibular apparatus. Samples for light microscopy were fixed in 3% glutaraldehyde for 3 hours, at 4°C temperature. Then the samples were washed in cacodylate buffer at pH 7.2, decalcified in Chelatone, again washed in cacodylate buffer and post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer, dehydrated in acetone and propyloxide and embedded in Durcupan ACM. For light microscopy we used the half-thin 1μm sections. We took photos with the light microscope (JENAMED) with an attached camera.

RESULTS

The observations on day 5 of age failed to detect any changes in the structure of sensory epithelium of the quails exposed to hypodynamy (Fig. 1), when compared to the controls (Fig. 2).

Under hypodynamic conditions, we observed changes in the structure of the sensory epithelium on day 14 of age, in comparison with the controls. On the 14th day of hypodynamy we detected damage to the structure of the sensory epithelium by examination of half-thin slices under a light microscope (Fig. 3). Changes in the shape and arrangement of hair cells were visible in the sensory epithelium of the *macula utriculi*. The arrangement of the sensory cells in the epithelium was irregular. The structure of the hair cells was damaged and their shape was changed due to extensive dilatation. The comparable structure of the sensory epithelium of the control birds can be seen in Fig. 4.

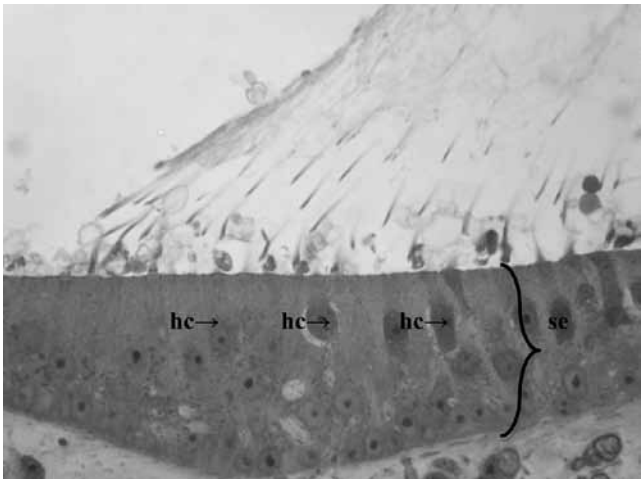

Fig. 1. Japanese quails – 5th day after exposure to hypodynamy
A section of parts of the sensory epithelium *macula utriculi*
– a half-thin section slice.
hc – hair cell, se – sensory epithelium, Magn. ×1 000

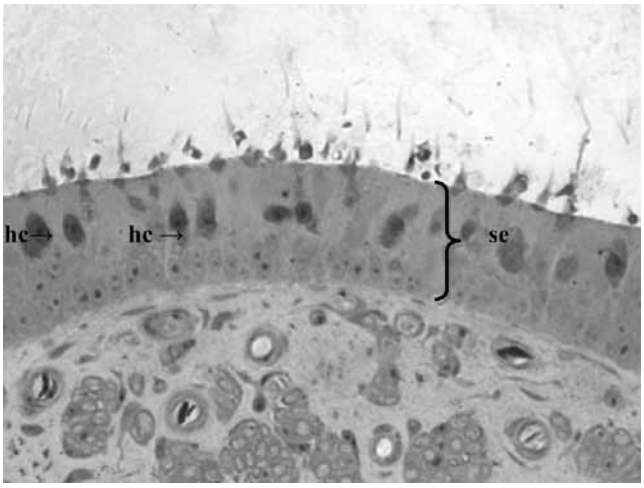

Fig. 2. Japanese quails – 5th day control sample
A section of portion of the sensory epithelium *macula utriculi* –
a half-thin section slice.
hc – hair cell, se – sensory epithelium. Magn. ×1 000

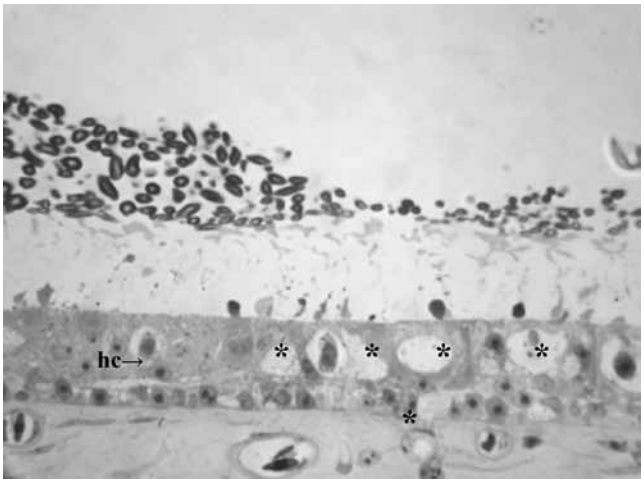

Fig. 3. Japanese quails – 14th day after exposure to hypodynamy
A section of parts of the sensory epithelium *macula utriculi* –
a half-thin section slice.
hc – hair cell, * – dilatation hair cells. Magn. ×1 000

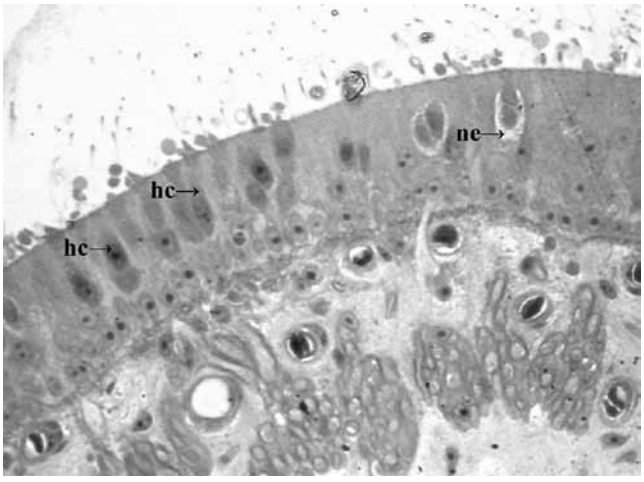

Fig. 4. Japanese quails – 14th day control sample
A section of parts of the sensory epithelium *macula utriculi* –
a half-thin section slice.
hc – hair cell, ne – nerve ending. Magn. ×1 000

DISCUSSION

We described the effects of simulated microgravity on the structure of the vestibular apparatus in Japanese quail. Our experiment showed that simulated microgravity induced changes in the shape and arrangement of hair cells in the sensory epithelium of *macula utriculi* at day 14 of age. Hypodynamy has an adverse influence on the structure of sensory organs in a vestibular apparatus. In our study, we observed that simulated microgravity caused morphological changes in the sensory epithelium of the vestibular apparatus of Japanese quails.

Our observations regarding the effects of microgravity on the sensory epithelium are similar to the results and findings of Lychakov *et al.* (2) and Ross (3). They observed the vestibular apparatus after a space flight of Japanese quail embryos in weightlessness from 1 to 12 days of age. Specific differences were observed in the structural organization of the *maculae* and *cristae* of the experimental and control embryos. Light microscopy revealed a marked symmetrical swelling of the calyx – like nerve endings throughout the sensory epithelium of the *maculae* and *cristae* of the flight embryos. Swollen cup-form nerve endings have been found in one adult rat after 7 days of space flight (2). One of the causative factors in the genesis of the swelling of the calyx – like nerve endings might be an altered ionic metabolism of the inner ear fluids in weightlessness (2).

Another study described similar differences in the rat vestibular system exposed to microgravity for 9 days aboard a spaceship. Synapses increased by approximately 41 % in type I cells and by approximately 55 % in the type II hair cells in the flight animals. We suppose that changes in the shape of the hair cells observed in our study were also caused by the increased number of ribbon synapses under the influence of the changed intensity of the altered gravitational forces. The mean number of synaptic ribbons in type II hair cells of the rat *macula utriculi* increased significantly in weightlessness. The *macula sacculi* had less ultrastructural complexity than the *macula utriculi* (6).

Seibert (7) observed rats during a 14-day space flight and detected a double count of ribbon synapses in type II hair cells, with a 50 % increase of synaptic ribbons in type I hair cells, and also an increased number of otoliths in comparison with the group of control rats on the Earth. When compared with animals exposed to an increased intensity of gravitation by means of cyclone separation/centrifugation, they expressed a decreased number of synapses.

Ross (5) observed that the synaptic mean for all type II hair cells of F13 flight rats (F13 – after 13 days aboard a spaceship) increased by 100 % and that for all sensory epithelium hair cells by 200 %. Type I cells were less affected, with a synaptic mean difference statistically insignificant in complete cells. Synapse deletion began within 8 hours upon return to Earth. Additionally, hair cell laminated rough endoplasmic reticulum of flight rats was reversibly disorganized on R0 (RO – upon return to the Earth). Two different space life science missions have demonstrated that the synapses of the hair cells of rat vestibular maculae increase significantly in microgravity.

In conclusion, this report is the first study examining the effects of simulated weightlessness on the structure of the sensory epithelium of the vestibular apparatus during postnatal development of Japanese quail males. This preliminary study about the effects of simulated microgravity on the structure of the vestibular apparatus showed changes not only in the shape and arrangement of the hair cells in the sensory epithelium of *macula utriculi* but also dilatations around the hair cells at day 14 of age.

ACKNOWLEDGEMENTS

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HAEMATOLOGICAL AND BIOCHEMICAL CHANGES FOLLOWING LEAD TOXICITY IN MALE WISTAR RATS

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ABSTRACT

The present work is devoted to study the toxicity of lead (Pb): in experimental Wistar male rats intraperitoneally exposed to 1% lead acetate; and to evaluate the risk on occupationally exposed workers to similar toxicity. A total of twenty five male Wistar rats were equally divided into five groups A, B, C, D and E. Group A served as the control group. Groups B, C, D and E were exposed daily to 0.5; 1.0; 1.5 and 2.0 mg $Pb^{2+}.kg^{-1}$ body weight respectively for a period of fourteen days. At the end of exposure, concentrations of blood lead, plasma sodium, potassium, calcium and magnesium were determined along with other haematological parameters. The results indicated that the exposure of animals to lead increases the blood lead concentrations. On the other hand, there was a significant decrease ($P < 0.05$) in the packed cell volume and haemoglobin concentration, especially in group E with concomitant decrease in plasma sodium, potassium and calcium concentrations. It was concluded that exposure to lead causes alterations in the haematological values and plasma electrolytes.

Key words: haemoglobin; lead toxicity; packed cell volume; plasma calcium; potassium, sodium

INTRODUCTION

There is considerable interest in defining the effects of low levels of lead on biological systems (14). Lead is a widely recognized ubiquitous, long-lived and pervasive environmental and industrial toxicant throughout the world (17, 23). The known broad spectrum of toxicological effects it induces, together with the substantial risk posed to the human population throughout the world, make lead exposure an important public health problem (7, 11). However, both occupational and environmental exposures remain a serious problem in many developing and industrializing countries (30). Lead has been found to produce a wide range of toxic-biochemical effects and these effects involve several organs, systems and biochemical activities (1, 18). The neurobehavioural, haematologic, nephrotoxic and reproductive effects of lead have been observed in humans and other animals (8, 12). Also, the immune system is not spared from the toxic effects of lead (4). Lead toxicity is closely related to its accumulation in certain tissues and it interference with the bioelements, whose role is critical for several physiological processes.

The maintenance of water homeostasis is paramount to life for all organisms. In mammals the maintenance of osmotic pressure and water distribution in the various body fluid compartments is primarily the function of the electrolytes. In addition to water homeostasis, electrolytes play an important role: in the maintenance of pH; regulation of proper heart and muscle function; involvement in oxidation and reduction reactions; and participation in catalysis as cofactors for enzymes (19).

Thus, it becomes quite apparent that abnormal levels of electrolytes may be either the cause or the consequence of a variety of disorders due to lead exposure. Therefore in this study, the effects of lead exposure on haematological factors and electrolytes of rats were investigated to evaluate the risk on persons who are environmentally and occupationally exposed to similar toxicity.

MATERIALS AND METHODS

The experiment was performed using male Wistar rats weighing about 200 g. The rats were maintained in rat cages in the laboratory animal shed of the College of Veterinary Medicine, Abeokuta, Ogun state. They were provided with laboratory animal feed (fat/oil 6 %, crude fibre 5 %, calcium 1 %, available phosphorus 0.4 %, lysine 0.85 %, methionine 0.35 %, salt 0.3 %, crude protein 18 %, metabolisable energy 2900 kcal.kg^{-1}, Manufactured by TOPFEEDS®, Lagos, Nigeria) and water provided *ad libitum*.

Experimental animals were acclimatized to their housing environment one month before the start of the experiments.

Experimental design

The experimental animals were divided into five groups of five animals each tagged group A (control), B, C, D and E. The rats in group A, served as the control, while the other groups were administered different concentrations of lead as lead acetate ($(CH_3.COO)_2Pb.3H_2O$, assay (ex Pb) 99–103 %, maximum limits of impurities, chloride (Cl) 0.005 %, copper (Cu) 0.002 %, a product of Cartivalues, England. The rats in groups B, C, D and E were administered intraperitoneally 0.5, 1.0, 1.5 and 2.0 mg Pb^{2+}.kg^{-1} body weight respectively daily for a period of fourteen days, while the control group received a daily dose of sterile distilled water intraperitoneally.

Animals of different groups were sacrificed under light anaesthesia one day after the end of the treatment. The blood was collected from the heart into the heparinised tubes for haematological, biochemical and blood lead determinations.

Haematological values

The packed cell volume, red and white blood cells counts were determined as described by Schalm *et al.* (21). The haemoglobin concentration was determined as described by Van Kamper (28) using a haemoglobin kit supplied by Cypress Diagnostics; Langdorp-Belgium.

Biochemical analyses

The electrolytes (sodium, potassium, calcium and magnesium) were analyzed in the plasma. The plasma sodium concentration was determined spectrophotometrically according to the method of Trinder (26) as described in the Cromatest® diagnostic kit manual.

The plasma potassium concentration was determined spectrophotometrically according to the method of Terri and Sesin (24) as described in the Cromatest® diagnostic kit manual.

The plasma calcium concentration was determined spectrophotometrically as described by Schmidt *et al.* (22) in the Cromatest® diagnostic kit manual.

The plasma magnesium concentration was determined spectrophotometrically according to the method of Abernethy and Fowler (3) as described in the Cromatest® diagnostic kit manual.

Blood lead analysis

The concentration of blood lead was determined by using the atomic absorption spectrophotometer (GBC Avanta Sigma; GBC Scientific Equipment PTY Ltd; Dandenog, Victoria, Australia) after digestion with a mixture of nitric acid and perchloric acid (6 : 1 v/v). The external standardization for lead was done by analyzing a certified Spex Lead Standard (Spex Industries Inc, Edison, New Jersey, USA) (taken through the digestion procedure) along with the samples.

Statistical analysis

The data are expressed as the mean ± S.D. and $P < 0.05$ was considered statistically significant. The significance of the differences was assessed by one-way analysis of variance (ANOVA). When significant effects were found among the groups, Tukey's test was used to assess which of the groups were significantly different from each others.

RESULTS

Table 1 shows the blood lead concentrations of animals administered intraperitoneally varying doses of lead daily during a period of two weeks. There was a linear significant ($P < 0.05$) increase in blood lead concentration of the animals exposed to varying doses of lead compared to the control group. The highest blood lead concentration was observed in group E which was about six times that of the control group.

Table 1. The mean blood lead concentrations of experimental animals

Experimental animals	Mean lead concentrations (μg.ml^{-1})
Group A (Control)	0.12 ± 0.0^a
Group B	0.16 ± 0.0^b
Group C	0.21 ± 0.00^c
Group D	0.25 ± 0.00^d
Group E	0.67 ± 0.02^e

Values in the same column with different superscript differ significantly ($P < 0.05$)

Table 2 shows the packed cell volume, haemoglobin concentrations, red and white blood cells counts and white blood cells differentials. There was a significant reduction in the packed cell volume of animals exposed to different doses of lead compared to the control group. The reduction was in the range of 80–85 % compared to the control group. There was a marked reduction ($P < 0.05$) in the haemoglobin concentration of rats exposed to the highest dosage of lead; the reduction was about 39 % compared to the control group. There were no significant changes in red and white blood cell counts as well as in lymphocyte and neutrophils.

Table 2. The mean packed cell volume, haemoglobin concentration, red and white blood cell count, lymphocytes and neutrophils

Experimental animals	Packed cell volume (PCV) %	Haemoglobin concentration ($g.dl^{-1}$)	Red blood cell (RBC) count × 10^6 mm^{-3}	White blood cell (WBC) count × 10^3 mm^{-3}	Lymphocytes (%)	Neutrophils (%)
Group A (Control)	49.75 ± 2.06^{b}	12.34 ± 2.75^{b}	7.08 ± 0.52^{a}	8.20 ± 1.78^{b}	66.75 ± 6.65^{a}	32.25 ± 6.24^{a}
Group B	41.25 ± 3.40^{a}	13.04 ± 2.65^{b}	6.80 ± 0.57^{a}	7.10 ±1.20^{ab}	63.75 ± 6.39^{a}	35.00 ± 7.70^{a}
Group C	40.00 ± 3.46^{a}	13.82 ± 0.51^{b}	7.23 ± 0.57^{a}	6.25 ± 0.69^{ab}	65.75 ± 8.30^{a}	34.25 ± 8.30^{a}
Group D	42.25 ± 2.63^{a}	16.12 ± 4.53^{b}	7.00 ± 0.39^{a}	5.20 ± 1.17^{a}	60.75 ± 6.99^{a}	38.50 ± 7.55^{a}
Group E	41.25 ± 1.89^{a}	7.52 ± 2.21^{a}	7.10 ± 1.28^{a}	6.80 ± 1.49^{ab}	66.00 ± 9.79^{a}	32.25 ± 7.85^{a}

Values in the same column with different superscript differ significantly ($P < 0.05$)

Table 3 shows the plasma sodium, potassium, calcium and magnesium concentrations of animals exposed to varying doses of lead. There was significant hyponatremia, hypokalaemia and hypocalceamia in all the animals exposed to varying doses of lead compared to the control group. The hyponatremia was more prominent in group E, which was about a 21 % reduction compared to the control group. The hypokalaemia was more prominent in group B which was about 16 % compared with the control group, while the hypocalcaemia was about 35 % compared with the control group.

Table 3. The mean sodium, potassium, calcium and magnesium concentration

Experimental animals	Sodium ($mmol.l^{-1}$)	Potassium ($mmol.l^{-1}$)	Calcium ($mg.dl^{-1}$)	Magnesium ($mg.dl^{-1}$)
Group A (Control)	145.71 ± 4.69^{b}	4.19 ± 0.18^{c}	8.67 ± 1.26^{b}	1.01 ± 0.45^{a}
Group B	137.86 ± 3.87^{b}	3.50 ± 0.61^{a}	5.64 ± 1.93^{a}	1.15 ± 0.61^{a}
Group C	118.14 ± 12.79^{a}	3.81 ± 0.29^{abc}	5.82 ± 2.35^{a}	1.37 ± 0.32^{a}
Group D	120.99 ± 11.75^{a}	3.63 ± 0.25^{ab}	7.82 ± 0.98^{ab}	1.65 ± 0.21^{a}
Group E	114.94 ± 9.44^{a}	4.09 ± 0.60^{bc}	7.86 ± 1.37^{ab}	1.65 ± 0.29^{a}

Values in the same column with different superscript differ significantly ($P < 0.05$)

DISCUSSION

Lead has been recognized as a biological toxicant and different doses have been used to study lead-induced alterations. Absorbed lead following intraperitoneal injection is carried via the blood to different parts of the body and 95% of the blood lead is transported on the erythrocytes as lead diphosphate (10). This might be the reason for the lead concentration increase in blood following intraperitoneal injection of lead. The results revealed a marked decrease of haemoglobin concentration and packed cell volume (PCV). This may be due to the high affinity of lead for the erythrocytes which make them more vulnerable to oxidative damage than many other cells. Studies have shown that lead toxicity facilitates the conversion of haemoglobin to methaemoglobin. During haemoglobin oxidation in the presence of lead, hydrogen peroxide is generated which may induce lipid peroxidation in the erythrocytic cell membrane (29). As a result, lead might induce the generation of reactive oxygen species by interacting with oxyhaemoglobin leading to peroxidative damage of erythrocytic membrane (20). Lead is known to interfere with haeme and haemoglobin synthesis which affects the erythrocyte morphology and survival (15).

In the present study we observed a lead induced hyponatriemia, hypokalaemia and hypocalcemia in lead treated rats compared to the control rats not exposed to lead. Hyponatriemia is a complex electrolyte disorder that results mainly from dysregulation of arginine vasopressin by osmotic and non-osmotic mechanisms (27). The hyponatriemia and hypocalcemia was similar to that observed by Okediran *et al.* (18) and Abam *et al.* (2) in lead-exposed occupational subjects. The hyponatriemia and hypokalaemia might be due to the inhibition of the enzyme sodium-potassium ATPase involved in the transport of sodium and potassium across the biological membrane resulting in the redistribution of these electrolytes into other tissues manifesting as the low concentration in the plasma. The hypocalcaemia observed in lead toxicity could be attributed to lead's chemical similarity to calcium; this similarity allows lead access to critical cellular pathways, particularly within the mitochondria and in second messenger systems, where it competitively antagonizes calcium action (9, 12). Both lead and calcium use the same transport system in erythrocytes and erythrocyte ghosts (5, 6).

Toxicants may therefore interfere with calcium and magnesium homeostasis by promoting their influx into or inhibiting their efflux from the cytoplasm. They also may diminish the efflux of these ions by inhibiting the ion pumps of their driving forces (13, 25). It is likely that lead blocks calcium and magnesium efflux from the erythrocyte membrane by substituting for these metals on the active site of calcium-magnesium ATPase. Alterations in chemical and physical characteristics of the erythrocyte membrane in lead exposure have been reported (16).

In conclusion, the exposure to lead possesses the potential to induce hazardous biological effects in rats. The main damaging role of the exposure to lead may be on red blood cells and haemoglobin coupled with the low plasma concentration of major electrolytes.

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CHARACTERIZATION OF THE BACTERIAL ISOLATES FROM TRANSMISSIBLE VENEREAL TUMOUR (TVT) LESIONS OF DOGS AND THEIR ANTIBIOGRAM RESISTANCE IN NSUKKA AREA, SOUTHEASTERN NIGERIA

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ABSTRACT

Bacterial isolates and their antibiogram resistance were studied using swab samples aseptically collected from TVT lesions within the vagina of ten dogs and also from the vaginal mucosa of another ten healthy dogs. The samples were inoculated on blood and nutrient agar plates and incubated aerobically for 24 hours at 37 °C and examined with a hand lens. The characteristic appearance of different colonies, size, shape, arrangement, colour, pattern of growth, spreading or not and consistency were noted. Also areas of haemolysis (α and β) were recorded. The cultures were then subjected to the Gram's stain reaction. Based on the colonial characteristics, Gram's stain and microscopic morphologies, organism suspected to be of significance were sub cultured on nutrient agar slants in bijoux bottles and incubated aerobically for 24 hours at 37 °C after which the bottles were stored at 4 °C for subsequent use. Antimicrobial resistance profiles of the organisms were determined using the disc diffusion method. *Staphylococcus* spp. (44.4%) and *Escherichia* spp. (66.6%) were isolates from the TVT lesions whereas *Staphylococcus* spp. (28.6%), *Proteus* spp. (14.3%), *Enterobacter* spp. (14.4%), *Escherichia* spp. (28.6%) and unidentified *Coliform* spp. (14.3%) were isolated from the normal dogs. All the *E. coli* isolates were resistant to rifampicin (100%) and showed the least resistance to ciprofloxacin (20%) and ofloxacin (20%) whereas the staphylococcal isolates from the TVT dogs were resistant to the tested drugs except for gentamycin (25%) and ofloxacin (25%). The study therefore revealed that TVT lesions do not encourage bacterial growth; however, high multidrug resistant organisms were found in both normal and TVT dogs.

Key words: antimicrobial resistance; bacterial isolates; dogs; transmissible veneral tumour

INTRODUCTION

Canine transmissible venereal tumour (CTVT) is a naturally occurring, coitally transmitted neoplasm of the dog that usually affects the external genitalia (17). It is seen most often in young, free roaming and sexually active dogs and has been reported in many regions of the world (3, 6). The tumour remains a big problem in countries where the mating of dogs is not under control. The tumour is usually located on the caudal part of the penis in the male dog, from the crura to the bulbus glandis or the area of the glans penis and occasionally on the prepuce. In the bitch, it is usually found in the posterior part of the vagina, often at the junction of the vestibule and the vagina. It sometimes surrounds the urethral orifice and if

it is just within the vagina, it may protrude from the vulva. Canine TVT may also develop at extra genital sites, such as the skin, nasal cavity or the mouth, even when there are no genital lesions. Metastasis is reported to occur in < 5 % of the cases. Metastasis of TVT to the skin, regional lymph nodes, tonsils, eyes, brain, pituitary, nose, tongue, lips, mammary region and thoracic and abdominal viscera has been reported (3, 6, 13). Various complications such as metastasis, urinary tract obstruction, dystocia in whelping bitch and urinary tract infection have been reported in affected dogs (5).

The urinary tract infection (UTI) typically results from normal skin and gastrointestinal tract flora ascending the urinary tract and overcoming the normal urinary tract defences that prevent colonization (10). Bacterial UTI is the most common infectious disease of dogs, affecting 14 % of all dogs during their lifetime. It is less common in cats and is seen only infrequently in large animals. Unlike humans, animals are often asymptomatic and the UTI may be an incidental finding. If symptoms such as painful or frequent urination with blood in the urine are present, as few as 100 uropathogenic bacteria per millilitre of urine may be considered significant (12). Cases of symptomatic bacteraemia are classified either as: cystitis, when the infection is limited to the bladder; or pyelonephritis, when the kidney is infected (7, 11). Cystitis, in the otherwise healthy dogs, generally resolves itself without sequelae. Pyelonephritis, on the other hand, can cause serious morbidity and can be fatal. Patients with abnormal or obstructed urinary tracts or with compromised immune systems are at a high risk of UTI (11, 16). These infections are often referred to as complicated UTIs (13). There is an increased risk in this group that a simple urinary tract infection may progress to a systemic infection. The consequences of untreated UTI included urinary tract dysfunctions, such as, urolithiasis, prostatitis, infertility, septicaemia and pyelonephritis, with rare but eventual kidney failure. Antimicrobials are the cornerstone of UTI therapy. Many animals with recurring UTI that are managed empirically with repeated antimicrobials may be predisposed as well as encouraged to develop resistant bacteria.

The objective of this study is to identify possible bacterial isolates from TVT lesions capable of causing ascending UTI in dogs and to characterize the isolates. The results of the study may provide some useful information on the role of TVT in ascending urinary tract infections of dogs. The results of the sensitivity study may provide information on the drugs of choice for both prophylactic and treatment in TVT cases complicated with ascending UTI in dogs.

MATERIALS AND METHODS

Animals Used

Twenty bitches aged between 8 months to 3 years were used for this study. Ten of them were naturally TVT infected dogs while others were normal dogs (Non-TVT infected dogs). The dogs were obtained from Ibagwa, and Orba markets, and the University of Nigeria Veterinary Teaching Hospital (VTH), University of Nigeria, Nsukka, as well as from private Veterinary Clinics near the University.

Sample Collection

After thorough restraint of the animals, swabs were aseptically collected using the sterile swab stick from the TVT lesions within the vagina; also swabs were taken from the vagina of normal dogs. The vulva lips were held laterally exposed, thus permitting the insertion of the sterile swab stick into the posterior vagina. The sterile swab stick was quickly withdrawn from the vaginal tract and put back into the sterile swab sheath and sent to the Veterinary Medicine laboratory, Department of Veterinary Medicine, University of Nigeria for analyses.

Isolation and identification of bacterial agents

The swab samples collected from the experimental dogs were used to inoculate Blood agar and MacConkey agar plates prewarmed to room temperature (2). The inoculum was spread with a sterile wire loop to obtain discrete colonies and the plates were incubated at 37 °C aerobically for 24 hrs after which the plates were examined and the colonies identified by sugar fermentation and biochemical tests (2).

Sensitivity/Antimicrobial Resistance

The antimicrobial resistance profiles of the organisms were determined using the disc diffusion method as described by Cheesbrough (2). Nutrient agar plates were inoculated by flooding with a 3 hour nutrient broth culture of test isolates. The inoculated plates were incubated at 37 °C overnight. Isolates with inhibition zones greater than or equal to 6 mm in diameter were considered susceptible, otherwise resistant.

RESULTS

Bacterial Agents Isolated

A total of nine (90 %) bacterial agents were isolated from samples obtained from the TVT dogs studied, while seven (70 %) were isolated from the samples obtained from the normal dogs. The distribution of the genera of bacteria strains isolated from both groups is shown in Table 1.

Table 1. Percentage of occurrence of isolates from the sampled dogs

Animal group	Isolates	Total number isolated	% of occurrence
TVT dog	*Staph.* spp.	4	44.4
TVT dog	*E. coli*	5	55.6
Normal dog	*Staph. aureus*	2	28.6
Normal dog	*E. coli*	2	28.6
Normal dog	*Proteus* spp.	1	14.3
Normal dog	*Enterobacter* spp.	1	14.3
Normal dog	Unidentified coliform	1	14.3
Total number of isolates		**16**	

$$\text{Percentage of occurrence} = \frac{\text{Actual number isolated} \times 100}{\text{Total number of isolates}}$$

Bacteria belonging to 2 genera were isolated from the TVT dogs while 4 genera were isolated from the normal dogs. Of the 9 isolates from the TVT dogs, 4 (44.4 %) were *Staphylococcus* spp. while 5 (55.5 %) were *Escherichia* spp. whereas 7 isolates from the normal dogs were *Staphylococcus* spp. (28.6 %), *Proteus* spp. (14.3 %), *Enterobacter* spp. (14.3 %), *E. coli* (28.6 %) and unidentified *Coliform* spp. (14.3 %).

Antibiogram of the bacterial isolates

The antibiogram of the isolates are shown in Table 2. From the table it is evident that *E. coli* isolates from the TVT dogs were all completely resistant (100 %) to rifampicin and showed least resistance to ciprofloxacin (20 %) and ofloxacin (40 %). Staphylococcal isolates from the TVT dogs were all completely resistant to tetracycline (100 %), ampicillin (100 %), norfloxacin (100 %), pefloxacin (100 %), sulfamethoxazole/trimethoprim (TMP-SMX) (100 %) and nalidixic acid (100 %) and least resistant to gentamycin (2 5 %) and ofloxacin (25 %).

Table 2. Percentage resistance of isolates from the TVT and Non-TVT dogs

Antibacterial agent	*E. coli*	Staph.	*E. coli*	*Staph.*	*Entero*	*Proteus*	Coliform?
	TVT				Non-TVT		
Genatmycin	0	1(25)	0	1(50)	0	0	1(100)
Tetracycline	4(80)	4(100)	2(100)	2(100)	1(100)	1(100)	1(100)
Ciprofloxacin	1(20)	2(50)	0	0	0	0	0
Streptomycin	2(40)	2(50)	0	2(100)	1(100)	0	1(100)
Chloramphenicol	2(40)	3(75)	0	1(50)	1(100)	1(100)	1(100)
Ampicillin	3(60)	4(100)	0	2(100)	1(100)	1(100)	1(100)
Norfloxacin	4(80)	4(100)	0	0	0	1(100)	0
Ofloxacin	2(40)	1(25)	0	0	0	0	0
Pefloxacin	4(80)	4(100)	2(100)	2(100)	1(100)	1(100)	1(100)
Rifampicin	5(100)	2(50)	1(50)	0	1(100)	0	1(100)
TMP-SMX	4(80)	4(100)	0	2(100)	1(100)	0	1(100)
Nalidixic acid	4(80)	4(100)	1(50)	2(100)	1(100)	1(100)	0

Table 4. Resistance pattern of Gram-negative bacteria isolates for both groups of dogs

Resistance pattern	Number of isolate(s)
CIP	1
GN	1
SXT, NA, OFX, R, NO, P	2
SXT, R, P, NA, TE, AM, C, S	2
SXT, P, R, TE, AM, C, S	4

GN – gentamycin; TE – tetracycline; CIP – ciprofloxacin; S – streptomycin; C – chloramphenicol; AM – ampcillin; OFX – ofloxacin; P – pefloxacin; R – refarmpicin; SXT – sulfamethoxazole/trimethoprim; NA – nalidixic acid

Table 3. Resistance pattern of Gram-positive (staphylococci) isolates for both groups of dogs

Resistance pattern	Number of isolate(s)
OFX	1
CIP, GN	1
R, C, S	1
TE, NA, SXT, P, AM, OFX	1
TE, NA, SXT, P, AM, CIP, GN	1
TE, NA, SXT, P, AM, R, C, S	1

GN – gentamycin; TE – tetracycline; CIP – ciprofloxacin; S – streptomycin; C – chloramphenicol; AM – ampcillin; OFX – ofloxacin; P – pefloxacin; R – refarmpicin; SXT – sulfamethoxazole/trimethoprim; NA – nalidixic acid

From the normal dogs, *E. coli* and staphylococci were the major isolates, while single isolates of *Enterobacter, Proteus* and unidentified *Coliform* spp. were also present. *E. coli* isolates were completely resistant (100%) to tetracycline and pefloxacin and showed least resistance to rifampicin (50%) and nalidixic acid (50%) while the staphylococcal isolates were completely resistant (100%) to tetracycline, streptomycin, ampicillin, pefloxacin, TMP-SMX and nalidixic acid. The rest of the isolates are also shown.

Resistance pattern of Gram-positive bacteria isolates

A total of 6 resistance patterns were recorded for the 6 Gram-positive isolates that were resistant to at least one antimicrobial agent (Table 3). Three (50%) of the 6 staphylococci isolates were resistant to one, two or three antimicrobial agents while 3 (50%) demonstrated multiple resistance to the antimicrobial agents used in the study.

Resistance pattern of Gram-negative bacterial isolates

A total of 5 resistance patterns were observed for the 10 gram negative bacteria isolates, resistant to at least one antimicrobial agent (Table 4). Two (20%) of the 10 isolates were resistant to only one antimicrobial agent while 8 (80%) showed multiple resistance to the antimicrobial agents used in the study.

DISCUSSION

This work has been able to demonstrate the common bacterial flora of a TVT infected dog relative to non TVT infected dogs (normal dogs).

From the results, out of the 10 TVT samples, 9 bacteria isolates were obtained, 4 coagulase negative *Staphylococcus* spp. and 5 *E. coli* strains. The 10 samples from the normal (non-TVT dogs) showed 7 isolates and had a more varied bacteria flora. Two were coagulase positive *Staphylococcus aureus*, 2 *E. coli* strains, a single *Proteus* species, one *Enterobacter* spp. and a single unidentified coliform.

The coagulase negative staphylococcus species has been shown to be a non-pathogenic specie and a harmless commensal organism on the body of man and other animals (4). Because of its wide spread occurrence on the body, air, dust and all kinds of formites, the organism is a common contaminant of clinical specimens and laboratory cultures (2). Some subclinical conditions such as UTI may account for the isolation of enterobacteria organisms such as *E. coli* and proteus as these organisms had been known to cause UTI in dogs (2). The presence of a subclinical UTI may constitute a complication of TVT in dogs. The enterobacterial isolate from the normal dog (non TVT dog) is inconsequential as this is a non-pathogenic and harmless commensal of the urinary tract (2). The presence of coagulase positive *Staphylococcus aureus* in the non-TVT dog (normal dog) may be inconsequential as *Staphylococcus aureus* may also be an opportunistic pathogen and a secondary bacterial invader.

The antimicrobial sensitivity test, revealed a high rate of drug resistance by most bacterial isolates studied. A single antimicrobial agent to which all bacterial isolates were susceptible was not identified. All the *E. coli* strains showed complete susceptibility to gentamycin. This may be due to the rational use and administration of it by experts. Ciprofloxacin and ofloxacine also showed low resistance by both *Staphylococcus* spp. and *E. coli*. These drugs are not used too frequently in veterinary practice. Apart from these three antimicrobials; gentamycin, ciprofloxacin and ofloxacin, all the rest of the antimicrobials were on the high side of resistance by the bacterial isolates.

For the *E. coli* strains, drugs to which resistance was most frequently identified are; tetracycline (86%), rifampicn (86%), pefloxacin (86%), nalidixic acid (71%), TMP-SMX (57%) and norfloxacin (57%). These high profile resistances shown by *E. coli* to the above antimicrobials are in consonance with the reports by Gyles *et al.* (8).

For *Staphylococcus* spp., total or complete resistance (100%) was shown to tetracycline, ampcillin, pefloxacin, TMP-SMX and nalidixic acid. These high profile resistances to these antimicrobials by the staphylococcal isolates are also in line with the report by Gyles *et al.* (8). High resistance to streptomycin (67%) and chloramphenicol (67%) by the staphylococcal organisms are also evident in this study.

From this study, it is seen very clearly that there is an alarming rate of multi-drug resistant organisms in the study area and probably in veterinary practice. This is in line with reports by Vogel *et al.* (18), Huycke *et al.* (9) and WHO (19). The most frequently administered group of drugs both by the human medics and veterinarians are the amino glycoside and the beta-lactams. The usage of these drugs are prone to wrong or indiscriminate use, hence the reason for their high profile resistance as seen in this study. The antimicrobials to which high rates of resistance were demonstrated are the ones that are most frequently or commonly requested, inappropriately prescribed and administered in veterinary and human medical practice (1, 15).

This work has been able to demonstrate the prevalent bacterial flora of TVT dogs in Nsukka area as well as their drug resistance status. It is evident from the study that significant difference does not exist between the bacterial flora isolates from both the TVT dogs and the non-TVT dogs (negative control). It is also evident from this study that there is an alarming rate of multi-drug resistant organisms in veterinary clinical practice. This is an axiomatic fact.

The study has provided information on antimicrobial drugs for use in the treatment of UTI in Nsukka area. There is an urgent need for the adherence to the WHO's guidelines and regulation on the use of antimicrobials which outlaws unnecessary, irrational, indiscriminate and inappropriate use of antimicrobial drugs.

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CHARACTERISTIC OF STAPHYLOCOCCI ISOLATED FROM COW'S MILK

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ABSTRACT

The examination of 300 milk samples by multiplex PCR confirmed 83 strains of *S. aureus*. All of the tested strains of *S. aureus* presented haemolysis on blood agar plates: 69 strains (83.13%) showed β-haemolysis; 14 (16.8%) α-haemolysis; and none of the isolates produced double haemolysis (α + β). All 83 isolates of *S. aureus* were sensitive to methicillin as detected by the interpretative criteria developed by the NCCLS (2002). For the 83 *S. aureus* isolates, we compared the methicillin susceptibility results, determined by the standardized agar diffusion assay, with the PCR assay for the detection of the antibiotic resistance *mec*A gene. For all isolates, we found a correlation between the results of the PCR and those of the classical resistance testing. The results obtained showed, that none of the isolates of *S. aureus* from the 83 individual milk samples from the experimental dairy farm were positive for the presence of the *mec*A gene coding for the methicilin resistance. None of the tested strains of *S. aureus* isolates were positive for the presence of the genes for enterotoxin (*sea, seb, sec, sed, see*) production.

Key words: cow's milk; PCR; *S. aureus*

INTRODUCTION

Milk as a highly nutritious food, is also ideal for microbial growth. Fresh milk easily deteriorates to become unsuitable for processing and human consumption. The milk from a healthy udder contains few bacteria but it may become contaminated by microorganisms from the surrounding environment during milking and milk handling, from water and milking equipment (12) as well as from the mastitis milk (13).

The genus *Staphylococcus* deserves special attention, because it includes many pathogens that may cause infections or produce toxins; *Staphylococcus aureus* is notorious in the dairy industry (9). *S. aureus* is a common worldwide cause of bovine intramammary infections which typically develop into chronic mastitis (13, 24). *S. aureus* is a contagious pathogen that spreads easily from cow to cow and usually manifests itself as chronic subclinical mastitis with elevated somatic cell counts (SCC) and occasional clinical flare-ups (8). In addition, the failure to disinfect stalls regularly or to replace bedding frequently, contributes to a high incidence of clinical *S. aureus* mastitis leading to an environmental epidemic of infections. Strain typing studies confirm that different clinical manifestations are associated with different strains of *S. aureus.* Some of the strains appear to be host adapted while others are more likely to be of environmental origin (33, 36).

Staphylococcus aureus is one of the most common agents in bacterial food poisoning outbreaks. Also milk and milk products have been reported as common foods that may cause staphylococcal food poisoning. *S. aureus* strains produce a spectrum of protein toxins and virulence factors thought to contribute to the pathogenicity of this organism (34). The staphylococcal enterotoxins (SEs) have been classified into many different types. These enterotoxins are heat-stable and resistant to the action of digestive enzymes (2). The most common types of these enterotoxins are SEA to SEE. Isolates carrying the toxin genes *sea* to *see* are responsible for 95 % of the staphylococcal food poisoning outbreaks. The remaining

staphylococcal food-borne disease outbreaks may therefore be associated with other newly identified SEs. Therefore, the presence of *S. aureus* in food can be considered a potential health risk (6).

The goal of this work was to study the collection of *S. aureus* isolates obtained from 300 milk samples collected from individual cows from a dairy herd located in Trebišov District in the Košice region of Eastern Slovakia for the following characteristics: haemolysins production; production of coagulase; sensitivity to chosen antibiotics; presence of gene *mec*A coding resistance to methicillin; and presence of genes coding staphylococcal enterotoxins *(sea, seb, sec, sed, see).*

MATERIAL AND METHODS

The cadastre area of the experimental farm situated in the Košice region is located on the boundary line of Slánske mountains and Podslánska hill-country merging into East Slovakia lowlands. The cattle breeding production schedule of the investigated farm is specialized.

Prior to milk sampling, the teats of the experimental animals were washed and dried, and the teat ends were swabbed with 70% ethyl alcohol. The initial milk stripped from each udder quarter was discarded. Approximately 10 ml of the subsequent milk was collected from each teat into sterile tubes, and then all samples from one cow were mixed together and examined as one sample (21). The samples were refrigerated until delivery to the laboratory.

For *S. aureus* detection, 0.1 ml of homogenate and of its subsequent dilutions were applied onto the surface of Baird-Parker agar (Hi Media, India) and spread evenly with a sterile bent glass rod until the surface appeared dry. The plates were incubated at 37 °C for 24 to 48 h. and duplicate plates were prepared. After incubation, *S. aureus* were determined as Gram-positive, catalase-positive, oxidase-negative, and tube coagulase test-positive cocci with rabbit plasma (Imuna Šarišské Michaľany, Slovak Republic) and gave positive results with cytidine deaminase test as described by Krasuski *et al.* (23). This test is a simple, rapid and accurate method for distinguishing *S. aureus* from other staphylococci.

The haemolytic activity was determined on blood agar (defibrinated sheep blood) at 37 °C for 24 h. The type of haemolysis was recorded as α-, β-, or double (α + β).

Methicillin susceptibility was determined by the standardized agar diffusion test on Müller-Hinton (Oxoid) using the discs of methicillin (10 μg). Isolates were categorized as susceptible, resistant, and intermediate resistant, based upon the interpretative criteria developed by the National Committee of Clinical Laboratory Standards (NCCLS, 29).

The colonies of *S. aureus* were confirmed by PCR (Polymerase Chain Reaction) amplification of the species-specific 420-bp fragment. The multiplex methods used in this study are according to Sharma *et al.* (32) and Strommenger *et al.* (33).

The PCR product was analyzed on 1.0% agarose agar (Gibko, BRL, USA) diluted in 1X TAE buffer (33), stained with ethidium bromide (Amresco, USA) in concentration of 0.1 $\mu g.ml^{-1}$ and viewed under the ultraviolet light. The 100 bp DNA ladder (Sigma) was used as a standard of the molecular weight.

The *S. aureus* isolates investigated in this study as the positive control strains (*S. aureus* CCM 5756 – *sea* gene, *S. aureus* CCM 5984 – *sec* gene, *S. aureus* CCM 5972-*see* gene) originated from the strain collection of the laboratory at the National Reference Centre for Microorganisms in Brno (Czech Republic).

RESULTS AND DISCUSSION

By examination of milk from 300 clinically healthy udders, we identified 83 strains of *S. aureus* by phenotypic tests (positive coagulase and cytidine-deaminase production) and confirmed them by the PCR reaction.

The results showed that out of 300 samples, *S. aureus* was detected in 58% of the cases. This is statistically significant at $P < 0.001$ compared to other identified staphylococcal strains. *Staphylococcus aureus* is repeatably associated as the etiology of many infections and intoxications in humans and other animals (as mentioned above) (4, 17, 19). Foster (10) reported that *S. aureus* is a widespread, very easily and rapidly changing bacterium in the environment.

Research on the evidence of *S. aureus* in raw cow's milk, including the study of haemolysins and enterotoxins production, and on positivity of *S. aureus* strains for the presence of a gene coding for antibiotic resistance (e.g. methicillin) is justified also today. This bacterium is pathogenic to the mammary gland and can cause outbreaks in humans due to staphylococci enterotoxins or raise problems during antibiotic treatment associated with increasing resistance to antibiotics in human medicine.

All the tested strains of *S. aureus* presented haemolysis on blood agar plates; 69 strains (83.13%) showed β-haemolysis, 14 (16.8%) α-haemolysis, and none of the isolates produced double haemolysis (α + β) (Table 1).

Table 1. Haemolysis in *S. aureus* isolates from individual milk samples

Haemolysis	Košice region (n = 83)	
	Number	(%)
α-haemolysis	14	16.87
β-haemolysis B	69	83.13
double (α + β)	0	0

n – number of samples evaluated

The majority of strains isolated from the milk samples showed a prevalence of β-haemolysis, while 14 strains produced α-haemolysis.

The prevalence of β-haemolysis in bovine *S. aureus* strains is in full agreement with other research papers (1, 25, 28). Our results contradict the findings of Morandi *et al.* (28) who found double haemolysis (α + β) in *S. aureus* isolated from cow milk and milk products, respectively.

The frequency of *se* genes in the *S. aureus* isolates is reported in Table 2. Of the 83 *S. aureus* isolates tested, none was positive for one or more *se* genes.

Table 2. Genes for staphylococcal enterotoxins production *(sea, seb, sec, sed, see)* in *S. aureus* isolates

Genes for staphylococcal enterotoxins production	Košice region (n = 83)	
	Number	(%)
Sea	0	0
Seb	0	0
Sec	0	0
Sed	0	0
See	0	0

n – number of samples evaluated

Numerous methods are based on the evidence of the enterotoxins directly in the food (ELISA, reversal passive latex agglutination and others). Burdová *et al.* (5) reported that these methods are capable of detecting nanograms of enterotoxins in one gram or one millilitre of food. The advantage of these methods is that enterotoxins are detected even if the producer, *Staphylococcus aureus,* cannot be identified by the conventional bacteriological procedure, because it is usually devitalized by the respective temperatures. Nowadays, this disadvantage can be eliminated by using DNA amplification methods (PCR) (39). It is because the PCR method can show the presence of enterotoxigenic strains of *Staphylococcus aureus* before expression of the enterotoxins on the basis of specific gene sequences and in this way detects the potential source of contamination (35, 37). The advantage of the PCR methods is that it is able to detect genes which code for the production of staphylococcal enterotoxins also from heat treated food, because the DNA remains unchanged (15, 36).

Our results do not agree with those presented by some other researchers because many authors reported positivity of *S. aureus* isolates for the presence of *se* genes. For instance, Morandi *et al.* (28) reported that 58 of 81 (72%) *S. aureus* isolates originating from cows were found to be positive for one or more *se* genes: *see*, and *sea, sed*, and *sej* were found more frequently. Twenty three of the 81 (27.03%) *S. aureus* isolates from cows did not produce staphylococcal enterotoxins.

In our experiment, of the 83 *S. aureus* isolates tested, none was found positive for one or more *se* genes. The *sec* (1; 4.8%) and *sed* (2; 9.5%) enterotoxin genes were detected also in three of 21 isolates of *S. aureus* from raw cow's milk samples taken from a farm in Eastern Slovakia (35). Similar results were also reported by other authors (37, 38).

For all of the 83 *S. aureus* isolates, we compared the susceptibility to methicillin (results determined by the standardized agar diffusion assay) with the PCR assay for the detection of antibiotic resistance *mec*A gene. For all isolates, we found a correlation between the results of the PCR and those of the classical resistance testing (Table 3).

Table 3. Correlation between phenotypic antibiotic resistance and PCR result of methicillin in *S. aureus* isolates from individual milk samples

Number of *S. aureus* strains tested	Resistance phenotype of methicillin	Presence of fragments	
		16S rDNA*	*mec*A
83	sensitive	+	-

n – number of samples evaluated
* – genus typical for *Staphylococcus* spp.

These results do not agree with those of Lee (26), who found 53% positive isolates for *mec*A gene in *S. aureus* strains isolated from cow's milk in Korea, and only a few cases of positive *S. aureus* milk samples obtained from cows showing clinical signs of mastitis. On the other hand, in Hungary, MRSA (Methicillin Resistent *S. aureus*) strains of *S. aureus* were isolated from the milk of cows suffering from subclinical mastitis (20).

The results of the *in vitro* sensitivity tests of *S. aureus* isolated from milk samples, as detected by the interpretative criteria developed by NCCLS (29), are given in Fig. 1.

According to Fig. 1, all isolates of *S. aureus* were sensitive to methicillin (as mentioned above) and oxacillin (100%). More than 90% of the isolated *S. aureus* strains showed sensitivity in amoxycillin-clavulanate testing (96.39%) while 80.72% and 75.90% isolates were sensitive to lincomycin and streptomycin, respectively. With respect to the other tested antibiotics, the sensitivity to penicillin of *S. aureus* isolates was reduced (59.03%).

Staphylococcus aureus is a common commensal of humans and its primary habitat is the moist squamous epithelium of the anterior nares (31). It was also reported, that about 20% of the population are always colonized with *S. aureus*, 60% are intermittent carriers, and 20% never carry the organism (7, 31). As there is considerable evidence that carriage is an important risk factor for invasive infections, it is surprising that so little is known about the bacterial factors that promote the colonization of squamous epithelial surfaces and the host factors that determine whether an individual can be colonized or not. Healthy individuals have a small but finite risk of contracting an invasive infection caused by *S. aureus*, and this risk is increased among carriers (10). Hospital patients who are catheterized or who have been treated surgically have a significantly higher rate of infection. In some, but not all, developed countries, many nosocomial infections are caused by *S. aureus* strains that show multiple resistance

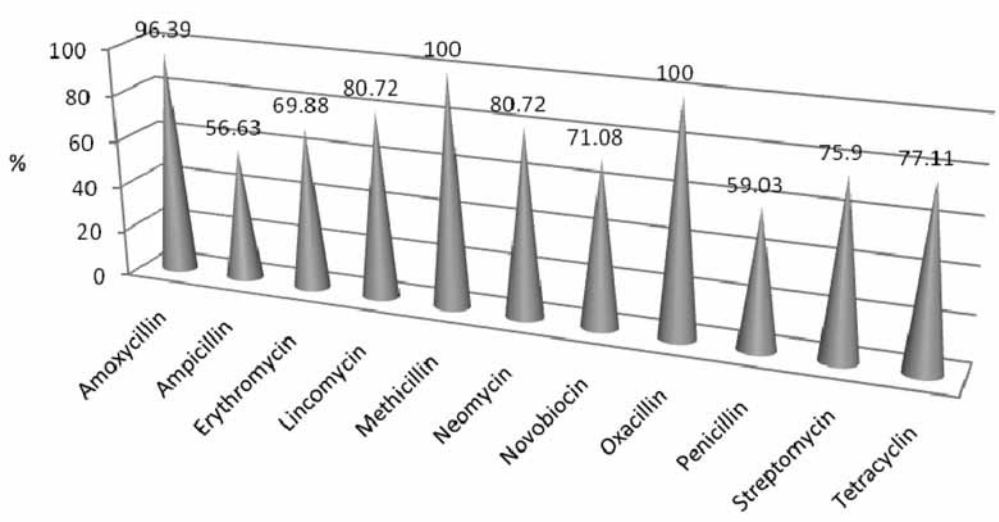

Fig. 1. Percentage of sensitivity of *S. aureus* strains isolated from milk samples of experimental animals to tested antibiotics

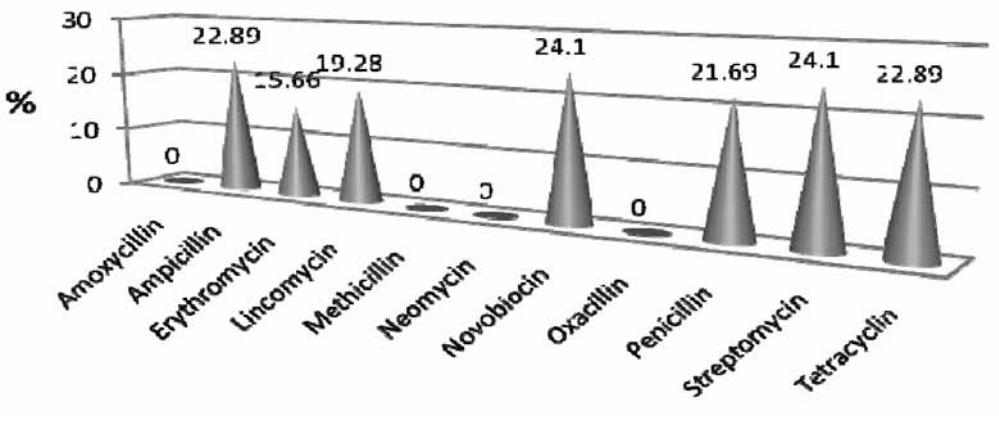

Fig. 2. Percentage of resistance of S. *aureus* strains isolated from milk samples of experimental animals to tested antibiotics

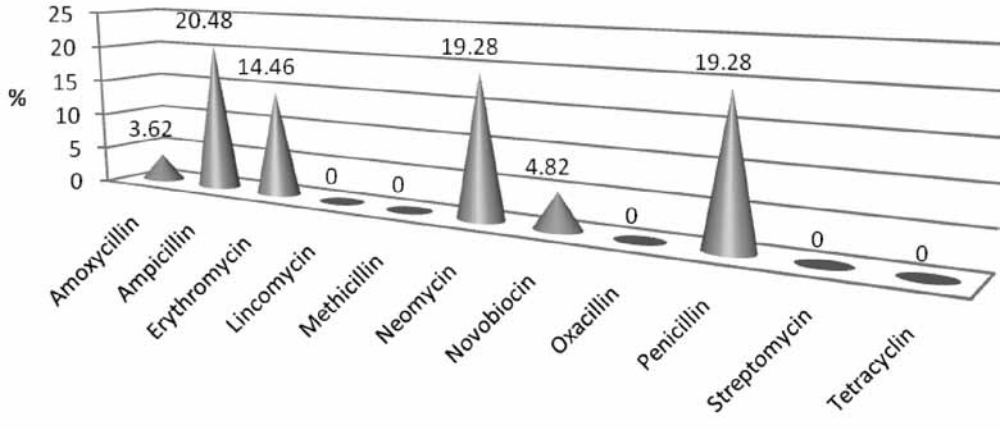

Fig. 3. Percentage of intermediate sensitivity of *S. aureus* strains isolated from milk samples of experimental animals to tested antibiotics

to antibiotics, known as methicillin-resistant *Staphylococcus aureus* (MRSA) (14, 16).

Resistance to penicillin and ampicillin was detected in 21.69 % and 22.89 % of the isolates of *S. aureus* from the milk samples of the experimental animals, respectively (Fig. 2).

The intermediate sensitivity of S. *aureus* strains isolated from the milk samples of experimental animals to tested antibiotics is illustrated in Fig. 3. According to this figure, intermediate sensitivity to ampicillin, erythromycin, neomycin, and penicillin of *S. aureus* isolates was observed in 20.48 %, 14.46 %, 19.28 % and 19.28 % of the tested isolates, respectively. Of the *S. aureus* isolates obtained from the milk samples, 3.62 % showed intermediate sensitivity to amoxicillin and 4.82 % to novobiocin.

In MRSA, the horizontally acquired *mecA* gene encodes a penicillin-binding protein, PBP2a, which is intrinsically insensitive to methicillin and all β-lactams that have been developed, including the isoxazoyl penicillins (e. g., oxacillin) that superseded methicillin, in addition to the broad spectrum β-lactams (third-generation cephalosporin's, cefamycins, and carbapenems) that were introduced primarily to treat infections caused by Gram-negative bacteria (16). That is why it is important to evaluate the strains of *S. aureus* isolated from milk also for the presence of the *mec*A gene.

Contaminated foods of animal origin, including milk, present a risk of MRSA infection rarely, although the potential risk exists mainly with respect to the transfer of MRSA strains in food workers (18). Isolates of MRSA in samples from milk and cheeses were reported also by other authors (9, 30). Kerouanton *et al.* (22) detected that two (6 %) out of 33 *S. aureus* strains isolated from humans and sheep in suspected alimentary outbreak were resistant to methicilin.

Multidrug-resistant staphylococcal isolates, such as methicillin-resistant *S. aureus,* were isolated primarily from human samples, but such isolates were detected also in animal samples (27). Additionally, a large scale outbreak of *S. aureus* foodborne-disease due to low-fat milk occurred in Japan in 2000 (3). Thus, the transfer of *S. aureus* between humans and cows may result in serious problems. Hata *et al.* (11) supported the hypothesis that a number of factors play a role in the adaptation of *S. aureus* isolates to specific hosts.

CONCLUSION

S. aureus remains a significant cause of both clinical and subclinical mastitis in cattle, which is in no small part due to the fact that the elimination of this infection from an infected lactating mammary gland can be difficult. There are no prizes for keeping a herd *S. aureus*-free, nor is there an extra income based on milk prices. A common approach to mastitis control programmes in general, which would apply equally well to *S. aureus* control, would be the monitoring of bulk samples (e. g. on a quarterly basis), combined with the sampling of CMT (California Mastitis Test) positive quarters from high SSC (right angle Side Scattering Cell counts) cows identified by routine composite cow SCC samples through monthly milk recordings. Also, other control measures like: keeping a closed herd and biosecurity; adequate post-milking dipping year round; good hygiene at teat preparation; improving the diagnosis of clinical mastitis by sampling; checking parlour functions; design and cleaning; dry cow therapy; etc., should be included.

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SEROTYPING OF FOOD AND ENVIRONMENTAL *LISTERIA MONOCYTOGENES* ISOLATES IN THE SLOVAK REPUBLIC IN 2008–2009

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ABSTRACT

The aim of this project was to study the serotypes of all isolates of the *Listeria monocytogenes* (*L. monocytogenes*) collected in the Slovak Republic in the period of 2008 and 2009. The serotyping was performed with the strains isolated in both veterinarian and public health laboratories and the results were compared. Not all strains of *L. monocytogenes* show the same pathogenicity (the most virulent serotype for humans is the serotype 4b). A major goal of this study was to find which of the strains circulate in the environment and in the foods distributed in the Slovak Republic most frequently and, thus, to determine their serogroups in order to assess the risk for consumers. Two hundred and five isolates of *L. monocytogenes* in 2008 (an incidence of 3.6%) and 94 isolates in 2009 (an incidence of 3.1%) were obtained from the National Reference Laboratory for *L. monocytogenes* in Dolný Kubín and from public health laboratories and state veterinary and food institutes in the Slovak Republic. Serotypes 1/2a, 1/2b and 4b were identified most frequently.

Key words: environmental samples; food; *Listeria monocytogenes*; serotyping

INTRODUCTION

The food-borne pathogen *L. monocytogenes* is commonly associated with fresh and frozen meats, soft cheeses, nonpasteurized dairy products and with processed foods (2). Listerias are bacteria which can survive in a variety of foods in spite of a high concentration of salt or low pH values. Listeriosis endangers especially pregnant women, children, elderly or immunosupressed people, as well as patients with malignant neoplasms, AIDS, diabetics or alcoholic liver disease (7).

Although more than 12 serotypes are known in *L. monocytogenes*, only a few of them can participate in food-borne outbreaks due to food contamination. The most frequent serotypes are 1/2a, 1/2b and 4b. The majority of outbreaks has been attributed to the serotype 4b; however, in surveys of foods or food-processing plants for *L. monocytogenes*, serotype 1/2a was found to be the most frequent (2, 3).

Based on the fact, that up until now, no serotyping of *L. monocytogenes* has been performed in Slovakia, this study should provide new information concerning the circulating strains. Both the Ministry of Agriculture and the Health Ministry of the Slovak Republic have indicated that the results of this study are highly desireable.

MATERIALS AND METHODS

Listeria strains were isolated according to the requirements of the government standards STN EN ISO 11290-1/A1 and 11290-2 (12, 13). Human strains were isolated in regional public health laboratories from ready-to eat foods (salads with mayonnaise or fish salads). The animal isolates were obtained from state veterinary and food institutes from various foods of animal origin (meat products, milk and dairy products), as well as from environmental samples (swabs from food-processing establishments). The isolates were collected and stored in the National Reference Laboratory (NRL) for *Listeria monocytogenes* in Dolný Kubín (the authorized laboratory

for *L. monocytogenes* in the Slovak Republic), where they were confirmed and submitted to further serotyping.

The confirmation of the isolates included: staining; CAMP-test; production of catalase, oxidase and other biochemical tests; as required by both reference methods (12, 13). The growth of suspected colonies was observed on the surface of prescribed selective Chromogenic *Listeria* agar OCLA (Oxoid, United Kingdom) and Rapid'L mono agar (Bio-Rad, USA).

The suspected colonies were further subcultured on the surface of blood agar and after a 24-hour incubation at 22 °C, their serotyping with *Listeria* antisera (Denka Seiken, Japan) was performed. Isolates of *L. monocytogenes* were divided into various serotypes based on the reactions of their somatic (O) and flagellar (H) antigens with a series of polyvalent and monovalent antisera. The O antigens (O I; I,II; IV; V,VI; VII; VIII; IX) divided the serotypes into groups 1/2, 3, 4 or 7; H antigens (HA, HAB, HC, HD) provided groups a, b, c, d, e or ab. The agglutination was performed by the mixing of each culture with the appropriate O and H antigens. A significant agglutination within one minute has been considered as a positive result. No or weak agglutination indicated negative results.

RESULTS

Table 1 shows the list of foods and the specific serotypes of *L. monocytogenes* isolated from them. During the year 2008, the presence of *L. monocytogenes* was determined in 205 samples (3.6 %) out of a total of 5 664 (including 2 199 swabs). Among them, 48 *L. monocytogenes* strains were isolated in regional public health laboratories (RPHL), 144 in the NRL for *L. monocytogenes*, and 13 in state veterinary and food institutes (SVFI). As demonstrated in Fig. 1, more than a half of the isolates (120) belonged to serotype 1/2a (58.5 %); 22.4 % to serotype 1/2b (46 isolates); 8.3 % to serotype 4b (17 isolates); 4.4 % to serotype 1/2c (9 isolates); 2.4 % to serotype 4ab (5 isolates) and 3.9 % were identified as the most dangerous serotypes for man; 4d (6 isolates) and 4c (2 isolates).

In 2009, the presence of *L. monocytogenes* was detected in 94 samples (3.1 %) out of a total of 3 025 (including 1 758 swabs); 56 of them being isolated in the NRL for *L. monocytogenes*, 33 in RPHL and 5 in SVFI. Serotype 1/2a was confirmed in the majority (48) of isolates (51.1 %) followed by 26 serotype 1/2b isolates (27.7 %) and 10 serotype 1/2c isolates (1.1 %). The presence of serotypes 4d and 4c was determined only in two samples (Table 2, Fig. 2).

The seasonal distribution of *L. monocytogenes* serotypes differed significantly within the year (Figs. 3 and 4). The lowest incidence of *L. monocytogenes* was noticed in the winter months, and the highest in the summer. During the summer months there was a wide range of serotypes (up to six). The most frequently found serotype within the whole year was that of 1/2a. Serotypes 3a and 4d were only confirmed in July and August.

DISSCUSION

Despite being of obvious benefits in the diagnostics and epidemiological investigation of listeriosis, the conventional typing method based on the slide agglutination is not performed routinely in clinical diagnostic laboratories. The main reason seems to be the requirement for the wide range of antisera needed. Thus, the serotyping of *L. monocytogenes* has been in many countries concentrated in national authorized laboratories.

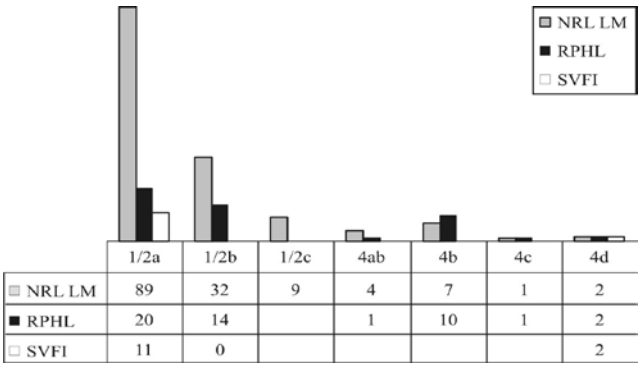
The National Reference Laboratory for *L. monocytogenes* in Dolný Kubín collects the samples of *L. monocytogenes* isolates in the Slovak Republic, performs their serotyping and

Table 1. Serotypes of *L. monocytogenes* isolates from food and swab samples in 2008

Serotypes Type of food	1/2a	1/2b	1/2c	4ab	4b	4c	4d	Σ
Raw meat	5	1	1	–	–	–	–	7
Meat products	29	7	1	1	3	1	3	45
Fermented meat products	1	1	1	–	1	–	–	4
Fish products	1	1	–	–	–	–	1	3
Sheep milk	1	–	–	–	–	–	–	1
Cheeses	27	3	2	1	1	–	1	35
Sheep cheese	7	5	1	–	2	1	–	16
Ready-to-eat products	27	22	–	2	7	–	1	59
Swabs	22	6	3	1	3	–	–	35
Σ	120	46	9	5	17	2	6	205

Table 2. Serotypes of *L. monocytogenes* isolates from food and swab samples in 2009

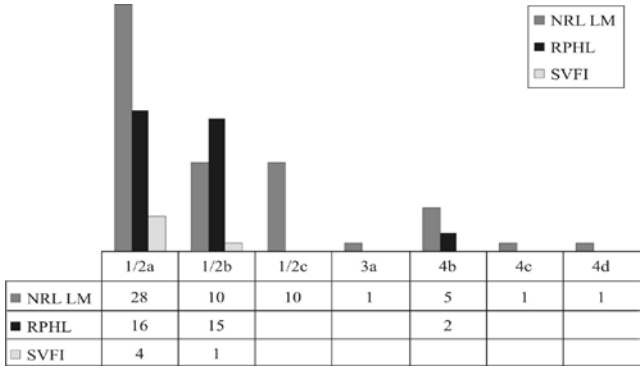
Serotypes Type of food	1/2a	1/2b	1/2c	3a	4b	4c	4d	Σ
Raw meat	1	–	–	–	–	–	–	1
Meat products	1	4	4	–	1	1	–	11
Fermented meat products	–	1	1	–	–	–	–	2
Fish products	–	3	–	–	–	–	–	3
Sheeps/cow milk	9	–	3	–	–	–	–	12
Cheeses	10	–	–	–	2	–	–	12
Sheep cheese	7	–	–	1	–	–	–	8
Ready-to-eat products	18	18	–	–	2	–	–	38
Swabs	2	–	2	–	2	–	1	7
Σ	48	26	10	1	7	1	1	94



	1/2a	1/2b	1/2c	4ab	4b	4c	4d
NRL LM	89	32	9	4	7	1	2
RPHL	20	14		1	10	1	2
SVFI	11	0					2

Fig. 1. Serotypes of *Listeria monocytogenes* isolates in Slovakia in 2008

NRL LM National Reference Laboratory for *Listeria monocytogenes*
RPHL Regional Public Health Institutes
SVFI State Veterinary and Food Institutes



	1/2a	1/2b	1/2c	3a	4b	4c	4d
NRL LM	28	10	10	1	5	1	1
RPHL	16	15			2		
SVFI	4	1					

Fig. 2. Serotypes of *Listeria monocytogenes* isolates in Slovakia in 2009

NRL LM National Reference Laboratory for *Listeria monocytogenes*
RPHL Regional Public Health Institutes
SVFI State Veterinary and Food Institutes

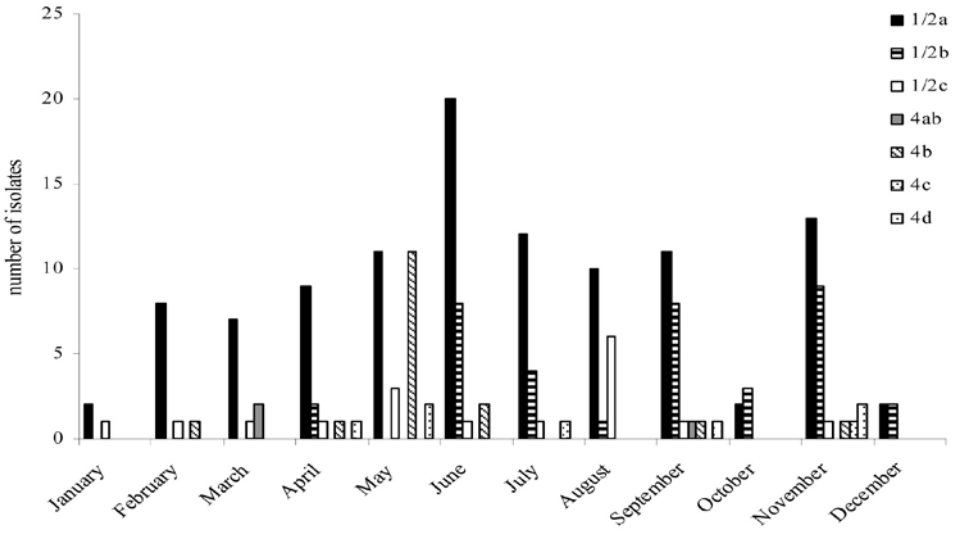


Fig. 3. Seasonal distribution of *Listeria monocytogenes* serotypes in Slovakia (2008)

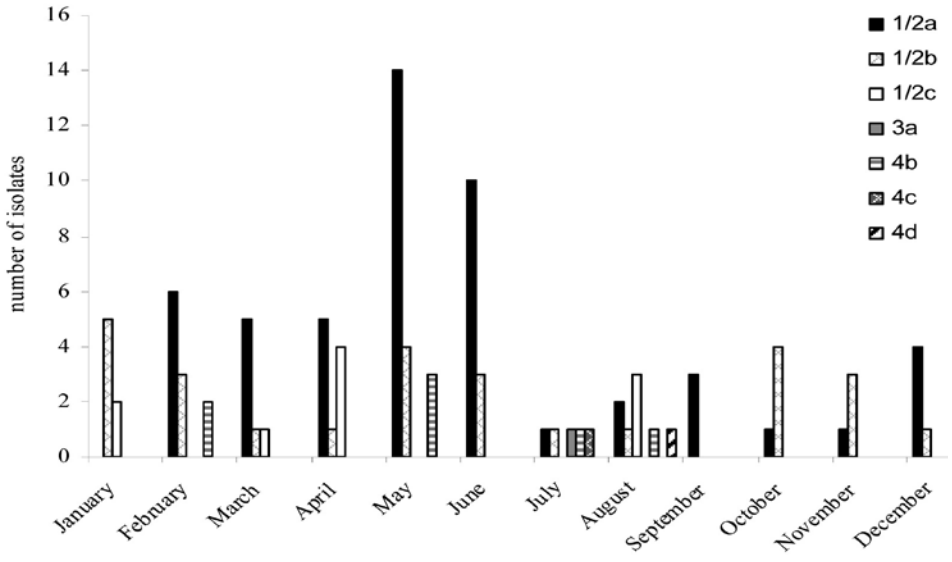


Fig. 4. Seasonal distribution of *Listeria monocytogenes* serotypes in Slovakia (2009)

stores them for further study of their properties important from the view point of food safety. The serotyping of *Listeria* spp. is a valuable tool for the identification of *L. monocytogenes* strains with the respect to their potential risk to human health. Although it is not required in the evaluation of common epidemiological situation, it could help to distinguish the potential risk of each isolate. However, misinterpretation must be avoided as it has been demonstrated that even the other *Listeria species* (*L. ivanovii* and *L. innocua*) can also give a positive agglutination with OV and VI antisera.

The results of this study confirmed the findings of numerous studies reporting on the prevalence of the 1/2 line in *L. monocytogenes* isolates (1, 2, 5, 14). Our positive findings were probably influenced by the number of samples taken within the year, as the higher temperatures are related to the occurrence of pathogen due to interruption of adequate refrigeration. Currently, several comparative studies with both human and food isolates of *L. monocytogenes* have been performed utilizing serotyping and other different molecular methods (3, 8). Palumbo *et al.* (9) compared the ELISA serotyping method with the slide agglutination method, using 101 different isolates of *L. monocytogenes*. The results obtained by the ELISA method matched those obtained by slide agglutination in 89 out of 101 isolates (88 %). For isolates of three clinically important serotypes (1/2a, 1/2b, and 4b), the ELISA method agreed with the slide agglutination method in 27 out of 27 strains (100 %), 10 out of 11 strains (91 %), and 38 out of 39 strains (97 %), respectively.

The growth of *L. monocytogenes* was observed within the range of a pH from 4.0 to 11.0. At pH-values below 4.6 the growth of the pathogen was considerably reduced. The addition of sodium chloride in an amount of 1–7 % did not inhibit the growth of *L. monocytogenes* (11). The storage of foods at chilling temperatures is the main requirement in order to maintain their quality and safety. However, it must be taken into account, that psychrotrophic bacteria (including *L. monocytogenes*) can grow and increase their counts under these conditions (4). The most risky foods are those which are raw or insufficiently cooked. However, in some outbreaks of food-born listeriosis even the pasteurized products have been involved, as reported in Finland where the butter was recognized as a source of the disease with a rare serotype of 3a (10).

From the results of this study it follows that the predominant serotypes of *L. monocytogenes* in samples of different foods and environmental swabs in Slovakia were those belonging to serogroup 1/2. In 2008, the presence of serotypes 4c and 4d were also confirmed in meat and milk products, those being most frequently involved into outbreaks of human listeriosis. The incidence of *L. monocytogenes* serotypes in food was significantly influenced by the season of the year. As *L. monocytogenes* is an important food-borne pathogen which cannot be eliminated completely from the environment, its presence in food must be monitored consistently in order to protect the consumer's health and to ensure food safety.

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DISTRIBUTION OF ELASTIC FIBRES IN THE GOAT MANDIBULAR SALIVARY GLAND

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ABSTRACT

The distribution of elastic fibres in the adult goat mandibular salivary glands was studied immunohistochemically. The accumulation of the elastic fibres was observed in the capsule and interlobular connective tissue septa where the elastic fibres were found surrounding the glandular ducts and the blood vessels. The amount and the thickness of the elastic fibres are related to the duct system, being greatest in the larger ducts. Around the extralobular ducts, the elastic fibres are distributed among the collagenous fibres in close contact with the blood vessels. With regard to the intralobular ducts, elastic fibres incompletely surround the striated and intercalated ducts in the form of fine circumferential fibres close to the basement membrane of the ductal epithelium. Among the secretory acini, only sparse elastic fibres are found. Our observations showed that the elastic fibres of the mandibular salivary gland constitute one of the main components of the connective tissue. The occurrence of the elastic fibres is related to their role as a supporting component and participant in the transport of the salivary product.

Key words: elastic fibres; goat; immunohistochemistry; mandibular salivary gland

INTRODUCTION

The mandibular, sublingual and parotid salivary glands have been the object of numerous studies, particularly ultrastructural studies (10, 18, 21, 24). Sato and Miyoshi (20) studied the topographical distribution of cells in the rat submandibular gland duct system with special reference to dark cells and tuft cells. The immunohistochemical identification of cytokeratins in the rat submandibular salivary glands during ontogenesis was studied by Dožič *et al.* (8).

Information about the presence of elastic components in salivary glands is scarce. Studies on the normal salivary glands revealed the presence of elastic fibres at the light and electron microscopy level (1, 6, 9, 13). It was found that the fine components of connective tissue are linked chemically. Their cohesion involves the binding of fibronectin to elastin microfibrils, collagen fibrils, cells, and proteoglycans (12). Suzuki *et al.* (22) observed elastic fibres around acini and intercalated ducts, with even more around the extralobular ducts. Elastic fibres were also found to be abundant in certain tumours of the human submandibular gland and in the adjacent normal gland tissue (3, 16). David and Buchner (7) and Nikai *et al.* (16) observed elastic fibres around the larger intralobular ducts in malignant salivary glands. Phang and Rannie (17) noted them around the extralobular ducts, and the larger intralobular ducts, with oxytalan around the smaller intralobular ducts.

In spite of various studies on the histological structure of the salivary glands, very little is known about the actual transport of the secretory products of exocrine glands. The presence of elastic fibres was related to the functional activity of the salivary gland, i.e., the transport of saliva. That secretory activity exists namely in the larger submandibular ducts in which, particularly during feeding, the rapid flow of profuse secretions would tense their walls. Elastic fibres in the flexible oral mucosa restore the tissue form after stretching (19).

The oral cavity and the salivary glands are open to the external environment and are thus exposed to a multitude of microbiological, chemical and mechanical influences (4). Moreover, the increasing environmental pollution may bring about various detrimental

effects of different xenobiotics, including heavy metals and pesticides, on the functioning of the living organism (15). The aim of the present study was to localize the elastic fibres in the goat mandibular salivary glands and to evaluate their involvement in the process of salivation.

MATERIAL AND METHODS

Samples of the mandibular salivary glands from five goats of both sexes were obtained and placed in 0.1 mol phosphate buffered 10 % formalin for twenty-four hours at room temperature, and then dehydrated and embedded in paraffin. Five μm sections were deparaffinised and rehydrated. For immunostaining, an avidin-biotin-peroxidase complex (ABC) method was used (11). The sections were pre-treated with 3 % H_2O_2 in methanol for 30 min to reduce endogenous activity and pre-incubated with 2 % goat serum to mask nonspecific binding sites. Afterwards, the sections were incubated at 4 °C overnight with monoclonal anti-elastin antibody (Sigma), dilution 1 : 5000. The sections were washed twice in a phosphate-balanced salt solution (PBS) and then incubated with biotinylated polyvalent secondary antibody for 45 min, washed in PBS, and incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame Calif., USA). After washing with PBS, the peroxidase activity was visualized with 0.05 % 3´,3´-diaminobenzidine (DAB) and 0.03 % v/v H_2O_2. Some sections were counter-stained with Mayer's haematoxylin. Negative controls were performed by omitting the primary antibody.

RESULTS

In the goat mandibular salivary glands, the elastic fibres were seen in the capsule, large connective tissue septa and particularly around the blood vessels and large ducts. In the capsule, the elastic fibres were distributed among the collagen fibres running longitudinally with the surface of the capsule. In the cross sections, the elastic fibres were seen as punctate profiles or appeared as short lines. The connective tissue septa contained elastic fibres with a longitudinal distribution (Fig. 1).

Variations in the distribution of elastic fibres were observed within the duct system of the gland. Altered proportions of variously oriented elastic fibres were present in different calibre ducts. Generally, there was a progressive diminution of elastic tissue in large, intermediate, and small extralobular ducts. The larger fibres were essentially confined to the main duct in its entirety and to the larger extralobular ducts. The position of fibres depended on the size of the duct, being oriented more spirally than longitudinally. In large extralobular ducts, angular-shaped longitudinal elastic fibres were commonly observed along large blood vessels. More peripherally, the ducts had a collagenous zone with large blood vessels. Here, the elastic fibres form thick elastic lamina in the adventitia of the vein, whereas around the arteries these elastic fibres are distributed more diffusely (Fig. 2 and 3). Next to the larger extralobular ducts, occasionally one or two layers of fine longitudinal elastic fibres were present. Fine prominent angular elastic and collagen fibres were seen between the blood vessels. This zone was bounded externally by longitudinal elastic fibres that were stratified as well as generally thicker and more numerous. The fibres were helical rather than truly longitudinal in arrangement.

The elastic fibres decreased progressively in number and diameter as the duct size decreased. In the intralobular ducts, the subepithelial elastic fibres were oriented circumferentially, usually in one layer localized under the basal lamina of the duct (Fig. 4). There were few circumferential and

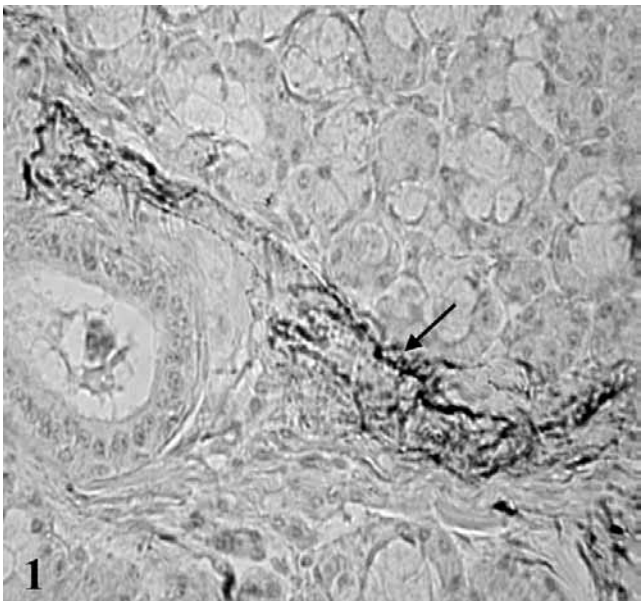

Fig. 1. A band of fine elastic fibres is accompanying a small blood vessel (arrow) in its longitudinal orientation. Magn. × 300

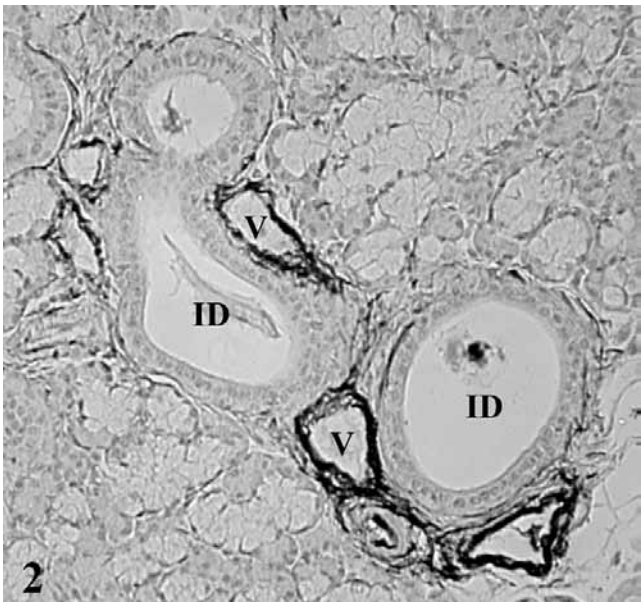

Fig. 2. Thick elastic fibres are very rich in the wall of the blood vessels; particularly in veins (V). Thin circumferential elastic fibres lie close and below the epithelium of the interlobular ducts (ID). Magn. × 150

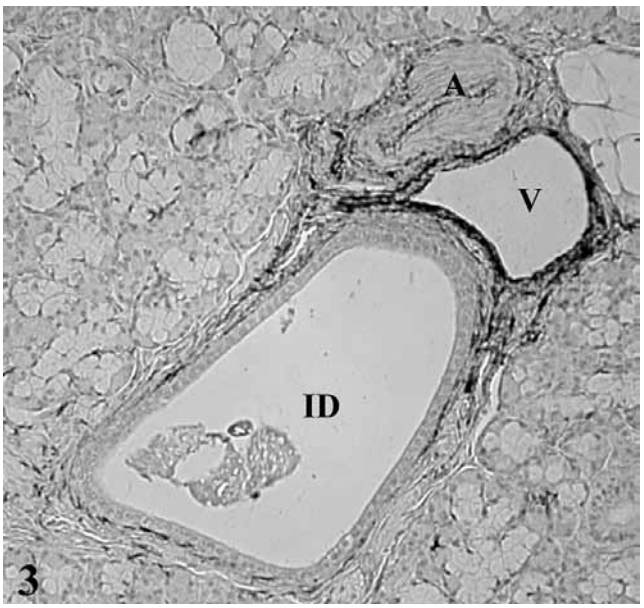

Fig. 3. Section of the interlobular duct that was accompanied by an artery and a vein. Circumferential elastic fibres surround the duct (ID). More elastic fibres areconcentrated around the artery (A) and vein (V). Magn. 100

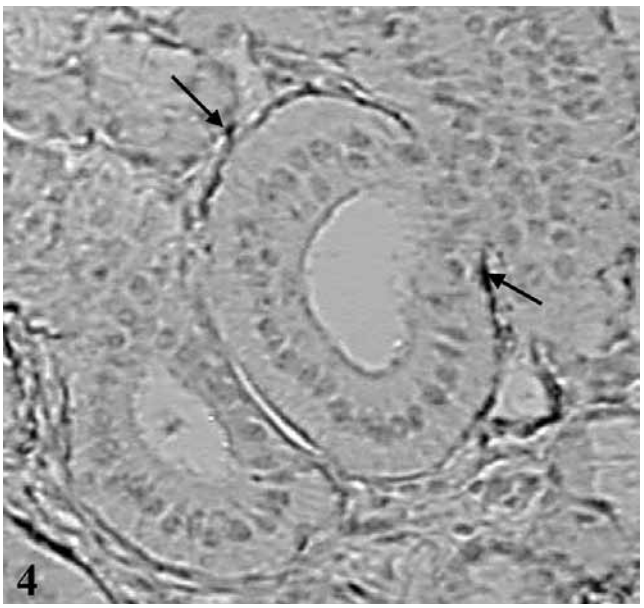

Fig. 4. One layer of circumferential elastic fibres (arrows) incompletely surrounds the epithelium of an intralobular duct. Magn. × 450

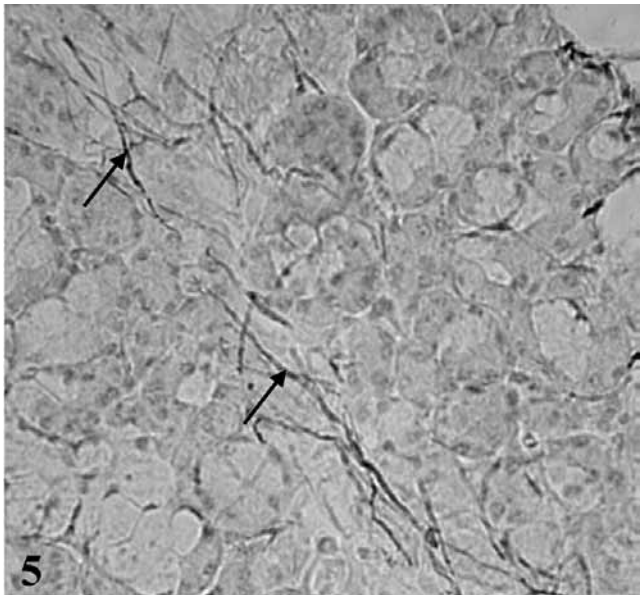

Fig. 5. Fine elastic fibres are coursing longitudinally among the glandular acini (arrows). Magn. 350

longitudinal elastic fibres in the intralobular ducts that were accompanied by arterioles and venules. In contrast to the fine fibres between capillaries and arterioles, the large angular elastic fibres, when present, were found usually peripheral to the longitudinal fibres. Small diameter elastic fibres were present in all locations possessing elastic fibres. In all areas, they were indistinct or absent from smaller ducts. A few elastic fibres bordered the smallest striated ducts or intercalated ducts, or the acini. Occasionally, though, a mainly stromal elastic fibre may be found among them (Fig. 5). Elastic fibres were also found in an unusual position. Some fibres extended up from the subepithelial connective tissue between the epithelial cells. Exceptionally, they went as far as reaching the apical border of the epithelium. Elastic fibres in the mandibular gland were also seen connected with the nervous structures, such as larger nerves and the autonomic ganglia and their ganglionic cells.

DISCUSSION

The elastic fibres are a stable component of the supportive tissue in the salivary glands. Our observations as well as those of Mansour and Sadeghi (14) showed that elastic fibres of the salivary gland constitute one of the main components of the connective tissue. Histological observations of Mansour and Sadeghi (14) showed that elastic fibres were encased in a relatively thin irregular fibrous connective tissue capsule. Altered proportions of the variously oriented elastic fibres were seen to be present in different calibre ducts; being most abundant in the extralobular ducts. Like in the rat salivary glands (13) (also in the goat) there was a progressive diminution of elastic tissue in large, intermediate and small intralobular ducts. In the rat submandibular gland, no elastic fibres bordered the smallest striated ducts or the granular or intercalated ducts (13). Also, in the goat, a few elastic fibres were seen around these ducts. A predominantly longitudinal type woven arrangement was observed which would have functional consequences. Circumferential and longitudinal elastic fibres were few and both types of angular fibres were essentially absent. According to Broom (5), as

the elastic fibres elongate, they become more aligned. Such arrangement would decrease the luminal diameters of their ducts impeding the flow. Most longitudinal elastic fibres form in the ducts in response to the foetal tongue movements that accompany swallowing. It is also probable that elastic fibres of all orientations would be augmented postnatally as suckling and feeding occurred, stimulating not only swallowing but salivary secretions (13). In the goat mandibular salivary glands, elastic fibres linkages between small ducts and vessels were loosely developed or absent.

In the goat mandibular salivary gland, extralobular and intralobular duct elastic fibres were accompanied by arteries and veins. In the rat submandibular salivary gland elastic fibres linkages between smaller extralobular ducts and vessels were absent (13). It has been suggested that elastic and collagen fibres permit large vessels to maintain high wall tension against the mass of blood within them. One can suppose that elastic and collagen fibres permit large vessels to maintain high wall tension against the mass of blood within them. This mechanism may also exist in the larger ducts in which, particularly during feeding, rapid flow of profuse secretion would tense the walls. The abundant occurrence of the elastic fibres observed in this area in the goat salivary glands supports this assumption.

Elastic fibres among the acinar cells were not seen frequently. Some fibres extended from the subepithelial connective tissue to penetrate among the epithelial cells. Perhaps the scarcity of elastic fibres in the parenchyma is a testimony to the effectiveness of damping so that the furthermost branching are not so stressed so as to engender appreciable formation of elastic tissue about them (13). In the glandular parenchyma of malignant salivary gland tumours, an increase of elastic fibres was observed around acini and intercalated ducts and even more in large intralobular ducts (2, 7). In salivary gland tumors, elastic deposits of varying amounts were clearly revealed on the basal-lamina-like material and/or masses of microfibrils in the matrix close to the neoplastic myoepithelium and, to a lesser degree, immediately beneath the nonneoplastic myoepithelium (16). One can assume a correlation with the finding that elastic fibres were also abundant in certain tumours of the human submandibular gland (3) and in adjacent normal gland tissue.

The presence of the elastic fibres was related to the functional activity of the salivary gland, i.e., with regard to the transport of the saliva. This mechanism exists in the larger ducts in which, particularly during feeding, the rapid flow of profuse secretion would tense their walls more than had occurred previously when the secretion was within small granular ducts, intercalated ducts, and acini. As a result, the tensile forces could modify the cell geometry and the calibres of the larger ducts lumens and intercellular spaces, thus affecting the flow and composition of salivary secretion (13).

The association of elastic fibres with salivary ducts might influence the dynamic interactions of fibroblasts, fibres, blood vessels, and the ductal epithelium. Tensile forces of elastic fibres may modify calibres of the larger duct's lumens and intercellular spaces and thus affect the flow and composition of salivary secretions. This function seems not to be unique. According to Tandler *et al.* (23) the presence of a variety of oxidative, acid hydrolytic, and transport enzymes in the excretory ducts, showed that, rather than simply acting as a passive conduit for saliva, these ducts may play an active metabolically role in gland functioning. This active physiological state may also play a role in the high grade of salivation following intoxication.

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DETECTION OF ANTIBODIES TO *Toxoplasma gondii* IN THE LIVESTOCK IN SLOVAKIA USING A COMPLEMENT FIXATION TEST

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ABSTRACT

This paper presents the results of a serological survey among livestock bred in Eastern Slovakia in order to detect the presence of antibodies to *T. gondii*. Blood specimens were collected from 413 asymptomatic animals. Tests were carried out using the CFT (complement fixation test) where all titres over 1 : 8 were considered to be positive. In total, 227 sera were positive (54.9%). Each group of the examined animals was divided into subgroups on the basis of their age. Out of the 233 sheep sera examined, 33 (14.1%) of the male and female lambs, 55 (23.6%) of the rams and 72 (30.9%) of the ewes were positive. In the group of goats, out of the 41 serum samples, 7 (17.1%) were positive in the subgroup of kids and 16 (39%) in subgroup of adults. From 63 examined pigs, 11 (17.5%) suckling piglets and 1 (1.6%) sow exhibited a positive serological reaction to *T. gondii*. From 76 cattle examined, 14 (18.4%) of the male and female calves, 2 (2.6%) of the heifers and 16 (21.1%) of the dairy cows were positive for the presence of overall antibodies to *T. gondii*. A statistically significant difference of the incidence of toxoplasmosis was noticed between the ewes and the two remaining subgroups of sheep ($P < 0.01$). These results point to the fact that toxoplasmosis is quite wide-spread among livestock bred in the study areas.

Key words: complement fixation test; livestock; parasitic zoonoses; seroprevalence; *Toxoplasma gondii*

INTRODUCTION

Toxoplasmosis is one of the most common parasitic zoonoses in the world afflicting a wide range of both mammals and birds. The aetiological agent is *Toxoplasma gondii* (*T. gondii*) whose definitive hosts are representatives of the family of Felidae infected by oocysts from the environment, or by tachyzoites and bradyzoites from intermediate hosts, such as all kinds of vertebrates, including humans. It is a pantropical cosmopolite and a facultative heterogenic coccidium (2).

The disease is mostly asymptomatic in animals. Toxoplasmosis may affect a number of organs, but it primarily affects the lungs, the CNS (central nervous system) and the eyes (1). In gravid animals, particularly in sheep and goats, the *T. gondii* infection causes embryonic mortality, fetal death or abortion, depending on the stage of gravidity in which the infection occurred (7).

As tissue cysts contained in the meat of domestic animals are a major ultimate source of infection in humans, attention should be primarily paid to pigs, beef cattle, sheep and goats in which *T. gondii* most frequently persists in the form of tissue cysts. Tissue cysts may develop as early as within 6 to 7 days after the infection of an intermediate host by oocysts or tissue cysts. Most probably, these persist in their hosts all their lives. In animals, tissue cysts are most commonly observed in infected pigs, sheep, and goats; less commonly in infected poultry, rabbits and horses (13).

From the epidemiological point of view, the most endangered

groups of humans are pregnant women, infants and immunosuppressed patients. An increased risk of infection has been observed in people working with raw meat, animals (veterinarians, animal keepers and cat breeders) and in those who have contact with the soil.

The objective of this study was to update the available information on the serological prevalence of *T. gondii* infection in livestock in the central and eastern parts of Slovakia.

MATERIAL AND METHODS

Animal sera. We examined 413 serum specimens of asymptomatic animals, out of which 233 were from sheep sera, 41 goat sera, 63 pig sera and 76 bovine sera. Blood serum specimens were taken from the *vena jugularis* of the beef cattle, sheep and goats and from the vena *cava cranialis* of pigs. The blood sera were stored at a temperature of –18 °C. The animals came from various farms in central and eastern Slovakia.

Group of animals. Each group of examined animals was divided into subgroups according to the age (sheep: female and male lambs up to 4 months of age, rams, ewes; goats: kids – young goats up to 4 months of age, adults – from 7 months of age; pigs: suckling piglets, sows; cattle: calves – up to 6 months of age, heifers, dairy cows).

Serological test. In order to detect the presence of overall antibodies to *T. gondii* we used the complement fixation test (CFT) according to Zástěra (16).

For diagnosis, Sevatest *Toxoplasma* antigen KFR, Sevatest *Toxoplasma* complement, (Sevapharma a. s., the Czech Republic) were used. All titres over 1 : 8 were evaluated as positive, while a twofold dilution was used.

Statistical analysis. The significance of the differences in prevalence of toxoplasmosis according to the age of animals was evaluated by Fisher Exact Test or a $\chi 2$ test and was considered as positive when the P value was less than 0.05.

Table 1. Detection of the presence of overall antibodies to *T. gondii* in serum specimens of livestock by means of a complement fixation test

Species (N)	Age groups	Positive samples (n/SP %)
Sheep (233)	lambs	33/14.1
	rams	55/23.6
	ewes	72/30.9
Goats (41)	kids	7/17.1
	goats	16/39
Pigs (63)	suckling piglets	11/17.5
	sows	1/1.6
Cattle (76)	calves	14/18.4
	heifers	2/2.6
	dairy cows	16/21.1
Total		**227/54.96**

N – number of examined animals; n – number of positive animals
SP – seroprevalence

RESULTS

In 2008 we examined 413 blood serum specimens for the presence of antibodies to *T. gondii*. A sample with a titre of 1 : 8 or higher was considered to be positive. Out of all of the specimens, the presence of antibodies to *T. gondii* was detected in 227 cases (54.96 %). Out of the 233 examined sheep sera 33 (14.1 %) of lambs, 55 (23.6 %) of rams and 72 (30.9 %) of ewes were positive. The frequency of sera positive for *T. gondii* was significantly greater in the group of ewes (30.9 %) than in the two remaining subgroups of sheep (14.1 % and 23.6 %) ($\chi 2$ test: $P < 0.01$). In the group of goats, out of the 41 serum samples, 7 (17.1 %) were positive in the subgroup of kids and 16 (39 %) in subgroup of adults. From 63 examined pigs, 11 (17.5 %) suckling piglets and 1 (1.6 %) sow exhibited a positive serological reaction to *T. gondii*. From 76 examined cattle 14 (18.4 %) calves, 2 (2.6 %) heifers and 16 (21.1 %) dairy cows were positive for the presence of overall antibodies to *T. gondii* (Table 1).

Figure 1 shows seroprevalence of antibodies to *T. gondii* with respect to titres detected in the examined animal species.

DISCUSSION

Toxoplasmosis has been found to be one of the most prevalent infections in livestock. The prevalence of this protozoan disease has been demonstrated by studies carried out to detect antibodies to *T. gondii* on farms in several European

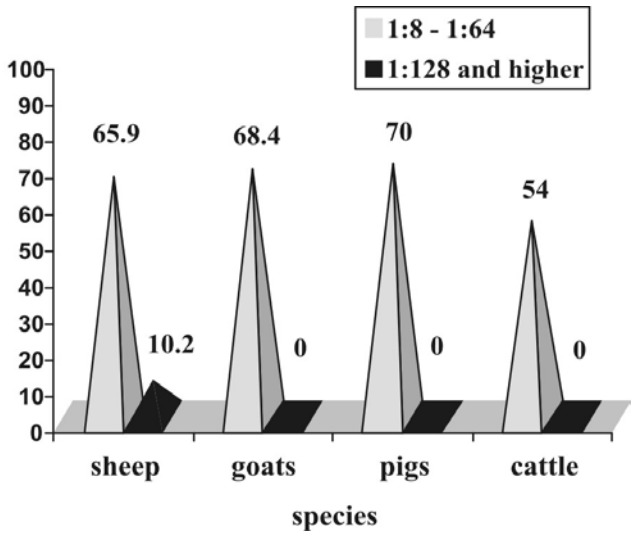

Fig. 1. Seroprevalence of antibodies to *T. Gondii*, CFT titres

countries. Fusco *et al.* (3) and Masala *et al.* (4) used serological methods to detect the presence of antibodies to *T. gondii* in sheep and goats.

Among laboratory diagnostic techniques a complement fixation test is one of the most frequently employed techniques for detecting antibodies to *T. gondii*. However, results acquired by this test may be influenced by different interpretation of such results. The level of overall antibodies in a CFT significantly correlates with the dynamics of IgM and IgA antibodies. A titre of overall antibodies 1 : 256–512 is significant for the acute phase of infection, whereas titres below 1 : 128 point to the chronic or latent course of the disease. With respect to the determination of a *Toxoplasma* infection in a serological examination, a CFT is of greater informative value in comparison to the same requirement related to IgG antibodies (5).

The prevalence of toxoplasmosis in animals in the Slovak Republic detected by the State veterinary and food institutes between 2001 and 2005 was 12.69 % in sheep, 32.82 % in goats, 15.16 % in beef cattle and 12.12 % in pigs (Gacíková, E., personal communication, June 6, 2006).

The presence of antibodies to *T. gondii* in humans in the Slovak Republic was approximately 9.1 % in 2003, while in 2005 it was 18.5 % and in 2006 it increased to 51.6 %. These results obtained by examinations carried out by the National Reference Laboratory indicate the increasing prevalence of this protozoan disease (11).

Fusco *et al.* (3) examined 117 flocks of sheep bred in pastures in the region of Campania, southern Italy. Blood and milk specimens were collected from 10 adult sheep (aged more than 18 months) from each of the flocks. The serum specimens were examined for the presence of IgG antibodies by an IFAT method (an indirect immunofluorescence antibody test). Out of the 1,170 examined sheep, 333 specimens were positive to *Toxoplasma* infection (28.5 %). Between 1999 and 2002 Masala *et al.* (4) analyzed 9,639 serum specimens and 815 abortion specimens (670 aborted fetuses and 145 placentas) from 964 sheep and goat farms in Sardinia. The collected sera were examined for the presence of IgG and IgM antibodies to *Toxoplasma gondii* using the indirect immunofluorescence method. Specific IgG antibodies were detected in 652 sheep (9 %). In France, the presence of specific IgG antibodies to *T. gondii* was detected in 22 % of the lambs and 65.6 % of the gravid sheep; in Sicily, the seroprevalence ranges between 70 and 90 %; in Switzerland, it is in approximately 58.6 % of sheep; while in Germany, it is only 19.1 % (14). Seroprevalence of toxoplasmosis were determined in 87 goats of the East Slovakia region. From these animals, antibodies were found in 43 goat sera (49.43 %). A statistically significant difference ($P < 0.0001$) was observed between the prevalence of antibodies against *T. gondii* in different age groups – goats up to 36 months of age and above 37 months of age (10).

Interesting results were obtained by examination of 787 pigs in the Czech Republic in 1999. They were kept on a modern large-capacity breeding farm in southern Bohemia. The examination by the complement fixation test revealed that only 4 pigs were positive for antibodies to *T. gondii* (15). In the Slovak Republic, the testing of 840 pig serum samples for *T. gondii* antibodies by ELISA detected 21 samples (2.5 %) positive (8).

Pleva *et al.* (6) published the results of the examination of 582 serum samples of cattle in 1996 and none of them was positive to *T. gondii*. In 2008, the examination of 85 samples of cattle in Slovakia by the ELISA detected anti-*Toxoplasma* antibodies only in 2 (2.35 %) of the samples (8). The seroprevalence of toxoplasmosis in cattle in Slovakia was monitored in 2009 by Spisak *et al.* (9) who examined 312 serum samples of cattle. The examination detected the presence of antibodies to *Toxoplasma gondii* in 31 (9.94 %) of the samples. The risk of the infection of domestic animals with *T. gondii* is very high. The potential preventive measures include: keeping meat-producing animals in captivity on farms throughout the feeding period; maintaining the stables without rodents, birds or insects; and paying closer attention to feeding the animals with non-contaminated fodder. By taking these measures it is possible to operate farms with a low prevalence of toxoplasmosis (12).

The results presented indicate that the prevalence of infections with *T. gondii* in livestock is closely related to the method of breeding. The risk of infection is higher for animals which are bred extensively and are allowed to graze on pastures, than in animals bred on farms with reduced or no contact with the outside environment. While grazing, the animals are exposed for long periods of time to the possibility of infections from the environment. Infected green fodder, soil and water are the most frequent sources of infection in these animals. If the animals are bred on farms where the basic animal hygiene rules are observed, animal contact with the outer environment is prevented and the fodder is well-stored, the risk of infection is reduced to the minimum and thus the seroprevalence is considerably lower.

CONCLUSION

Examination of blood specimens collected from 413 asymptomatic animals (sheep, goats, pigs, cattle) for the presence of overall antibodies to *T. gondii* by complement fixation test showed that toxoplasmosis is quite widespread among livestock bred in the study area. In total, 227 sera were positive (54.9 %). A statistically significant difference of the incidence of toxoplasmosis was noticed between the ewes and the two remaining subgroups of sheep ($P < 0.01$).

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GENOTYPING OF MEDICALLY IMPORTANT SPECIES OF MICROSPORIDIA AND THEIR GEOGRAPHIC DISTRIBUTION

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ABSTRACT

In the past several years microsporidia have become recognized as another important group of opportunistic infectious agents of humans and other animals, especially the immunocompromised patients. The detection and genotyping within microsporidial species is a prerequisite for the elucidation of their currently unknown reservoirs and their mode of transmission. In addition, the study of the genetic variability itself contributes to the discovery of new genotypes, such as in the species *Enterocytozoon bieneusi*. Also a new species *(Encephalitozoon romaleae)* was only discovered in 2003. It was named and classified only in the past year. Other new genotypes are discovered in various parts of the world year after year. Genetic variations have been described only in the species *Encephalitozoon cuniculi, Encephalitozoon hellem* and *Enterocytozoon bieneusi*. Their genotyping has been based only on molecular methods because their genotypes cannot be distinguished morphologically. The Internal Transcribed Spacer (ITS) region of the rRNA genes is the most frequently used genetic marker, mainly due to the high degree of diversity of this region in individual genotypes. This study describes the individual molecular differences between genotypes of medically important species of microsporidia *(Encephalitozoon cuniculi, Encephalitozoon hellem and Enterocytozoon bieneusi),* their geographical distribution and host diversity. The characterisation of microsporidian species at the genotype level may help to identify their sources and gain better understanding of the ways in which they infect humans.

Key words: *Encephalitozoon; Enterocytozoon;* genotypes; geographical distribution; microsporidia

INTRODUCTION

Microsporidia are a diverse group of obligate intracellular parasites capable of infecting a wide spectrum of invertebrates and vertebrates (including humans). Currently, more than 1 300 microsporidian species included in ~160 genera (102) have been identified. Of these, 14 species are capable of infecting humans (22). The species *Enterocytozoon bieneusi (Ent. bieneusi), Encephalitozoon cuniculi (E. cuniculi), Encephalitozoon hellem (E. hellem)* and *Encephalitozoon intestinalis (E. intestinalis)* have been described as opportunistic pathogens in Human Immunodeficiency Virus (HIV)-infected patients and other immunocompromised patients, such as transplant recipients. Infections with microsporidia in immunocompetent individuals such as travelers, children, elderly and contact lens wearers have also been described (22, 66, 71). In HIV-infected patients, *Ent. bieneusi* and *E. intestinalis* can cause a severe, persistent diarrhoea, and the species have frequently been isolated from stool specimens (30, 33, 34, 68, 96). Microsporidia are classified as a Category B organisms on the National Institute of Allergy and Infectious Diseases (NIAID) Category A, B & C Priority Pathogens List. Originally, it was assumed that microsporidia were primitive protozoa, but in the 1990's, molecular analysis showed their relationship to the kingdom Fungi. Adjustments of microsporidia to the parasitic way of life led to several principal alterations which resulted in an apparent paradoxical mixture of properties. On the one hand, using extremely complicate and elaborate mechanisms, they can attack their host and avoid their defences but, on the other hand, their metabolism is reduced to such a degree that they are, to a considerable extent, dependent on the metabolism of nutrients

and energy of the host. Although microsporidia are true eukaryotes (they have the typical eukaryotic nucleus, cytoplasmic membrane system, cytoskeleton), their molecular and cytological properties resemble prokaryotes. This involves: functions of their translation apparatus; genome size (corresponding to the size of bacteria; from 2.9 Mb to 19.5 Mb); absence of mitochondria, peroxisomes and the typical Golgi apparatus as well as ribosomes the individual parts of which have prokaryotic features, namely low sedimentation coefficients (ribosomes 70S; ribosome subunits 30S and 50S; rRNA 5S, 16S and 23S); high content of rRNA (60 %) and absence of free 5.8S rRNA gene (13, 44, 98).

The aim of this study was to describe individual molecular differences between the genotypes of medically important species of microsporidia *(Encephalitozoon cuniculi, Encephalitozoon hellem and Enterocytozoon bieneusi),* their geographic distribution and host diversity. Characterisation of microsporidian species at the genotype level may help to identify their sources and gain better understanding of the ways by which they infect humans.

Genus *Encephalitozoon* spp.

Three species of the *Encephalitozoon* genus were identified as human pathogens: (i) *E. cuniculi*, with a wide host range in mammals (11) and, particularly, the worldwide spread among domestic rabbits; (ii) *E. hellem*, the species differentiated in 1991 from *E. cuniculi* (27) and detected in several cases in birds in the USA and Indonesia; (iii) *E. intestinalis* (originally named *Septata intestinalis*), described for the first time in 1993 (9) and diagnosed in stool of farm animals worldwide and also in other mammals.

All three species produce spores morphologically indistinguishable from each other. Genetic variations have been described only in *E. cuniculi* and *E. hellem*.

The fourth species of this genus, *Encephalitozoon lacertae* (11, 49), was identified only in reptiles and is closely related to *E. cuniculi*.

In 2003 there was discovered a new species infecting insects of species *Romalea microptera* in Louisiana, in the USA. Only in 2009 was this newly discovered species classified as *Encephalitozoon romaleae* (50, 51), which is the fifth species included in the genus *Encephalitozoon*. Analysis of the ITS region proved its homogeneity with *E. hellem* species in 91 % of those examined.

Genotypes of *Encephalitozoon cuniculi* and their geographic distribution

Determination of the *E. cuniculi* genotypes is based on the ITS region located among rRNA genes (SSU and LSU rRNA genes), the size of which is much smaller in this species than in *Ent. bieneusi* (~243 bp) and ranges between 33 to 41 bp depending on the genotype. Immunological and molecular methods proved the existence of three genotypes of *E. cuniculi* (genotypes I, II and III) differing in the number of repeat sequences 5'-GTTT-3' in the ITS region. Genotype I ("rabbit genotype") has three 5'-GTTT-3' repeats and

Table 1. Ribosomal RNA intergenic spacer region sequences of the genotypes of *Encephalitozoon cuniculi* (26)

Genotypes *E. cuniculi*	ITS region	P*
Genotype I (rabbit)	5'-TGTTGTTGTGTTTTGATGGAT - - - - **GTTTGTTTGTTT**GTG-3'	37
Genotype II (mouse)	5'-TGTTGTTGTGTTTTGATGGAT - - - - - - - - **GTTTGTTT**GTG-3'	33
Genotype III (dog)	5'-TGTTGTTGTGTTTTGATGGAT**GTTTGTTTGTTTGTTT**GTG-3'	41

P* – number of base pairs in ITS region

Table 2. Differences among *Encephalitozoon cuniculi* genotypes in the repetitive region of the SWP-1 gene (104)

Genotype	PCR product length (bp)	Repeat length (bp)	No. of repeats	Repeat order*
Ia	339	255	10	36, 15, 36, 15, 36, 15, 36, 15, 36, 15
Ib	450	306	12	36, 15, 36, 15, 36, 15, 36, 15, 36, 15, 36, 15
II	363	219	9	15, 36, 15, 36, 15, 36, 15, 36, 15
	414	270	11	15, 36, 15, 36, 15, 36, 15, 36, 15, 36, 15
IIIa	348	204	8	15, 36, 15, 36, 36, 15, 36, 15
	429	285	12	15, 36, 15, 36, 15, 15, 15, 36, 36, 15, 36, 15
IIIb	429	285	12	15, 36, 15, 36, 15, 15, 15, 36, 36, 15, 36, 15

bp – base pairs
* – 36, 36 – bp repeat; 15, 15 – bp repeat

Table 3. ***Encephalitozoon cuniculi*** **genotypes identified by the internal transcribed spacer (ITS) sequence and reported in humans**

Genotypes	Hosts	Origin	Reference(s)
Genotype I	Human	Switzerland	19, 48, 61, 99
		Italy	77
		USA	104
Genotype II	–	–	–
Genotype III	Human	Italy	94
		England	1, 41, 42
		Spain	15
		Canada	64
		USA	12, 14, 25, 63, 93
		Mexico	19, 37, 61

Table 4. ***Encephalitozoon cuniculi*** **genotypes identified by the internal transcribed spacer (ITS) sequence and reported in animals**

Genotypes	Hosts	Origin	Reference(s)
Genotype I	Rabbit	Switzerland	61, 67
		Germany	Deplazes *et al.* (unpublished data)
		USA	47
		Australia	26
		Japan	36
		Bird Czech	45
Genotype II	Mouse	USA	104
		Czech	26
	Rabbit	Slovakia	Valenčáková *et al.* (unpublished data)
	Rat	Switzerland	65
	Fox	Norway	62
		Finland	2
	Bird	Czech	45
		Slovakia	Malčeková *et al.* (unpublished data)
Genotype III	Dog	USA	26, 87
		South Africa	26
	Rabbit	Slovakia	Valenčáková *et al.* (unpublished data)
	Tamarin	Germany	39, 73, 101
	Pig	Germany	72
	Monkey	Japan	3
	Bird	Czech	46
		Czech	45

the size of the ITS region is 37 bp, genotype II ("mouse genotype") has two 5'-GTTT-3' repeats and the size of its ITS region is 33 bp and genotype III ("dog genotype") has four 5'-GTTT-3' repeats and the size of its ITS region is 41 bp (26; Table 1).

The repetitive sequence 5'-GTTT-3' in the ITS region is a reliable and widely usable genetic marker of *E. cuniculi* species. The recent multiloci analyses brought up additional markers for determination of genotypes, namely the gene encoding Polar Tube Proteins (PTP gene; 70) and gene encoding spore wall protein 1 gene (SWP-1 gene; 104). Analyses of the nucleic sequences of the PTP gene also divided *E. cuniculi* to three genotypes. Segregation of genotypes corresponded to the results of the analysis of the ITS region. The nucleic sequences of 1 076 bp fragment from the PTP gene observed in all three genotypes differed from each other by the number of copies of 78 bp repeat and by point mutations (104). The genotype II sequence had four pair bases differing from those of genotype I and genotype III had one deletion of one copy of 78-bp repeat and two base pairs different from genotype I. Similar results were obtained for the SWP-1 gene with the difference that on the basis of variants of the number of repeats of 15 bp and 36 bp in this gene, the genotypes I and III were divided into several sub-genotypes (104). All together, 5 genotypes of *E. cuniculi* were identified; however, even two heterogeneous copies of the SWP-1 gene appeared in some isolates. The length of the PCR products ranged between 348 bp and 450 bp due to the different number of the mentioned repeats. The genotype Ia generated one PCR product with 10 repeats, genotype Ib also one product with 12 repeats and genotype II generated two PCR products, one with 9 repeats and another with 11 repeats; genotype IIIa generated two PCR products, one identical with the PCR product of genotype IIIb with 12 repeats and another PCR product with 8 repeats. The sizes of the individual PCR products are shown in Table 2.

These genotype differences are helpful in explaining the complicated epidemiological situation of *E. cuniculi* in various hosts and in different parts of the world.

Genotypes determined in the species *E. cuniculi* were named according to the host in which they were identified for the first time. Thus, the genotype I is referred to as "rabbit genotype" discovered in rabbits, genotype II is called "mouse genotype" identified in mice and the last genotype III is "dog genotype" named according to its host. Individual genotypes are not restricted to their primary hosts but, according to the current knowledge, were detected also in other animal species, even in the man. Tables 3 and 4 present host species and the geographic origin of those *E. cuniculi* isolates which were identified at the genotype level and for which there is available complex information about the host.

Table 5. Sequence differences among genotypes of *Encephalitozoon hellem* in the SSU rRNA gene (103)

Genotype	Nucleotide at position									
	119 (119)	160 (160)	– (162)	171 (172)	505 (506)	517 (518)	538 (539)	539 (540)	952 (953)	1013 (1014)
1A	A	G	–	G	A	G	T	A	A	G
1B	A	G	–	G	A	G	T	A	A	G
1C	A	G	G	G	A	G	T	A	A	G
2A	A	T	–	T	T	A	G	G	A	A
2B	A	T	–	T	T	A	G	G	A	A
2C	T	T	–	T	T	A	G	G	G	A

Table 6. Sequence differences among genotypes of *Encephalitozoon hellem* in the ITS rRNA region (103)

Genotype	Nucleotide at position
1A	TGTTGATTTGATTATTTT - - - GTGGGGATTT T T AGTTTTTTAGTT - - - - - - - - - - TTTCTTTCT
1B	TGTTGATTTGATTATTTT - - - GTGGGGATTT T T AGTTTTTTAGTT - - - - - - - - - - TTTCTTTCT
1C	TGTTGATTTGATTATTTT - - - GTGGGGATTT T T AGTTTTTTAGTT - - - - - - - - - - TTTCTTTCT
2A	TGTTGATTTGATT**G**TTT - - - - GTGG - **T**ATT**GAG** AGTTTTT- AGTT**TTTTTTCTTTC**TTTCTTTCT
2B	TGTTGATTTGATT**G**TTT - - - - GTGG - **T**ATT**GAG** AGTTTTT- AGTT**TTTTTTC** - - - - TTTCTTTCT
2C	TGTTGATTTGATT**G**TTT**GTTT**GTGG - **T**ATT**GAG**AGTTTTT- AGTT**TTT** - - - - - - - - TTTCTTTCT

Dashes depict nucleotide deletions. Genotypes 1A, 1B and 1C – genotype 1 (60); genotype 2A – genotype 2 (60); genotype 2C – genotype 3 (60)

Genotypes of *Encephalitozoon hellem* and their geographic distribution

The primary studies focused on the analysis of SSU rRNA gene and ITS region which resulted in conclusion that the *E. hellem* isolates are genetically homogeneous (17). These conclusions were refuted by Mathis *et al.,* 1999 (60), who proved the existence of three genotypes (genotype 1, genotype 2 and genotype 3) on the basis of the differences in the ITS regions. Karyotype analysis (5, 17) with multiloci analysis focused on coded (PTP gene and SSU rRNA gene) and uncoded (ITS region) regions which proved the presence of six different genotypes, named 1A, 1B, 1C, 2A, 2B and 2C (Tables 5 and 6; 103). Differences between the individual genotypes in the SSU rRNA gene and ITS regions, based on sequence differences and deletions, are presented in Tables 5 and 6. Genotypes are most frequently identified on the basis of the ITS region. Genotype 1 is identical with genotypes 1A, 1B and 1C; genotype 2 was renamed to 2A and genotype 3 to 2C (Table 6). Four various sizes of PCR products were amplified from the PTP gene. Genotype 1A generated the smallest amplicon of size, only 1 253 bp, and the lengths of the other genotypes, 1B, 1C and 2B, had lengths 1 313 bp, 1 373 bp and 1 421 bp respectively. Different lengths of sequences resulted from different numbers of copies of the 60-bp repeats. Genotype 1A had six copies of repeats, 1B seven copies and 1C eight copies. Genotype 2B had besides three copies of 60-bp repeats also five copies of 66-bp repeats and in one copy of the 66-bp repeat the sequence of size 6-bp (GGAAGC or GGAAGT) occurred as one copy at the beginning of the 60-bp repeat. In addition to that the genotype 2B had an 18-bp insert in front of the region of repetitions (103). A uniform size of markers IGS-TH (193 bp) and IGS-HZ (230 bp) was recorded in all genotypes with only sequence differences (40, 103).

Summary of these studies prove the extensive genetic diversity of *E. hellem.* In the past the study of its genetic diversity was limited only to the ITS region but the analyses of PTP gene and additional markers provided further confirmation.

Up to the present, the presence of *E. hellem* was proven only in human and avian populations (Table 7). The genotypes described were identified by the sequence analysis of the ITS region. Some genotypes were identified employing also other markers as described in Table 7.

Currently, the number of *E. hellem* is very limited and does not allow one to compare meaningfully the division of genotypes between human and avian population. The recent data indicate significant segregation of certain genotypes.

Encephalitozoon intestinalis

Contrary to other species of the genus *Encephalitozoon,* in which there were identified various genotypes with different molecular biology and epidemiology, *E. intestinalis* seems to be a very homogenous species. A number of analyses (Western blot and electrophoresis) of five isolates revealed only small differences (16) insufficient for defining of genotypes. Contrary to isolates of species *E. cuniculi* and *E. hellem,* showing considerable intraspecies variability of karyotypes (4, 5, 17, 88), karyotypes of *E. intestinalis* isolates are identical (5). We recorded no variations in the ITS region in 16 *E. intestinalis* isolates (24, 55).

Genus *Enterocytozoon* spp.

The family *Enterocytozoonidae* includes two genera: (i) *Nucleospora*, which includes *Nucleospora salmonis,* an intracellular parasite of salmonids (28) and *Nucleospora secundo*, a parasite of African fish of hot water (59) and (ii) genus *Enterocytozoon* with species *Ent. bieneusi*, infecting the cytoplasm of enterocytes and other epithelial cells of humans and other mammals (21). *Ent. bieneusi* is a microsporidian species most frequently causing diseases in humans. It was described for the first time in 1985 as a HIV-associated, opportunistic intestinal pathogen and its morphological character was obtained by electron microscopy (21). In 1996, morphologically identical spores were found in pig faeces (20) and subsequently also in the faeces and intestinal tissues of other mammals.

Genotypes of *Enterocytozoon bieneusi* and their geographic distribution

After 20 years, *Ent. bieneusi* has been characterised as an ubiquitous intestinal parasite with extensive genetic diversity. Viewing *Ent. bieneusi* as a complex of many genotypes with unique biological attributes may help to provide an important perspective allowing one to judge better the diversity of its host specificity and pathogenicity. Currently, 81 genotypes differing in the ITS region (243 bp) have been described.

The genotyping of *Ent. bieneusi* is based only on molecular methods because genotypes of *Ent. bieneusi* cannot be differentiated morphologically. The sequence analysis of the ITS region of the rRNA genes is a standard method for genotyping *Ent. bieneusi* owing to the high diversity of this region in individual genotypes.

Different terminologies used by individual authors when naming genotypes of *Ent. bieneusi* have caused problems with the identification of both the already discovered and new genotypes. The lack of acceptable nomenclature at genotype naming resulted in several names for the same genotype and thus some genotypes have more than one name. This applies, for example, to genotype D, which

Table 7. *Encephalitozoon hellem* genotypes reported in humans and other animals

Hosts	ITS region	SSU rRNA gene	PTP gene	Origin	Reference(s)
Humans	1A	1A	1A	Puerto Rico	97
	1A	1A	1A	USA	25, 27, 35, 85, 97, 105
	1A	1A	1B	USA	27, 35, 85, 97
	1A	1A	1B	Spain	69
	1A	1A	1B	Italy	83, 84, 97
	1A	1C	1C	Italy	38, 82, 83, 97
	2A	–	–	Tanzania	60
	2A	–	–	Switzerland	60
	2B	2B	2B	Switzerland	97, 100
	2C	–	–	Switzerland	60
Bird	1A	–	–	Czech	45
	2A	–	–	Czech	45
	Genotype 1	–	–	Indonesia	92
	Genotype 1	–	–	USA	86

Table 8. ***Enterocytozoon bieneusi*** **genotypes reported only in humans (78)**

Host	Primary genotype name (GenBank accession number)	Synonyms (GenBank accession number)	Reference(s)
	A (AF101197, AY168419[a], AY357185 – AY357204, DQ683750 and DQ683753)	**Peru1** (AY371276)	7, 45, 54, 76, 90
	B (AF101198 and DQ683754)	**Typ I** (AF242475)	7, 56, 76
	C (AF101199)	**Typ II** (AF242476)	56, 76
	Q (AF267147)[b]		74
	R (AY945808)		53
	S (AY945809)		53
	T (AY945810)		53
	U (AY945811)		53
	V (AY945812)		53
	W (AY945813)		53
	Typ III (AF242477)		56
	Typ V (AF242479)		56
Humans	**Peru3** (AY371278)		90
	Peru7 (AY371282)		90
	Peru8 (AY371283)		90
	Peru11 (AY371286)	**Peru12**(EF014428[a])[c]	90
	Peru13 (EF014429)[a]		
	Peru15 (EF014431)[a]		
	CAF1 (DQ683746)		7
	CAF2 (DQ683747)		7
	CAF3 (DQ683748)		7
	CAF4 (DQ683749 and DQ683757)		7
	HAN1 (EF458627)		31
	NIA1 (EF458628)		31
	UG2145 (AF502396)		95
	Genotyp 17 (EU140500)[a]		

[a] – Unpublished; [b] – Genotype Q has 245 bp

[c] – Genotypes Peru11 and Peru12 differ in a nucleotide at position 3 in the LSU rRNA gene (G *vs.* A)

Table 9. ***Enterocytozoon bieneusi*** **genotypes reported in humans and other animals (78)**

Host	Primary genotype name (GenBank accession number)	Synonyms (GenBank accession number)	Reference(s)
Humans, beaver, fox, muskrat, raccoon	**WL15** (AY237223)[a]	**WL16** (AY237224) **Peru14** (EF014430[b])	90, 91
Humans, beaver, cattle, dogs, falcons, fox, macaque, muskrat, pigs, raccoon	**D** (AF101200, DQ793213, DQ683751, DQ683755 and AF023245[c])	**PigITS9** (AF348477) **WL8** (AY237216) **Peru9** (AY371284) **PtEb VI**(DQ885582) **CEbC** (EF139197)	7, 8, 43, 52, 57, 75, 90, 91
Humans, birds, cattle, dogs	**Peru6** (AY371281 and DQ154137)	**PtEbI** (DQ425107) **PtEbVII**(DQ885583)	57, 58, 81, 90
Humans, beaver, fox, muskrat, pigs, otter, raccoon	**EbpC** (AF076042 and U61180[c])	**E** (AF135832)[d] **WL13** (AY237221) **WL17** (AY237225) **Peru4** (AY371279)	6, 20, 74, 90, 91
Humans, cats	**Peru10** (AY371285 and DQ836342)		80, 90
Humans, guinea pigs	**Peru16** (EF014427)		10
Humans, cats, fox	**WL11** (AY237219)	**Peru5** (AY371280, DQ836344 and EU650271)	79, 80, 90, 91
Humans, cats, cattle, dogs	**Typ IV** (AF242478)	**K** (AF267141, DQ836343, DQ683752, DQ683756 and EU650272) **Peru2** (AY371277) **PtEb III**(DQ885579) **BEB5** (AY331009) **BEB5-var** (AY331010[b])[e]	7, 18, 56, 57, 79, 80, 89, 90

[a] – Genotypes WL15 and WL16 / Peru14 differ in a nucleotide at position 25 in LSU rRNA gene (G *vs.* A); [b] – Unpublished;
[c] – It was not named in the publication or GenBank;
[d] – Genotypes E / WL13 / Peru4 and WL17 differ in a nucleotide at position 25 in the LSU rRNA gene (A *vs.* G);
[e] – Genotypes K(AF267141 and DQ836343) / Peru2(AY371277) / PtEb III(DQ885579) / BEB5(AY331009) and BEB5-var(AY331010) differ in a nucleotide at position 1225 in the SSU rRNA gene (A *vs.* T)

has as many as six different names (Table 9). This extensive genetic diversity of the species *Ent. bieneusi* (only on the basis of the ITS region) contributed to uncertainty regarding the role of *Ent. bieneusi* as an infectious agent because it is especially difficult to identify the differences in the action of individual genotypes in the host.

The first attempt to establish a classification system was made by Drosten *et al.* in 2005 (29) and subsequently the sequences of the ITS region were made available in the GenBank database. The classification system was based on three principal groups and one remote group with the three groups containing genotypes only from one animal host species. For example group 2 contained only cattle, group 3 muskrat and group 4 (remote group) only racoons. Contrary to groups 2, 3 and 4, group 1 contained a number of host species and some subgroups that were associated only with humans or only with pigs. The zoonotic potential of *Ent. bieneusi* was supported phylogenetically by the species diversity within host subgroups in group 1 which contained (i) humans, macaques, pigs, cats, foxes, and beavers; (ii) humans, cattle, llamas, cats, foxes, beavers, and muskrats; (iii) humans, pigs, racoons, and muskrats (29). Re-evaluation of this system in 2008, showed that for individual genotypes, stored in GenBank database, there were identified several host species in all proposed groups which made the grouping based on host specificity invalid.

In 2009, there was proposed a new classification of *Ent. bieneusi* genotypes based on the ITS region and, subsequently, individual genotypes were summarised into new groups (78). The majority of

Table 10. ***Enterocytozoon bieneusi*** **genotypes reported in livestock (78)**

Host	Primary genotype name (GenBank accession number)	Synonyms (GenBank accession number)	Reference(s)
Cattle	**I** (AF135836)	**BEB2** (AY331006), **CEbE** (EF139199)	52, 74, 89
	J (AF135837)	**BEB1** (AY331005), **PtEb X** (DQ885586), **CEbB** (EF139196)	52, 57, 74, 89
	M (AF267143)		18
	N (AF267144)		18
	PtEb XI (DQ885587)		57
	BEB3 (AY331007)		89
	BEB4 (AY331008)		89
	BEB6 (EU153584)		32
	BEB7 (EU153585)		32
	CEbA (EF139195)		52
	CEbD (EF139198)		52
	CEbF (EF139194)		52
	4948 FL-2 2004 (DQ154136)		81
Pig	**EbpB** (AF076041)		6
	EbpD (AF076043)		6
	G (AF135834)		74
	H (AF135835)		74
	O (AF267145)		18
	PigEBITS1(AF348469)		8
	PigEBITS2(AF348470)		8
	PigEBITS3(AF348471)		8
	PigEBITS4(AF348472)		8
	PigEBITS5(AF348473)		8
	PigEBITS6(AF348474)		8
	PigEBITS7(AF348475)		8
	PigEBITS8(AF348476)		8
Cattle, pigs	**EbpA** (AF076040)	**F** (AF135833)	6, 74

Table 11. ***Enterocytozoon bieneusi*** **genotypes reported in companion animals (78)**

Host	Primary genotype name (GenBank accession number)	Synonyms (GenBank accession number)	Reference(s)
Cats	**L** (AF267142)		18
	PtEb IV (DQ885580)		5
	PtEb VIII (DQ885584)		57
	D-like (DQ836345)		80
	AF118144[a]		60
Dogs	**PtEb IX** (DQ885585, AF059610 and EU650273		57, 60, 79

[a] – It was not named in the publication or GenBank

Table 12. ***Enterocytozoon bieneusi*** **genotypes reported in miscellaneous hosts (78)**

Host	Primary genotype name (GenBank accession number)	Synonyms (GenBank accession number)	Reference(s)
Beaver	**WL7** (AY237215)		91
	WL9 (AY237217)		91
Bird	**PtEb II** (DQ425108)		58
Kudu	**PtEb V** (DQ885581)		57
Llama	**P** (AF267146)		18
Marmoset	**PtEb XII** (DQ885588)		57
Muskrat	**WL4** (AY237212)	**WL5** (AY237213)[a]	91
	WL6 (AY237214)		91
	WL10 (AY237218)		91
	WL14 (AY237222)		91
Raccoon	**WL1** (AY237209)		91
	WL2 (AY237210)		91
	WL3 (AY237211)		91
Beaver and otter	**WL12** (AY237220)		91

[a] – Genotypes WL4 and WL5 differ in a nucleotide at position 29 in the LSU rRNA gene (G *vs.* T)

primers presented by various authors amplify at *Ent. bieneusi* genotyping the ITS regions containing in most cases 243 base pairs. Some sequences of the mentioned genotypes include beside the ITS region also parts of SSU and LSU rRNA genes. In this study, we summarize genotypes with complete ITS regions (excluded genotypes with incomplete ITS regions: EF661812-EF661814, F010879-EF010883, AY668952, AY668953, AY298730, AY315716, L20290 and AH012971).

Up until 2009, there were identified 81 genotypes of *Ent. bieneusi*: 26 genotypes identified only in humans (Table 8); 8 genotypes in humans and other hosts (Table 9); 27 identified exclusively in cattle and pigs (Table 10); 6 identified exclusively in cats and dogs (Table 11); and 14 identified in various hosts (Table 12). Some genotypes with identical ITS regions have different names due to nucleotide differences in regions flanking the ITS regions (specifically of SSU and LSU rRNA genes), for example Peru11 and Peru12 (Supplement 1) or WL15 and WL16/Peru14 (Supplement 2). The first published genotype name (together with the number in the GenBank database), has the priority and becomes the principal name in all other publication reporting genotyping based on sequence analysis of the ITS region and the synonyms should be published along with this name.

Contrary to the situation in microsporidia of the genus *Encephalitozoon,* no other genetic markers are available for the species *Ent. bieneusi*. A big disadvantage of only one marker is that the genotypes which are considered genetically identical may not be such which raises some doubts about the past published results. Because of that, there is a need to identify in the future, the new genetic markers and use them to analyse whether or not the genotypes identified on the basis of the ITS region are coincident with the new genetic markers. This would contribute to the better identification of sources and help to assess the host spectrum and pathogenic potential of the isolates. New sets of markers could become a very useful tool for evaluation of the epidemiological relationships between individual genotypes of *Ent. bieneusi*.

CONCLUSION

After the discovery of the genetic variability of the species *E. cuniculi, E. hellem* and *Ent. bieneusi* there has been an increasing interest in genotyping of these species. The identification of pathogens at the genotype level can become a useful tool in searching for and identifying reservoirs of these pathogens and gaining better understanding of the ways in which the human infections are contracted.

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DETERMINATION OF TYLOSIN IN FOOD OF ANIMAL ORIGIN BY LIQUID CHROMATOGRAPHY

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ABSTRACT

A modified, rapid and precise high-performance liquid chromatographic method (HPLC) in combination with solid phase extraction (SPE) for the determination of tylosin residues in the food of animal origin (muscle, milk, and eggs) was developed. The method consists of a two steps process. The first step is based on the solid phase extraction on a C_{18} cartridge and a liquid-liquid extraction with phosphate buffer and dichloromethane. The second one is a HPLC analysis on a reversed phase column C_{18} using a mobile phase consisting of acetonitrile and 0.01 M phosphoric acid (60 : 40 v/v). UV detection was performed at 287 nm with a detection limit of 10 μg.kg^{-1}. The average recoveries of tylosin from the poultry and pork muscle tissues were comparable (66.7–78.8 % and 69.9–77.7, resp.), the recoveries from eggs ranged from 57.5 to 62.0 %, and from milk 86.5–94.1 %.

Key words: eggs; HPLC; milk; muscle; residues; tylosin

INTRODUCTION

The commonly used macrolide antibiotics are characterised by a common chemical structure including a macrocyclic lactone ring usually bearing from 12 to 16 atoms with one or more deoxy neutral and/or amino sugars linked via glycosidic bonds (8). Tylosin is a broad-spectrum macrolide antibiotic, which has been developed specifically for veterinary use (11, 14). Macrolide antibiotics constitute a very important class of antibacterial compounds highly active against Gram-positive and Gram-negative cocci (8).

Antibiotic residues may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or may indirectly cause problems through the induction of resistant strains of bacteria (6). The abuse and misuse of antibiotics in human and veterinary practices has lead to a significant increase of antimicrobial resistance and therefore poses a risk for human health. In parallel, the quality of food products has become a major concern of consumers and producers in the European Union in regards to protecting consumer health. Hence the control of the origin and quality of food demands the development of reference analytical methods to enforce the relevant Commission policies (8). Regulation (EC) No 470/2009 of the European Parliament and of the Council lays down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin (15). Pharmacologically active substances and their classification regarding maximum residue limits are set by Commission Regulation (EU) No 37/2010 (3) establishing the respective MRLs as follows: 100 μg.kg^{-1} for meat, 200 μg.kg^{-1} for eggs, and 50 μg.kg^{-1} for milk.

Simple and reliable analytical methods are required for monitoring of these drugs in the edible tissues of livestock animals. According to Korsrud *et al.* (9) standard microbiological methods were not sufficiently sensitive for the detection of tylosin in food of animal origin with respect to the maximum residue limit (9). However, microbial inhibitions assays were the earliest methods used for the detection of antibiotic residues and they are still widely used. They are very cost-effective and in contrast to, for example, immunological or receptor-based tests, they have the potential to cover the entire antibiotic spectrum within one test (13).

To determine withdrawal periods, the European Commission (EC) demands validated methods for the quantification of the target compound in different tissues (2). The method of high-performance liquid chromatography (HPLC) is selective and sensitive.

Macrolides are commonly extracted from biological matrices into organic solvents and further preconcentrated by solid-phase extraction, always after the elimination of proteins and fat. However, recovery is affected by the type of biological matrix (5). Reversed phase liquid chromatography with silica-based columns is the most usual approach for macrolide separation. Current mobile phases consist of a mixture of acetonitril (ACN) and an aqueous phosphate or acetate buffer (10). Tylosin is detected by UV-VIS detector at 280 or 287 nm (7, 12) or by HPLC combined with mass spectrometry(1). They give limits of detection lower than those stated for MRL.

The aim of this work was to develop a modified, rapid and precise high-performance liquid chromatographic method (HPLC) in combination with solid phase extraction (SPE) for the determination of tylosin residues in food of animal origin.

MATERIALS AND METHODS

Tylosin tartate was purchased from Sigma Chemical Co. (St. Louis, MO, USA); ammonium acetate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, phosphoric acid were supplied by Lachema (Brno, Czech Republic); dichlormethane, methanole, and acetonitrile HPLC-grade from Merck (Darmstadt, Germany); water was distilled and of HPLC-grade; SPE cartridges, SEP-Pak Vac C_{18}, 500 mg, (Waters, Milford, MA, USA) and Bond Elut $C_{18,}$ 500 mg, (Varian, Harbor City, CA, USA).

Phosphate buffer solution (pH 6) was prepared by dissolving 2 g of dipotassium hydrogen phosphate and 8 g of potassium dihydrogen phosphate in water and diluting it to 1 liter. 0.1 M ammonium acetate solution was prepared by dissolving 0.7708 g of ammonium acetate in 100 ml of methanol.

The following equipment was used: LHM 20 homogenizer (ČSAV Brno, Czech Republic), centrifuge BR4i (Jouan, St Herblain Cedex, France), vacuum evaporator Laborota (Heidolf, Kelheim, Germany).

Chromatography was performed by a liquid chromatograph Hewlett Packard (HP) (Avondale, PA, USA), Series 1050, with a quaternary pump, autosampler, variable wavelength detector, and diode array detector.

Tylosin standard solutions: Tylosin stock solution – 1 mg.ml^{-1} was prepared by dissolving 10 mg tylosin tartrate in 10 ml methanol and stored at –18 °C. The solution remains stable for 1 month. Tylosin working solution for fortification was prepared by dilution of tylosin stock solution with water, and the working solution for HPLC by diluting 1 μg.ml^{-1} in the mobile phase. Fresh solutions were prepared daily.

The fortification of meat (poultry muscle, pork muscle): Meat not containing tylosin was ground and 5 g was weighed as a sample. The tylosin standard solution for fortification was prepared by dissolving in water. Spiked samples were prepared after the mixing of the tylosin standard solution to muscular substances to obtain final concentrations 1, 0.5 and 0.1 μg.g^{-1} of meat.

Fortification of milk: Spiked samples were prepared by mixing milk with the tylosin standard solution to obtain concentrations 1, 0.1 and 0.05 μg.ml^{-1} of milk.

Fortification of eggs: Egg (white and yolk) was homogenised. Spiked samples were prepared by mixing the egg mass with tylosin solution to obtain concentrations 1, 0.5 and 0.1 μg.g^{-1} of egg.

Sample preparation

Samples of muscle tissues: 5 g of ground fortified muscle tissue was mixed with 5 ml water. 20 ml acetonitrile was added to the sample and the mixture was homogenised for 10 min. Then the sample was centrifuged for 15 min at 3 000 rpm and 4 °C. The extracted solvent layer was then decanted and diluted to 100 ml with water and applied to the Bond Elut C_{18} cartridge.

Ewe milk sample: 30 ml of fortified ewe milk was centrifuged for 10 min at 3 000 rpm and 4 °C to separate the fat layer. From the lower skim layer 10 ml was transferred to another centrifuge tube and 20 ml acetonitrile was added. The solution was mixed and centrifuged for 10 min at 3 000 rpm and 4 °C. The extracted solvent layer was then decanted and diluted to 100 ml with water and applied to the SEP-Pak Vac C_{18} cartridge.

Hen egg sample: 5 ml of n-hexane was added to 5 g of homogenised fortified egg sample. Then the mixture was centrifuged for 10 min at 3 000 rpm and 4 °C to separate the fat layer. The sample without the fat layer was then prepared as the samples of muscle tissue.

The cartridge (C_{18}) was activated with 2 ml of methanol and 5 ml of water. The supernatant containing tylosin was added to the SPE cartridge and filtered under vacuum at < 5 ml.min^{-1}. The cartridge was washed with 20 ml of water and dried under vacuum. The tylosin residue was eluted by 4 ml 0.1 M ammonium acetate solution in methanol. The eluate was mixed with 6 ml 0.01 M phosphate buffer (pH 6.0) in a separatory funnel and dichloromethane (20 ml) was added to the solution. The mixture was shaken for 5 min. The organic layer was separated and evaporated to dryness by a vacuum evaporator. The dried residue was dissolved in 2 ml of the mobile phase. The final solution was stored in a glass minivial.

Liquid chromatographic analysis

For the chromatographic analysis we used Hypersil BDS C_{18} (3 μm, 100 × 4 mm) column (HP) with Lichrospher 100 RP-18 (5 μm, 100 × 4 mm) guard column (Merck, Germany). The mobile phase was acetonitrile – 0.01 M phosphoric acid (pH 2.4) in the ratio of 60 : 40. The flow rate was 0.75 ml.min^{-1}. The volume injected was 20 μl. UV detection was performed at 287 nm.

RESULTS AND DISCUSSION

The method described allows tylosin to be extracted in a simple and reliable way from all the target tissues. Acetonitrile, in all matrices, was used for precipitating the proteins and n-hexane for extraction of fats in eggs. The traditional strategies for extracting of antibiotics include as a first step, the precipitation of proteins with organic solvents, either alone or in combination with inorganic or trichloracetic acids (5). Different solvents, such as acetonitrile or chloroform-ethyl acetate, at alkaline pH, are recommended. In some studies, acidic pH was used to make the union of the molecule to column solid-phase extraction easier (16).

Reversed-phase HPLC has often been used for the determination of tylosin in biological substances. In our experiment Hypersil BDS C_{18} reversed-phase column was used. The mobile phase selected involved acetonitrile – 0.01 M phosphoric acid at pH 2.6 (60 : 40 v/v) in an isocratic system. The acetonitrile content and pH of the mobile phase strongly af-

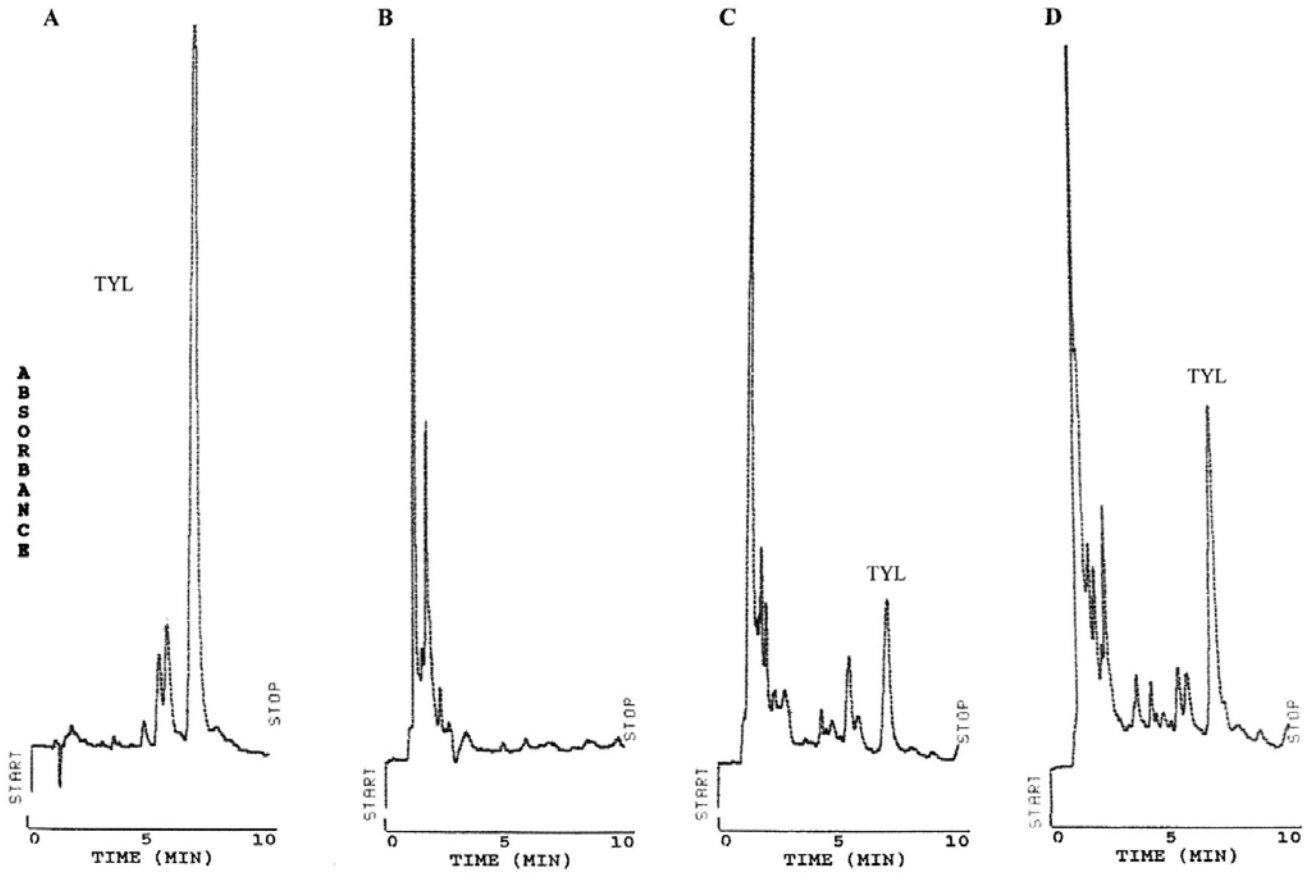

Fig. 1. Representative HPLC chromatograms: (A) tylosin standard (0,02 μg.g^{-1}) (B) blank muscle sample, (C) milk sample spiked with tylosin (0.05 μg.ml^{-1}) and (D) therapeutic milk sample. HPLC conditions: Hypersil BDS C_{18} column (3 μm, 100 × 4 mm), acetonitrile – 0.01 M phosphoric acid (60 : 40 v/v), flow rate 0.75 ml.min^{-1}, UV detection at 287 nm. Volume injected 20 μl

fect the chromatographic behaviour of the analyte. Although no significant effect on the retention times was observed in the 2–4 pH range, the peak shape improved as pH decreased and pH 2.5 was chosen in order to preserve the column life (10). Tylosin was successfully detected within 8 min when the flow rate was 0.75 ml.min^{-1}.

Chromatograms in the place of elution of tylosin did not contain interference peaks which could influence the quantitative results. In close nearness of the tylosin peak, before or after it, a small peak was observed, the so-called impurities (parts of tylosin tartate, tylosin A, B, respectively) (12). The measurement was conducted at 287 nm, which gave the maximum absorbance for tylosin. The minimum detectable amount (signal-to-noise ratio > 5) was 0.17 ng.

The chromatograms of the tylosin standard, milk blank, representative spiked milk samples and therapeutic sample are shown in Fig. 1. Samples were processed according to the procedure given under Material and Methods. Extraction with acetonitrile is a simple and effective procedure for extraction of tylosin from biological materials and avoids the extreme pH (17).

For the recovery study samples not containing tylosin residue were used. Quantification was based on a peak area. Table 1 summarizes the average recoveries of tylosin from the poultry and pork muscle tissues, eggs and milk spiked samples at three different fortification levels (1, 0.5, 0.1 μg.g^{-1} for muscle and eggs, respectively 1, 0.1, 0.05 μg.ml^{-1} for milk). As shown in Table 1, recoveries were comparable and there were no significant differences between spiked poultry and pork muscle. Recoveries from eggs were from 57.5 to 62.0 % and they were lower. Recoveries from milk ranged from 86.5 to 94.1 %. The recoveries from milk were higher than those from muscular tissues and eggs. The decreasing recoveries were caused by a variety of matrix (e. g. protein content, fat content, etc.). The spectrophotometric detection was shown to be suitable for the determination of tylosin. Indeed, De Liguoro *et al.* (4) presented a method for the determination of tylosin in different tissues at MRL level. Recoveries were estimated in tissues fortified at three different concentration levels (0.05, 0.1, 0.2 μg.g^{-1}). The mean recoveries obtained ranged from 69.9 to 85.3 % depending on the matrix.

The limit of detection (LOD), defined as the average background of samples (fluctuations of the baseline) plus three times the SD, was 10 μg.kg^{-1}. The limit of quantification (LOQ), defined as the average background of samples plus ten times the SD, was 30 μg.kg^{-1}. In 2001, Prats *et al.*

Table 1. Recovery of tylosin from fortified samples (n = 5)

Parameter	Poultry muscle		
Spiked level ($\mu g.g^{-1}$)	1	0.5	0.1
Average recovery (%)	66.7	78.8	77.6
R.S.D. (%)	11.3	8.2	11.5
Parameter	**Pork muscle**		
Spiked level ($\mu g.g^{-1}$)	1	0.5	0.1
Average recovery (%)	69.9	77.7	76.9
R.S.D. (%)	2.7	7.4	9.0
Parameter	**Eggs**		
Spiked level ($\mu g.g^{-1}$)	1	0.5	0.1
Average recovery (%)	59.2	62.0	57.5
R.S.D. (%)	8.4	10.1	9.7
Parameter	**Milk**		
Spiked level ($\mu g.ml^{-1}$)	1	0.1	0.05
Average recovery (%)	94.1	89.0	86.5
R.S.D. (%)	4.3	7.5	9.2

R.S.D. — relative standard deviation, n — number of measurements

(13) published a HPLC method capable of detecting and quantifying tylosin residues in several tissues from different animal species also using a spectrophotometric detector. The analysis of tylosin was performed on a C_{18} column using the isocratic elution of the mixture of acetonitrile and phosphate buffer at pH 2.4. The detector, set at 287 nm, was demonstrated to be sufficiently sensitive and selective. For limits of quantification at 50 $\mu g.g^{-1}$ for poultry, pork, egg and 30 $\mu g.l^{-1}$ for milk samples the precision and accuracy were considered adequate.

CONCLUSIONS

The proposed method for determination of tylosin in muscle tissues, eggs and milk offers short analysis time (total < 1.5 h per sample), low organic solvent consumption (total 22 ml per sample), high precision (RSD < 11 % in the recovery test) and the LOQ is lower than MRL (100 $\mu g.kg^{-1}$ for meat, 200 $\mu g.kg^{-1}$ for eggs, and 50 $\mu g.kg^{-1}$ for milk). The results obtained demonstrate that this method is useful for the routine determination of tylosin residues in food of animal origin.

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