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# IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL STUDIES OF *MYCOPLASMA HYOPNEUMONIAE* STRAIN IN NATURALLY INFECTED PIGS IN NIGERIA

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## ABSTRACT

**Enzootic pneumonia caused by *hyopneumoniae* (MHYO) remains a serious concern to the swine industry in many countries including Nigeria. MHYO strains isolated from pigs from different countries and geographical locations are known to vary in pathogenicity. There is a paucity of information on the pathogenicity of the MHYO strain affecting pigs in Nigeria. This study investigated the pathogenicity of the MHYO strain in naturally infected pigs using immunohistochemistry and electron microscopy. Two hundred and sixty four lungs of slaughtered pigs were randomly collected from abattoirs at Abeokuta, Ibadan and Lagos, in Southwest Nigeria. A sub-sample of 104 pneumonic and 20 apparently normal lungs was selected, processed for routine histopathological examination and immunohistochemistry, while 3 lung tissues samples were selected for ultrastructural studies. The most significant microscopic changes observed were suppurative broncho-interstitial pneumonia associated with varying degrees of lymphoid hyperplasia of the bronchus-associated lymphoid tissue (BALT) and thickened alveolar septa due to cellular infiltration consisting predominantly of neutrophils and a few mononuclear cells. Immunohistochemically, MHYO antigen was detected in 86/104 (82.69%) of MHYO-infected lung tissues and typically exhibited a granular brown reaction on the bronchial and bronchiolar epithelial lining, mononuclear cells in the BALT and luminal cellular exudates within the airways. Transmission electron microscopy revealed numerous *Mycoplasma* organisms in the lumina of the airways, in between**

**degenerated cilia, while a few *Mycoplasmas* were located within the alveoli. It was concluded that the MHYO strain detected in this study was pathogenic to pigs and capable of inducing pneumonia, and therefore implicated in the pathogenesis.**

**Key words: Immunohistochemistry; *Mycoplasma hyopneumoniae*; Nigeria; pathogenicity; pigs; pneumonia; ultrastructural changes**

## INTRODUCTION

Pneumonia remains one of the most challenging problems in intensive pig production systems [8, 24, 32]. *M. hyopneumoniae* is a known primary pathogen and initiator of enzootic pneumonia (EP) in pig herds [6, 22, 25, 40, 41], and contribute significantly to the development of porcine respiratory disease complex [5, 6, 22, 29, 31]. EP, a contagious, prevalent and chronic respiratory disease of pigs, constitutes a significant threat to swine health and productivity and reported to be responsible for substantial financial losses to swine producers in a number of countries [9, 20, 23, 35, 39] and has been considered as the most economically significant respiratory disease of finishing pigs [9, 39, 42]. There have been a number of publications on histopathology [15, 37] and immunohistochemical findings [37, 38] of infection with *M. hyopneumoniae* in pigs. The ultrastructural changes and the pathogenesis have also been well documented [10, 11, 17]. The appearance of lesions in different areas of the lung tissue sections has been associated with the presence of MHYO, as detected by immunohistochemistry [12, 37, 38], in-situ hybridization [18, 19], immunofluorescence [2] and transmission electron microscopy [4, 10, 36, 38]. In addition, a number of studies have demonstrated that MHYO colonizes the respiratory tract at the level of ciliated epithelial cells by attaching specifically to respiratory epithelium of the pig [10, 36, 47, 48]. The colonization of respiratory cilia by MHYO has been reported to result in ciliostasis, clumping and loss of cilia over the affected epithelium [11, 45], and colonization resulted in a significant reduction in the efficiency of the clearance of debris and the invading pathogens by the mucociliary apparatus [12]. Previous studies reported that adherence of the organism to the respiratory epithelium is a prerequisite step for colonization, pathogenicity and development of pneumonia [12, 17, 45]. This action had been shown to require a cilia binding epitome of the MHYO of which P97 adhesin (a membrane protein) has been identified and characterized [1, 15, 16, 27, 44, 48]. P97 protein has been reported to be expressed during infection with MHYO [1] and has been classified as one of the immunogens responsible for the immune response in the respiratory tract of swine [1, 16, 26, 27]. Furthermore, several studies have shown that different strains of MHYO are antigenically and genetically diverse [26] and vary in pathogenicity for pigs in different countries and geographical locations [27, 48]. Moreover, the course of EP depends on the virulence of the *M. hyopneumoniae* strain affecting the pigs [44]. This may be due to the existence of differences in virulence of the MHYO strains infecting pigs [28, 44]. Higher pathogenicity in high-virulence strain has been attributed to a high capacity to multiply in the lung tissues [36] and the induction of a more severe inflammatory process [25]. There has not been a study on the pathogenesis of EP and pathogenicity of MHYO strain affecting pigs in Nigeria. This study, therefore, investigated the pathological and ultrastructural changes in pneumonic lungs collected from slaughter-age finishing pigs with an intent to determining the pathogenicity of the MHYO strain infecting pigs in Nigeria as well as to further extend the knowledge of the pathogenesis of EP.

## MATERIALS AND METHODS

### Study location and sample collection

A total of 264 lung samples consisting of 60 apparently normal lungs (control group) and 204 grossly pneumonic lungs (case group) were randomly collected irrespective of the age, sex and breed from abattoirs located at Abeokuta, Ibadan and Lagos in southwest Nigeria, as well as carcasses submitted for postmortem examination at Department of Veterinary Pathology, Federal University of Agriculture, and Abeokuta. Samples were collected for a period of two years (2015—2017) on 12 occasions, 8 weeks apart. Grossly, pneumonic lungs had lesions characterized by cranioventral pulmonary consolidation (CVPC). Cases of CVPC were defined as those with pneumonic lesions affecting a minimum of three cranioventral lung lobes (i. e. the apical, cardiac and intermediate lobe). All the lung samples collected were immediately fixed in 10 % neutral

buffered formalin and left to fix for 48—72 hours. A subsample of 104 pneumonic and 20 apparently normal lungs of the formalin-fixed samples was processed for histopathological examination, while 104 samples were selected and used for immunohistochemistry.

**Histopathological technique**

One hundred and twenty four formalin-fixed lung tissues (104 pneumonic and 20 apparently normal lungs) were embedded in paraffin, sectioned at 3—5 μm, and stained with haematoxylin and eosin stain (H&E) for light microscopic examination to evaluate the following structures in the section: bronchi, bronchioles, bronchus- associated lymphoid tissue (BALT) and alveolar septa. The classification of histological lesions followed the semi-quantitative criteria according to Hansen et al. [15]. BALT hyperplasia was graded as mild (+) moderate (++), marked (+++) and extensive (++++).

**Immunohistochemistry protocol**

An immunohistochemical (IHC) test was used to detect MHYO-specific antigens on selected 104 formalin-fixed, paraffin-embedded lung tissues. Tissues were sectioned at 3—5 μm and processed for immunohistochemical staining. The IHC test was performed by the use of the heat-induced epitope retrieval technique using the citrate base antigen retrieval unmasking solution (Vector Lab., USA). Paraffin-embedded tissue sections were dewaxed by heating the unstained slides at 65 °C for 20 minutes using a hybridization incubator (Robbins Scientific® model 1000, Robbins Scientific Inc. USA). Paraffin wax was removed by washing the tissues in Hemo-De 3 times for 10 minutes each. Slides were air dried for about 20 minutes until the tissues became white. Deparaffinized tissue sections were pen-circled using PAP marker (Vector Lab., USA) and placed in antigen retrieval solution (Citra, BioGenex, CA, USA) in a plastic stander and were kept in a microwave for 20 minutes.

After cooling for about 20 minutes, slides were laid on a humid chamber, flooded with 3 % $H_2O_2$ (Fisher scientific®, UK) and incubated at room temperature for 15 minutes (2 times) to quench endogenous peroxidase activity. After washing 3 times (5 minutes each) in phosphate-buffer saline (PBS, pH 7.4; 0.01 M) containing 0.1 % Tween 20, sections were treated with power block, 1X blocking antibody (Universal Blocking Reagent, BioGenex, CA, USA) for 20 minutes to saturate nonspecific protein-binding sites.

After draining the excess blocking serum, sections were incubated with MHYO monoclonal antibody of 100 % specificity (Identification number D79DI-7, Dr Chris F. Minion, Iowa State University, Ames, USA) diluted to 1 : 500 in PBS (pH 7.4; 0.01 M) and kept in a humidified chamber at 4 °C overnight. After washing with PBS 3 times, sections were treated with biotinylated anti-mouse IgG made in goat secondary antibody (Vector Lab. Inc., CA, USA), applied at 1 : 250 dilution for one hour at room temperature in a humidified chamber.

The sections were washed 3 times and further treated with a labeled peroxidase-conjugated streptavidin-biotin complex (Vectastain®, Elite ABC, Vector Lab. Inc., CA, USA) for one hour.

After another PBS bath (3 times), the sections were incubated with 3,3 diaminobenzidine tetrahydrochloride (DAB) (Vector Lab. Inc., CA, USA). Sections were finally washed in running tap water, counterstained with Gill haematoxylin (Vector Lab. Inc., CA, USA), dried and covered with VWR micro cover glass (VWR®, USA). Lung tissues from known *Mycoplasma hyopneumoniae*-infected pig (known MHYO-positive) and *Mycoplasma hyopneumoniae*-free pig (known MHYO-negative) (Courtesy, Dr Uriel Blas-Machado, Athens Veterinary Diagnostic Laboratory, University of Georgia, USA) were used as positive and negative controls respectively.

**Transmission electron microscopy (TEM)**

TEM sample preparation was carried out at the Georgia Electron Microscopy Laboratory, University of Georgia, Athens, USA. Three paraffin-embedded lung tissue blocks (IB5, 020 and AB2) were selected for electron microscopy. From each block, tissue pieces were cut out of the paraffin-embedded blocks and excess paraffin wax trimmed off. Cut tissues were placed in glass petri dish on a hot plate to melt off excess paraffin after which they were placed in 100 % xylene overnight. Tissues samples were placed in several fresh xylene every 30 minutes for a total of 5 hours, followed by a gradual rehydration from 100 % ethanol to water. Tissues samples were processed using standard transmission electron microscopy protocol as described by Cheville and Stasko [7]. Tissues were post fixed in 1 % $OsO_4$ (aqueous) for 1½ hours followed by 4 rinses in deionized water; 15 minutes for each step. The tissues were dehydrated in a graded alcohol series, and then cleared in

propylene oxide before infiltration and embedment in epoxy resin (Embed 812, Electron Microscopy Sciences Hatfield, PA, USA). The embedded tissues were polymerized at 60 °C overnight. Approximately an 0.5 µm thick section was cut from each block with a glass knife, stained with Toluidine blue, and examined under light microscope to ensure that the bronchial or bronchiolar epithelium, and BALT or parenchymal tissue were present (Fig. 1). Several ultrathin sections of 50—70 nm thickness were cut on ultracut sectioning machine (Reichert Ultramicrotone, Leica Microsystems, Wetzlar, Germany) using a diamond knife (Diatome LTD, US), placed on 200-mesh CU Hex grid (Electron Microscopy Sciences Inc., Hatfield, PA, US), post-stained with ethanolic uranyl acetate and lead citrate, and examined with a JEOL JEM 1011 (JEOL Inc. Peabody, MA, US) electron microscope.

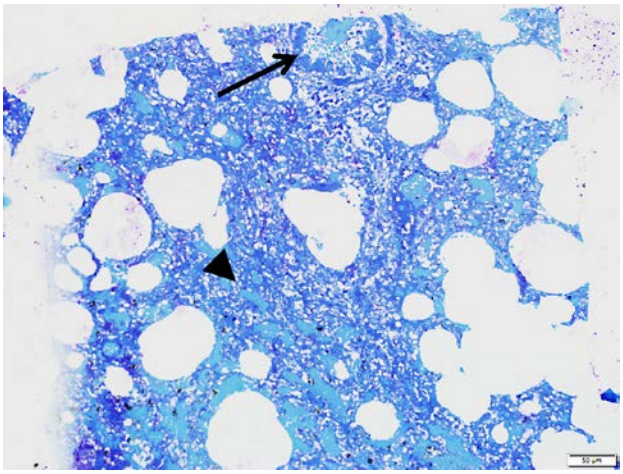

**Fig. 1. Toluidine blue stain photomicrograph of 0.5 µm thick section of pig lung showing a bronchiole (arrow) and thickened alveolar septa (arrow head) Bar = 50 µm**

## RESULTS

### Histopathological changes

Histologically, the most conspicuous lesions were massive peribronchiolar, and perivascular mononuclear cells infiltration (Fig. 2), varying degrees of lymphoid hyperplasia of BALT (mild to extensive) with formation of lymphoid nodules (Fig. 3), and thickening of alveolar septa due to cellular infiltration consisting predominantly of neutrophils with intraluminal cellular exudate (Fig. 4A). Lesions were divided into acute (8/124, 6.45 %), subacute (22/124, 17.74 %) or chronic (94/124, 75.81 %) cases of broncho-interstitial pneumonia. All acute cases were mainly suppurative broncho-interstitial pneumonia (BP) having a severe bronchitis, bronchiolitis and in some acute cases, the alveoli were collapsed and filled with inflammatory cells and oedema fluid 9 (Fig. 4B). The chronic cases were subdivided into non-suppurative (66/94, 70.21 %), and mixed (28/94, 29.79 %) broncho-interstitial pneumonia. There was moderate to severe parenchymal lymphoplasmocytic infiltration especially in the chronic stage of the infection (Fig. 5).

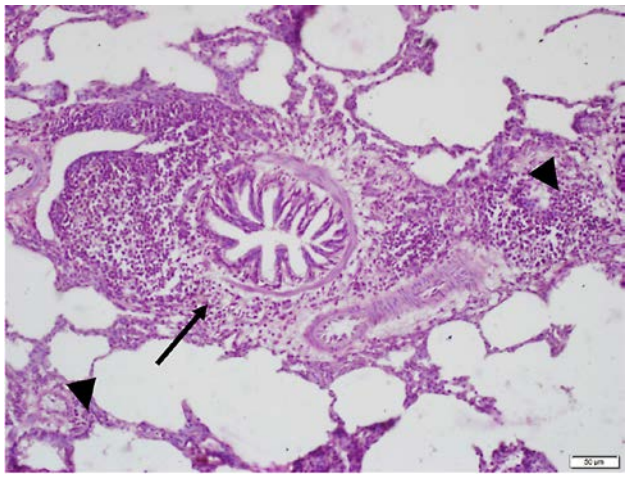

**Fig. 2. Photomicrograph of pig lung section showing massive peribronchiolar (arrow), and perivascular mononuclear cells infiltration (arrow head). H&E stain; Bar = 50 µm**

### Immunohistochemical findings

One hundred and four lung tissue sections were selected for IHC studies. *Mycoplasma hyopneumoniae* antigens were immunolabelled as a granular brown reaction at the luminal surface of bronchial or bronchiolar epithelial cells of all positive lung tissues (Fig. 6A, 6B). There was intense immunolabelling of mononuclear cells in the BALT (Fig. 7C), and of cellular exudates within the airways (Fig. 6B).

Sections were scored ranging from 0—3 based on: no signal detectable (0), weak labelling of the ciliated epithelium lining of a least one airway (1), weak to moderate labelling on the surface of a low number of airways (2), and intense labelling on the surface of several airways (3). In total, 86/104 (82.69 %) of the lung tissue sections showed immunolabelling to MYHO antigen. Out of 86 positive lung tissues, 56 (65.12 %) showed strong immunolabelling, 14/86 (16.28 %) showed moderate labelling, 16/86 (18.60 %) weak labelling, while immunosignal was not detected in 18/104 (17.31 %) lung sections.

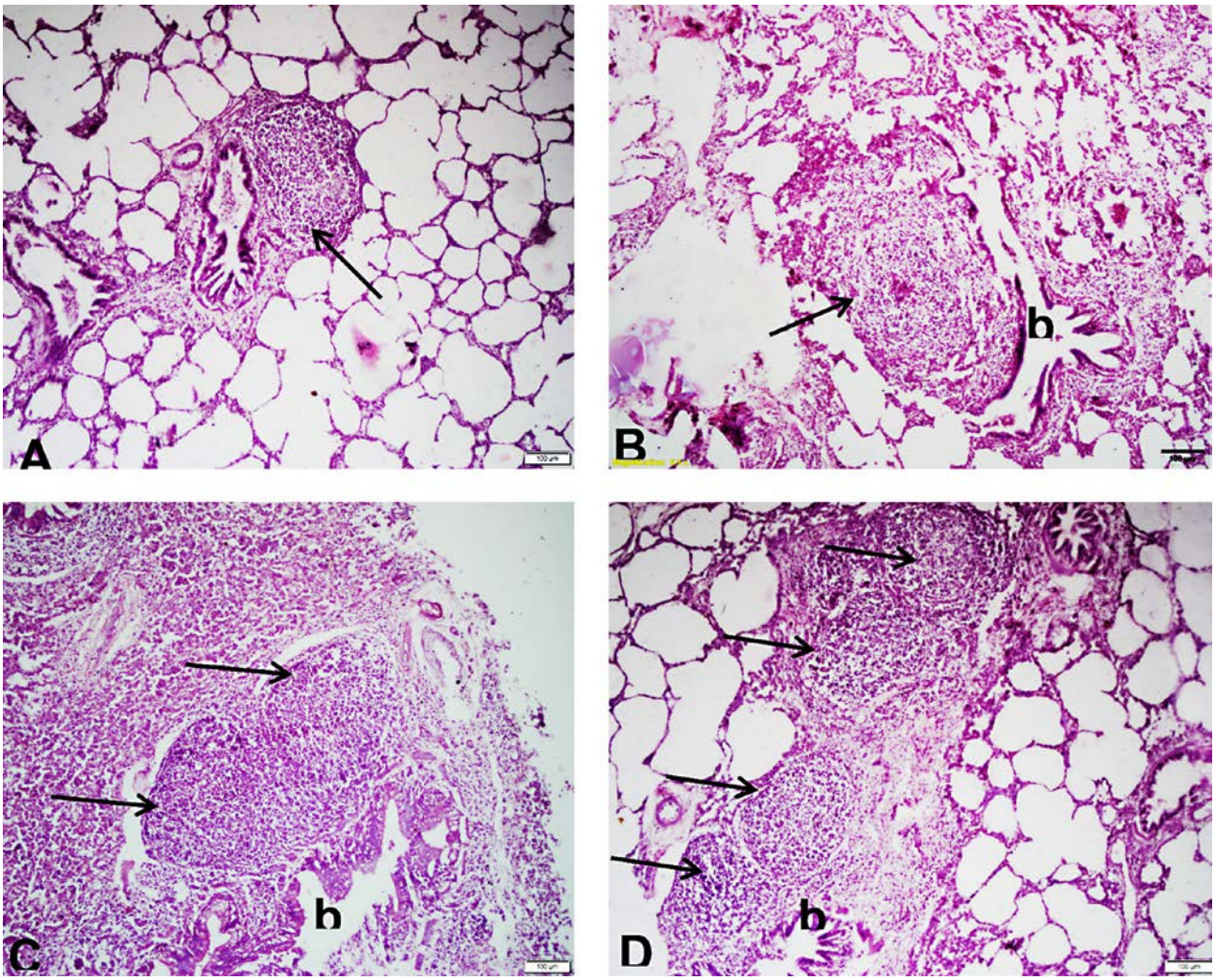


**Fig. 3. Photomicrograph of pig lung sections showing varying degrees of BALT hyperplasia (A): An example of BALT hyperplasia graded as +, with lymphocytes infiltrating the muscularis mucosa of a bronchiole and formation of a lymphoid node (arrow). (B): An example of BALT hyperplasia graded as ++, with a greater diffuse infiltration of lymphocytes with formation of a bigger lymphoid node (arrow) and a markedly compressed bronchiolar lumen (b). (C): An example of BALT hyperplasia graded as +++, with the presence of a few lymphoid nodules (arrows), a compressed bronchiole (b) can be seen. (D): An example of BALT hyperplasia graded as ++++, with the presence of numerous lymphoid nodules (arrows), a large portion of the lung parenchyma is affected while a marked compressed bronchiole (b) can be seen, H&E stain, Bar = 100 µm**

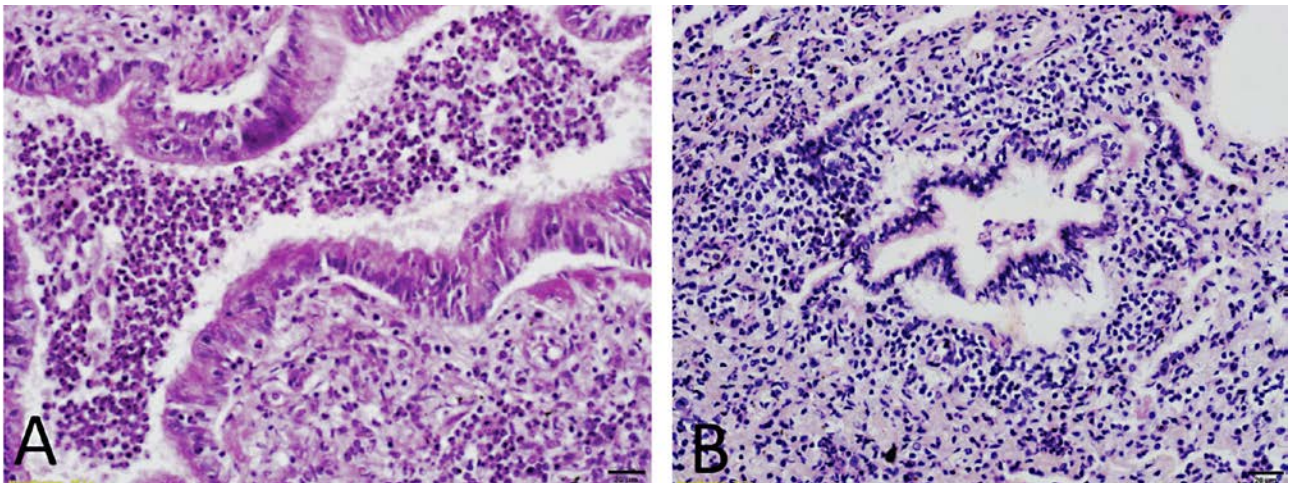


**Fig. 4. Photomicrograph of a pig lung section showing suppurative bronchiolitis with concurrent epithelial hyperplasia and intra-luminal cellular exudate consisting predominantly of neutrophils and cellular debris (A). Alveoli filled with inflammatory cells and oedema fluid in the acute phase of the infection (B). H&E stain, Bar = 20 µm**

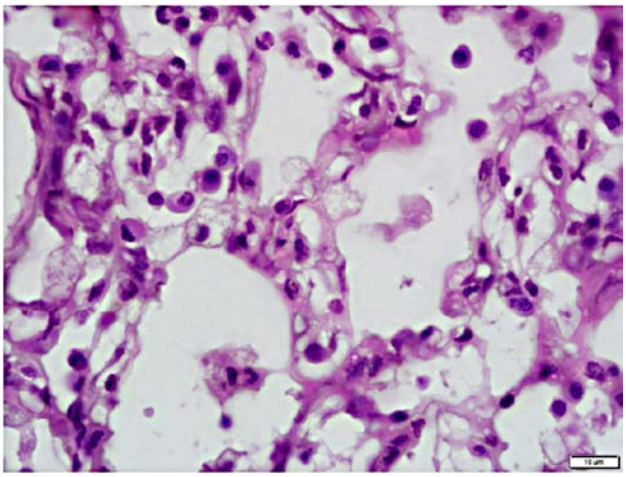

**Fig. 5. Photomicrograph of a pig lung section showing interstitial pneumonia as a result of lymphoplasmocytic infiltration in the chronic stage of the infection. H&E stain, Bar = 10 µm**

### Ultrastructure of *Mycoplasma hyopneumoniae* and its interaction with the respiratory epithelium

In thin sections prepared from three formalin-fixed, paraffin-embedded tissue blocks, there was interaction of *Mycoplasma hyopneumoniae* with the bronchiolar and bronchial epithelial lining as observed by the electron microscopy. Numerous *Mycoplasmas* were present in the lumina of the bronchioles and bronchi. They were found mainly between degenerated cilia, but many were lying freely in the lumen and were not found in contact with the cell surface (Fig. 7). Loss of cilia over the affected epithelial cells was commonly observed. Most of the *Mycoplasmas* were oval or round in shape and varied in sizes and one was seen to be undergoing the process of binary fission (Fig. 7B, inset). A few numbers of *Mycoplasmas* were present in the alveoli (Fig. 8).

## DISCUSSION

To the best of our knowledge, this is the first investigative study on the pathogenicity of MYHO strain in growing-finishing pigs in Nigeria. This study demonstrated that the histopathology of EP is complex, as nearly all pulmonary reaction patterns were observed. However, the most consistent microscopic lesion was suppurative bronchointerstitial pneumonia. The MYHO lesions were markedly influenced by secondary bacterial infections, stress, poor air quality, and also bad management [5, 13, 41]. This was characterized by peribronchial, peribronchiolar and perivascular cells infiltration consisting predominantly of

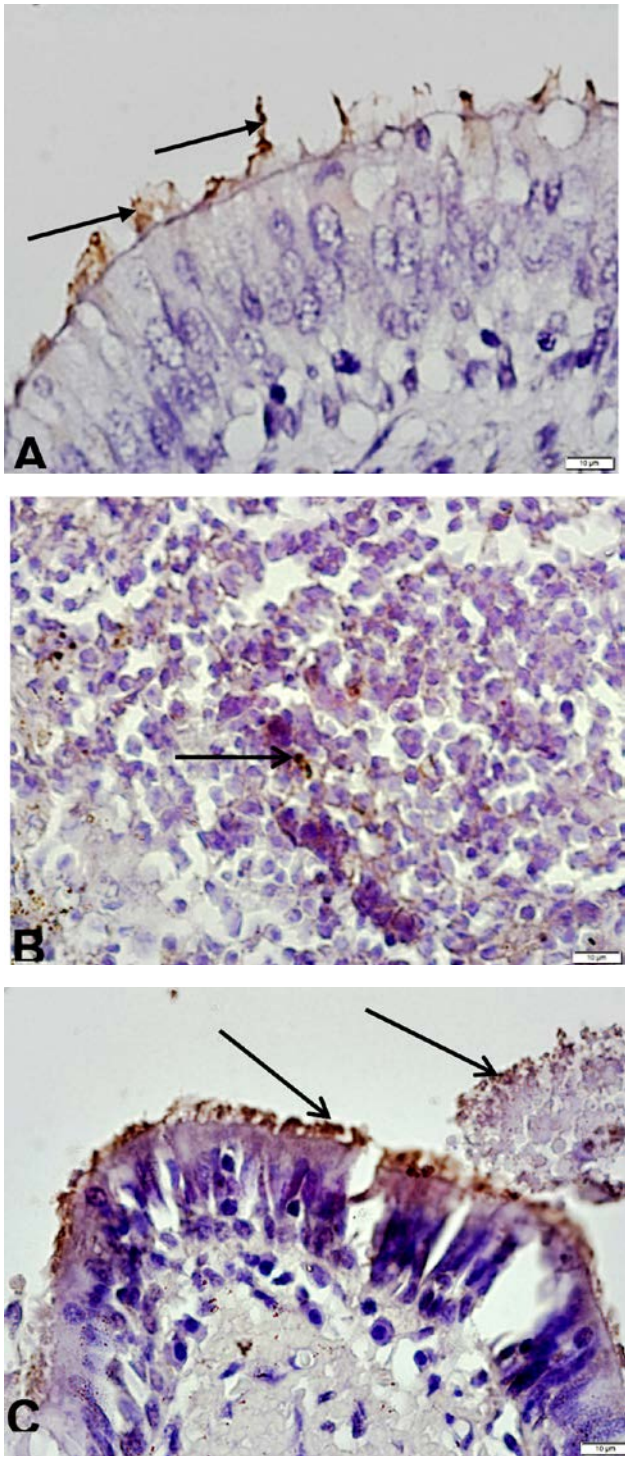


**Fig. 6. Photomicrograph of pig lung sections showing immunolabelled MYHO antigen (arrows) on the apical surface of bronchiolar epithelial cells (A). IHC, Gill haematoxylin counterstain, Bar = 20 µm. Intense immunolabelling of alveolar macrophage and mononuclear cells in the BALT (arrowed) and (C) cellular exudates within airways (arrowed) (B). IHC, Gill haematoxylin counterstain. Bar = 10 µm**

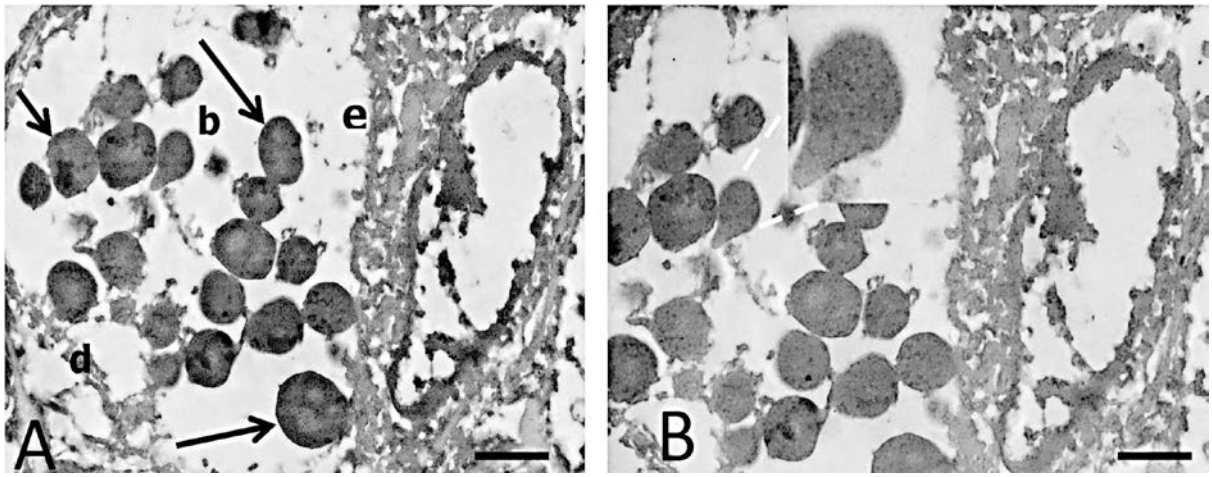


**Fig. 7. Transmission Electron Micrograph (TEM) of a thin lung section of a pig showing the loss of cilia over the epithelial surface (e), degenerated cilia (d) and Mycoplasma organisms (arrowed) in the lumen of a bronchiole (b) (A). Ethanolic uranyl acetate and lead citrate stain. Bar = 500 nm. Mycoplasma undergoing the process of binary fission in the bronchiolar lumen (Fig. 7B, inset). Ethanolic uranyl acetate and lead citrate stain; Bar = 600 nm**

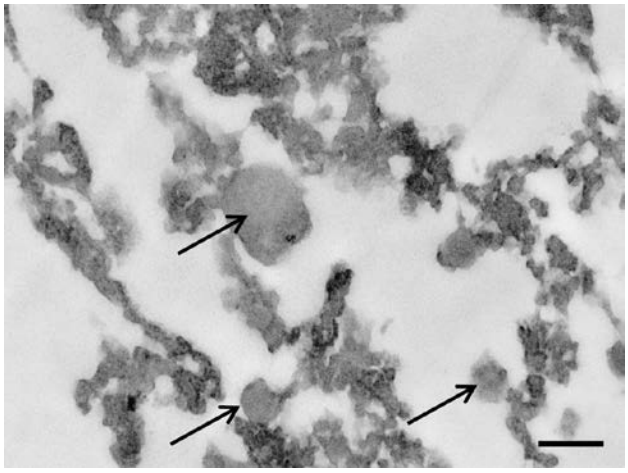

**Fig. 8. TEM of the lung section showing a few Mycoplasma hyopneumonae (arrowed) within the alveoli of the infected pig. Bar = 200 nm**

lymphocytes, varying degrees of lymphoid hyperplasia of the BALT with the formation of lymphoid nodules which resulted in the obliteration of the lumina of many bronchioles. There was also enlargement of alveolar septa in many cases due to the infiltration of plasma cells, lymphocytes and macrophages. These lesions have been reported by previous workers in both naturally occurring MHYO-infected pigs [18, 38] and in experimentally induced infections in pigs [8, 14, 21, 37]. The partial or complete obliteration of bronchiolar lumina associated with these lesions resulting in the collapse of surrounding alveoli, observed in this present study has been previously described [14, 23, 37]. This has been attributed to the presence of hyperplastic lymphoid aggregates [23, 37, 38], accumulation of mucus and inflammatory exudate in the bronchial and bronchiolar lumina due to decreased ciliary activities [14], increased activities of mucus secreting cells and altered glycoprotein [33] and the release of pro-inflammatory chemical mediators such as TNF-α, IL-1, IL-6 and INF-γ by alveolar macrophages [43]. The present study revealed a granular brown immunoreaction in MHYO-infected lung tissues on the luminal surfaces of bronchial and bronchiolar epithelial cells. The localization and distribution of antigens recorded in this study agrees with earlier findings obtained with an indirect immunoperoxidase method [21, 37, 38] and by in-situ hybridization [18, 19]. However, MHYO antigens were not detected in the pulmonary parenchyma in our study; this has been reported by previous workers [30]. In this study, MHYO antigens were detected in the BALT and cellular exudates in the bronchiolar lumen, which contradicts the previous report of Asai et al. [3], Kwon and Chae [18] and Kwon et al. [19]. These discrepancies may be due to infection with different strains of MHYO [18, 19]. The presence of MHYO antigens in the cellular exudates within airways recorded in this study, may suggest a local cellular immune response to MHYO infection as earlier reported [21].

A wide variety of ultrastructural changes occurring in the respiratory epithelial cells of pigs with enzootic pneumonia have been previously described [4, 10, 12]. The most significant ultrastructural change recorded in this study was a loss of cilia over the ciliated epithelial lining of the respiratory tract; *Mycoplasmas* were seen confined to the surface structure in the lumen of bronchioles and were found predominantly in the spaces between degen-

erated cilia, confirming the observation of Blanchard et al. [4]. The present ultrastructural study demonstrated and confirmed that *M. hyopneumoniae* adheres to cilia, and this adherence is associated with degenerative and/or necrotic changes and eventual loss of cilia. The exact means by which *M. hyopneumoniae* caused these cytopathic changes could not be determined by the current morphological study. However, such a change has been reported to reduce mucociliary action that would make removal and clearance of MHYO from an infected area slower and hindered thus tending to perpetuate the lesion and eventual establish the disease and its lesions [12, 17] as well as rendering the tract susceptible to secondary bacterial infection [10, 11, 17]. The study of Park et al. [33] had earlier elucidated the pathogenic mechanism of *Mycoplasma hyopneumoniae*-infected pigs and reported that the organism altered and increased fucosyl glycoconjugate in *M. hyopneumoniae*-infected pigs as a potential factor that enhanced colonization and adherence of *Pasteurella multocida* type A in bronchial and bronchiolar ciliated epithelial cells which led to enzootic pneumonia in infected pigs.

In this study, a few numbers of *M. hyopneumoniae* were present within the alveoli; this is in contrast to the previous report of Irigoyen et al. [17] who noted that *Mycoplasmas* are rarely present in the alveoli. However, this result supports a recent report of Raymond et al. [36] that showed that approximately 8% of *M. hyopneumoniae* cells reside intracellularly within the porcine epithelial cells where the organism was reported to associate with integrin β1 on the surface of epithelial cells via interactions with surface-bound fibronectin and initiates signaling events that stimulate pathogen uptake into clathrin-coated vesicles (CCVs) and caveosomes. These early events thus allow *M. hyopneumoniae* to exploit an intracellular lifestyle by commandeering the endosomal pathway [36]. Such discrepancies may be due to infection with a highly virulent and pathogenic strain of MHYO which has a high capacity to multiply in the lung tissues [34]. Differences in virulence could also be due to variation in the expression levels of virulence-associated genes like adhesins [36]. It is plausible to suggest from this observation that the alveolar lesions recorded in this study may be due to direct pathogenic action of the *M. hyopneumoniae* that resulted in severe structural and functional impairment of the respiratory tissues as a result of a severe inflammatory response.

## CONCLUSIONS

In the present study, the localization and distribution of *M. hyopneumonia* antigens in the lung tissues reported in this study provided some insight into the pathogenesis of enzootic pneumonia, investigated the pathogenicity of MHYO strain affecting pigs in Nigeria and further expounded the pathogenesis of enzootic pneumonia in pigs.

Therefore, it is concluded that the MHYO strain detected in this study induced pulmonary pathology including broncho-interstitial pneumonia and loss of cilia over the ciliated epithelial cells of the airways. These have been reported to be prerequisites for the development of pneumonic lesions. Further studies are warranted particularly a molecular study of the MYHO strain detected in this study.

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# IMMUNOHISTOCHEMICAL STUDIES OF αSMA IN THE EPIDIDYMIS OF AFRICAN FOUR-TOED HEDGEHOG (*ATELERIX ALBIVENTRIS*)

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## ABSTRACT

**The epididymis plays an important role in sperm maturation, storage, transport and in the secretion of enzymes and proteins into the tubular lumen. In this study, we examined the histology, microstereology and immunohistochemical localization of alpha smooth muscle (αSMA) in the three regions of the epididymis of the African four-toed hedgehog (*Atelerix albiventris*). Ten adult males were captured from the wild in Ibadan, Nigeria, between May and October, 2016. The animals were euthanized and the epididymis (caput, corpus and cauda regions) were retrieved and fixed in buffered neutral formalin ahead of the paraffin technique, following standard procedures. The duct of the epididymis was lined by pseudostratified columnar epithelium comprising basal, principal and apical cells as well as intraepithelial lymphocytes in proximity to basal cells. The principal cells, the major cells encountered within the epididymal epithelium of the animal, decreased in population from the caput to the cauda epididymidis while the apical cells were more abundant in the cauda epididymidis. Positive reactions to αSMA were observed in the peritubular muscular coat of the epididymal duct as well as blood vessels across the three regions of the epididymis with the caput and cauda epididymidis showing stronger positive reactions compared to the corpus epididymidis. This study demonstrated that the histology, microstereology as well as the cellular constituents of the epididymal duct of the *Atelerix albiventris* are similar to those of other mammals with a slight variation. It has also highlighted variation in the localization of αSMA across the regions of the epididymis of the animal.**

**Key words: African four-toed hedgehog; epididymis; histology; immunohistochemistry**

## INTRODUCTION

The African four-toed hedgehog (*Atelerix albiventris*) is a relatively small nocturnal mammal, possessing a short grooved brown or grey spine covering the dorsum of the body with a band of whitish fur running across its fore-

head [9]. It belongs to the order Insectivora and family Erinaceidae. They are widely distributed in the West African plains, savannas, and grasslands [23]. Unlike other species of hedgehogs having a fifth toe on their hind feet, the fifth toe of the African four-toed hedgehog is reduced to a vestigial hallux, hence the name "four-toed" [11].

The mammalian epididymis is a channel for the transportation, concentration and storage of spermatozoa. The epithelium of the epididymis, under the influence of androgens secretes proteins into its intra-luminal compartment in order to enhance the maturation of the spermatozoa in the tail of the epididymis [1]. Hence, the tail of the epididymis, apart from storing spermatozoa until ejaculation also specifically prepares the sperm for fertilization by the regulation of temperature, oxygen tension, pH and available energy substrate within its ducts [13].

While an appreciable number of research documentations exist on the biology of the European hedgehog (*Erinaceus europaeus*) [18, 19, 28, 29]; very few of such reports exist on the hedgehogs of African origin: the African hedgehog (*Atelerix pruneri*) [10]; the central African hedgehog (*Erinaceus albiventris*) [8]; and the African four-toed hedgehog (*Atelerix albiventris*) [5]. The contractile cells of the peritubular coat of the mammalian epididymis are important in the transport of spermatozoa from the caput to the cauda epididymis where spermatozoa are stored. The smooth muscle cell, a major contractile apparatus of the epididymis, has been shown to be immunopositive for α-smooth muscle actin, being present in the peritubular coat as well as the vascular tissue of the mammalian epididymis [2]. However, there are no reports on the patterns of the distribution of α-smooth muscle actin in the various segments of the epididymis in the epididymis of the African four-toed hedgehog.

To bridge the existing knowledge gap, the immunohistochemical localization of alpha smooth muscle actin (α-SMA) in the various segments of the epididymis of the African four-toed hedgehog (*Atelerix albiventris*) was investigated.

## MATERIALS AND METHODS

### Experimental Animals

Ten (10) adult male African four-toed hedgehog were used for this study. They were captured from the wild in Ibadan, Oyo State, Nigeria, between the months of May and October, 2016, being the wet season of the year. The animals were euthanized using excess dosage of ether anaesthesia and the epididymis (caput, corpus and cauda regions) were retrieved immediately and fixed in buffered neutral formalin ahead of the paraffin technique. All procedures were carried out according to the guidelines for the care and use of experimental animals National Institute of Health (NIH), USA.

### Histology and microstereology

Epididymal samples were fixed in buffered neutral formalin and embedded in paraffin blocks. The sections (2—4 μm thick) were stained with Haematoxylin and Eosin (H&E) [4] and the slides were then studied under a light microscope (Olympus BX63 with a DP72 camera). Quantitative histomorphometric measurements of the three regions of the epididymis were taken from the H&E- stained sections, using a stereological module of computer-assisted digital image analyser (CellSens® dimension software version 1.6) attached to a computer.

### Immunohistochemistry

Immunohistochemistry was carried out as previously reported [21]. Briefly, paraffin-embedded epididymal tissues were cut and mounted on slides pre-coated with polylysine. They were deparaffinised, rehydrated and heat-treated for antigen retrieval. To reduce endogenous peroxidase activity, sections were incubated for 5 min in hydrogen peroxide (3 % in distilled water). In order to block non-specific binding sites, the slides were rinsed in a 0.01 m phosphate buffered saline solution (PBS, pH 7.4), containing bovine serum albumen, for 5 minutes. Prior to immunostaining, validation trials for α-smooth muscle actin antibodies in this species were carried out using four different dilutions (1 : 50, 1 : 100, 1 : 200 and 1 : 400). Immunostaining of slides were carried out for 1 hour at room temperature, using the LSAB-plus kit (Dakocytomation, Glostrup, Denmark) monoclonal antibodies against α-smooth muscle actin at dilutions of 1 : 400. The slides were then rinsed in PBS followed by incubation for 15 minutes in a linked antibody (Biotinylated secondary antibody, LSAB-plus kit; Dakocytomation) and then in peroxidase-labelled streptavidin. This was followed by the addition of 3,3'-diaminobenzidine tetrachloride solution (DAB) from the LSAB+® kit to visualize antigen localization. Negative

controls involved the primary antibody replaced by bovine serum albumen. Smooth muscle was used as a positive control for α-smooth muscle actin. Sections were counterstained with haematoxylin for 30 seconds, washed in water, dehydrated through graded ethanol, cleared in xylene, mounted with DPX permanent mounting media (Sigma-Aldrich, St. Louis, MO, USA) and examined under a light microscope (Olympus BX63 with a DP72 camera). Immunoreactivities to α-smooth muscle actin were designated as absent/negative (-), moderately positive (++) and strongly positive (+++) based on visual examination [21].

### Statistical analysis

Quantitative data were recorded as the means and standard deviation. The comparison of means was performed using a one-way ANOVA with the aid of the GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, California, USA). Statistical significance among parameters was considered at $P < 0.05$.

## RESULTS

### Histological and microstereological observations

The ductus epididymis of the African four-toed hedgehog is surrounded by a relatively thin sheath of connective tissue composed of blood vessels (Fig. 1, A—D). The duct contained clumps of spermatozoa and was lined by pseudostratified columnar epithelium surrounded by a thin lamina propria as well as a peritubular muscle coat (PMC)

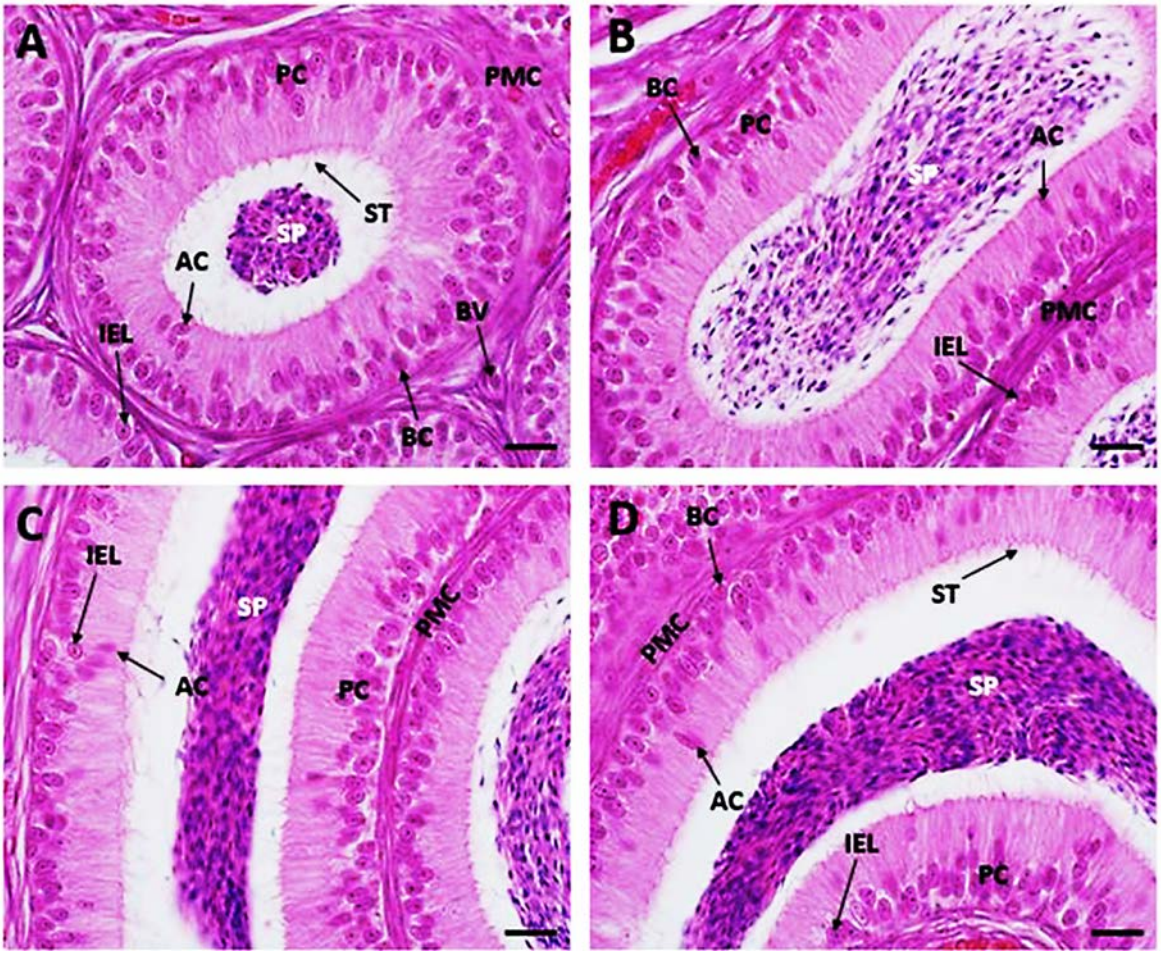


**Fig. 1. Representative histological sections of the epididymis of the African four-toed hedgehog (*Atelerix albiventris*). Scale bars = 20 µm (H & E)**

**A.** *Caput epididymidis*. AC: apical cell; BC: basal cell; PC: principal cell; BV: blood vessel; ST: stereocilia; SP: spermatozoa; PMC: peritubular muscular coat; IEL: intraepithelial lymphocyte. **B.** A. *Corpus epididymidis*. AC: apical cell; BC: basal cell; PC: principal cell; SP: spermatozoa; PMC: peritubular muscular coat; IEL: intraepithelial lymphocyte. **C.** A. *Cauda epididymidis*. AC: apical cell; BC: basal cell; PC: principal cell; SP: spermatozoa; PMC: peritubular muscular coat; IEL: intraepithelial lymphocyte. **D.** A. *Cauda epididymidis*. AC: apical cell; BC: basal cell; PC: principal cell; ST: stereocilia; SP: spermatozoa; PMC: peritubular muscular coat; IEL: intraepithelial lymphocyte

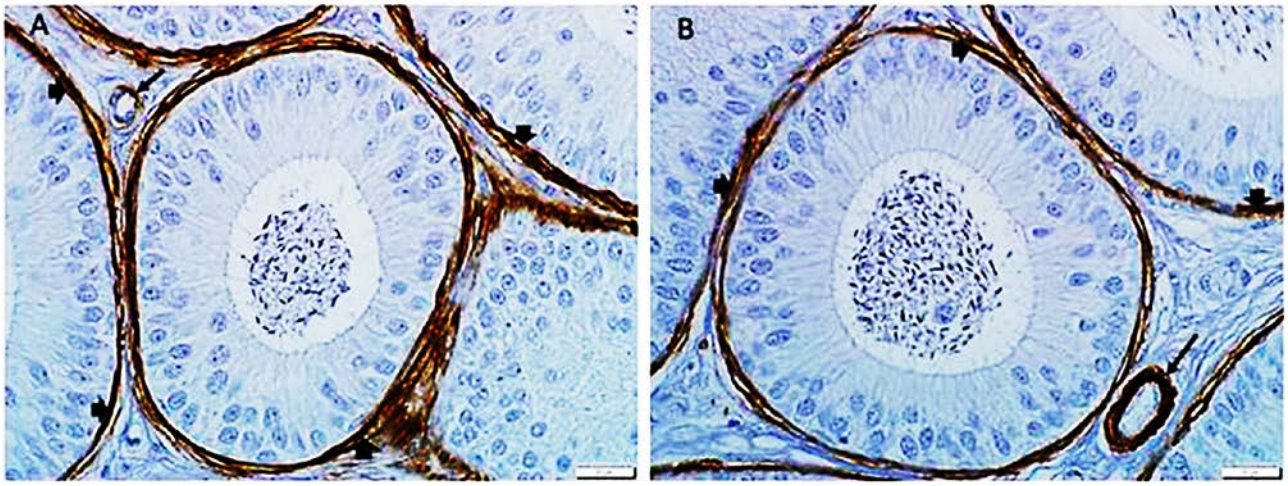


**Fig. 2. Immunohistochemical localization of alpha smooth muscle actin (αSMA) in the caput epididymidis of the African four-toed hedgehog (*Atelerix albiventris*). Strongly positive reactions seen at peritubular muscular coat (arrow head) and blood vessel (arrow). Scale bars = 20 µm**

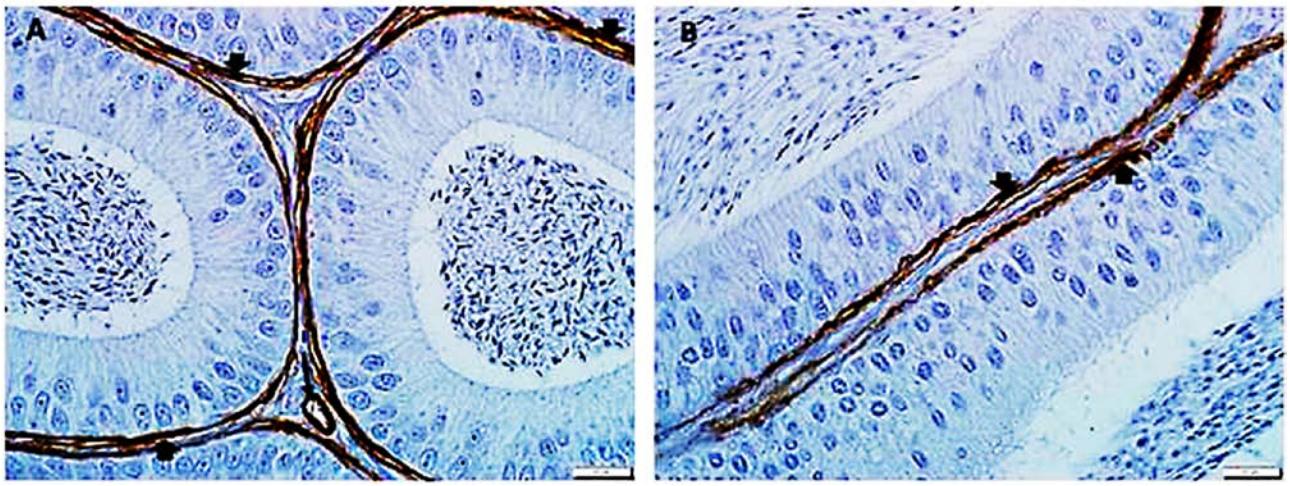


**Fig. 3. Immunohistochemical localization of alpha smooth muscle actin (αSMA) in the corpus epididymidis of the African four-toed hedgehog (*Atelerix albiventris*). Moderately positive reactions seen at peritubular muscular coat (arrow head) and blood vessel (arrow). Scale bars = 20 µm**

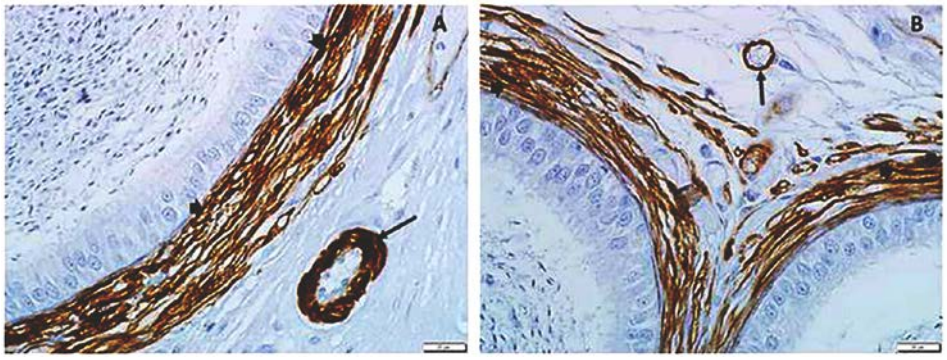


**Fig. 4. Immunohistochemical localization of alpha smooth muscle actin (αSMA) in the cauda epididymidis of the African four-toed hedgehog (*Atelerix albiventris*). Moderately positive reactions seen at peritubular muscular coat (arrow head) and blood vessel (arrow). Scale bars = 20 µm**

(Fig. 1, A—D). The epithelium comprised basal, principal and apical cells as well as intraepithelial lymphocytes (Fig. 1, A—D). The principal cells formed the major cells encountered within the epididymal epithelium decreasing in population from the caput to cauda epididymidis. However, apical cells were more encountered in the cauda epididymidis than other segments of the epididymis. From the epithelial surface projected stereocilia measuring about 3—5 μm. The lengths of these stereocilia varied across the three segments of the epididymis, with the cauda epididymidis being the longest, although not significantly different ($P > 0.05$) from others (Table 1). In terms of the quantity of spermatozoa contained in the ducts of the epididymis across the three segments, the caput epididymidis had the least clumps of spermatozoa while the cauda epididymidis had the highest. The ductal diameter, the luminal diameter and epithelial height varied from the caput to cauda regions of the epididymis with the epithelial height being highest at the caput and lowest at the cauda epididymidis. However, the luminal diameter was highest at the cauda epididymidis and lowest at the caput epididymidis (Table 1).

**Immunohistochemical observations**

Immunostaining revealed positive reactions for α-smooth muscle actin (αSMA) at the PMC as well as blood vessels of the epididymis while the epididymal epithelium did not react to αSMA (Figs. 2—4, Table 2). The caput epididymidis showed strong positive reactions to αSMA (Fig. 2 A—B). The corpus epididymidis showed moderately positive reactions to αSMA (Fig. 3 A—B). The cauda epididymidis showed strongly positive reactions to αSMA (Fig. 4 A—B). However, the cauda epididymidis had more layers of circularly oriented smooth muscle cells compared to those of the other two segments (Fig. 3 A—D).

**Table 2. Distribution of α-smooth muscle actin (αSMA) in the epididymis of the African four-toed hedgehog**

| Region of Epididymis | PMC | BV | EE |
|---|---|---|---|
| **Caput** | +++ | +++ | – |
| **Corpus** | ++ | ++ | – |
| **Cauda** | +++ | +++ | – |

PMC: peritubular muscle coat; BV: blood vessel; EE: epididymal epithelium; Negative reaction (–); moderately positive reaction (++); strongly positive reaction (+++)

## DISCUSSION

The results of the present study revealed that the epididymal epithelium of the African four-toed hedgehog is similar to those earlier reported for mammals including the Wistar rat [1], the boar [13], camel [3], deer [22], donkey [2], African greater cane rat [25] and African giant rat [24]. The populations of the basal, principal and apical cells found within the various regions of the epididymis of the African four-toed hedgehog were similar to those earlier reported in the African giant rat [24], camel [3] and donkey [2]. In the epididymis of mammals, the principal cells constitute approximately 80 % of the total epithelial cell population in the caput epididymidis and gradually decreases to 65 % of the total epithelial cell population in the cauda epididymidis [30]. The principal cells have been demonstrated to be responsible for the majority of the proteins that are secreted into the epididymal lumen and they have been shown to be directly involved in the control of luminal protein concentrations as evidenced by the blebs of cytoplasm emanating from the apical cell surface [12,

**Table 1. Microstereology of the epididymis of the African four-toed hedgehog (*Atelerix albiventris*)**

| Parameter | Caput | Corpus | Cauda |
|---|---|---|---|
| Ductal Diameter [μm] | $201.18 \pm 14.25^a$ | $226.10 \pm 17.16^a$ | $343.19 \pm 26.19^b$ |
| Luminal Diameter [μm] | $98.69 \pm 18.27^a$ | $116.38 \pm 15.67^a$ | $149.31 \pm 17.26^b$ |
| Epithelial Height [μm] | $57.14 \pm 11.29^a$ | $51.04 \pm 13.32^a$ | $31.26 \pm 12.14^b$ |
| Stereocilia height [μm] | $3.017 \pm 0.19$ | $3.35 \pm 0.10$ | $4.71 \pm 0.6$ |

Means with different superscript within rows differ significantly ($P < 0.05$)

31]. Hence, it can be inferred that the concentration of epididymal luminal protein would be highest at the caput and lowest at the cauda epididymidis. The proximity of basal cells to intraepithelial lymphocytes across the three segments of the epididymis in the study suggests that basal cells could be responsible for certain immune functions. This confirms earlier report on the role of basal cells as being positively involved in epithelial immune system and in the regulation of electrolytes by the principal cells [12]. The greater frequency of apical cells in the cauda epididymidis is suggestive of their role in spermatozoa storage and retention. Apical cells are said to be involved in the production of enzymes of the carbonic anhydrase family which have been shown to play an important role in the regulation of spermatozoa retention [20].

In our study, the morphometric relationships observed for the luminal diameter and epithelial height across the three regions of the epididymis all follow similar patterns already reported in the epididymis of mammals [1, 2, 25, 26, 27]. The height of the epithelium at the caput epididymidis may be connected with the synthesis of dihydrotestosterone (DHT). DHT, a metabolite of testosterone, is synthesized in the epididymis, predominantly in the caput epididymidis when testosterone is acted upon by the enzyme 5α- reductase [7]. As reported by previous studies [24, 27], the increasing lengths of stereocilia from the caput to cauda epididymidis of the African four-toed hedgehog can be linked to the roles of the various regions of the epididymis in relation to spermatozoa motility.

Research documentations have shown that spermatozoa from all segments of the epididymis have different levels of motility, with the percentage of motile cells and the progressive swimming pattern of cells being higher in the cauda epididymidis [6, 16]. Also, it has been demonstrated that spermatozoa, upon leaving the corpus epididymidis, are bound with "forward motility protein" which allows the cauda spermatozoa to move progressively rather than in circles or thrashing, as earlier reported for spermatozoa in the caput epididymidis [14]. Also, the ductal lumen of the epididymis being widest at the cauda epididymidis can be linked to the role of the cauda epididymidis in spermatozoa storage and retention. The cauda epididymidis of mammals not only stores the sperm but also provides the proper conditions for retaining spermatozoa in a quiescent state of metabolism [7, 15]. However, the length of the stereocilia observed in this study is shorter than those reported in the African greater cane rat [25] as well as in the African giant rat [24]. This difference, is presumed to be related to species specificity than function.

Alpha smooth muscle actin is a contractile protein mainly found in cells with contractile function. It is an important marker in the investigation of differentiation of smooth muscle cells in physiological and pathological conditions [32]. The positive reactions of the peritubular muscle coat of the epididymal duct as well as the blood vessels of the epididymis observed in this study are consistent with previous reports on the localization of αSMA in mammalian epididymis [2, 3]. In this study, the observed greater intensity of αSMA staining in the caput and cauda epididymidis compared to that of the corpus is indicative of a more contractile function in these regions. Hence, the involvement of smooth muscles in the movement of spermatozoa within the epididymis is more intense at the caput and cauda epididymidis. Also, immunohistochemical staining with αSMA revealed the multilayers of circularly oriented smooth muscles of the cauda epididymidis in the African four-toed hedgehog. This may be due to the contractile demands in the movement of retained spermatozoa from the cauda epididymidis into the ductus deferens when the need arises. However, the observed pattern of the orientation of smooth muscles involving only circular smooth muscles in the cauda epididymidis of the African four-toed hedgehog is unlike the pattern described in the dromedary camel being both circularly and obliquely oriented smooth muscles cells [3]. This difference is also suggestive of species specificity. In our study, there were no reactions with αSMA at the epididymal epithelium. This is particularly so because smooth muscle cells are not among the cellular constituents of the epididymal epithelium of mammals. This is in conformity with previous reports on the localization of αSMA in mammalian epididymis [2, 3, 17]. Also, the increased vascular reactions to αSMA in the cauda epididymidis of the African four-toed hedgehog shows that more smooth muscles are involved in vascular contractile activities at that region of the epididymis than other regions. This finding correlates positively with those of previous authors [2, 3].

## CONCLUSIONS

This study has shown that the histology, histomorphometric parameters as well as cellular constituents of the

epididymal duct of the African four-toed hedgehog all bear close similarities to those of other mammals with a slight variation. It has also revealed the variation in the localization of αSMA across the regions of the epididymis of the animal. This study therefore presents vital information needed in the comparative regional anatomy of the histology and immunohistochemistry of hedgehogs and mammals in general. However, ultrastructural studies of the epididymal epithelium as well as the use of Western Blot to further investigate αSMA expression across the regions of the epididymis is hoped to provide more details on the characterization of the epididymal duct in this animal.

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# REVERSAL OF DIABETIC COMPLICATIONS IN ANDROLOGY PARAMETERS OF ALLOXAN-INDUCED DIABETIC MALE WISTAR RATS TREATED WITH *CLEOME RUTIDOSPERMA* LEAVES

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## ABSTRACT

**Male infertility is one of the complications associated with *diabetes mellitus*. This study reported the effect of managing experimentally-induced diabetes in male Wistar rats with *Cleome rutidosperma* leaf extract at doses of 100 mg.kg$^{-1}$, 200 mg.kg$^{-1}$ or 400 mg.kg$^{-1}$ for 14 days. Further deterioration or amelioration of reproductive derangement was assessed by changes in sperm morphology, sperm characteristics, and testicular histopathology. Andrology profile of diabetic untreated rats showed significant ($P < 0.05$) derangements compared to normoglycaemic rats. The profile of diabetic rats treated with the extract of *C. rutidosperma*, especially at the 200 mg.kg$^{-1}$ dose showed a significant decrease in abnormal sperm cells, increases in sperm motility, live/dead ratio and count compared to the untreated diabetic rats. Histopathology showed spermatogenic arrest and degeneration of Sertoli cells in the diabetic untreated rats, but was restored in the *C. rutidosperma* treated rats. This study showed evidence of infertility associated with diabetes and the ameliorative ability of *C. rutidosperma* on infertility demonstrated by improved andrological parameters. Traditional management of diabetes with *C. rutidosperma* leaves should therefore be encouraged in both humans and other animals, especially males with reproductive intentions.**

**Key words: andrology; *Cleome rutidosperma*; *diabetes mellitus***

## INTRODUCTION

Up to now medicinal plants have provided mankind with remedies for disease prevention, treatment and/or

management. The role of plants in folklore medicine has been attributed to the presence of phytochemicals which are either nutritive or non-nutritive plant chemicals with disease preventive or curative properties [1, 20]. *Diabetes mellitus* has been successfully managed traditionally with medicinal plant preparations [5, 16]. Diabetes, usually characterized by persistent hyperglycemia, may lead to tissue/organ damages in the cardiovascular system, kidney, eye, nerve and reproductive organs [34]. Reproductive complications in the male associated with diabetes include disruption of male fertility, impotence, retrograde ejaculation and hypogonadism. The male hypothalamic-pituitary-gonadal axis was found by researchers to be regulated by pancreatic insulin, and this is essential for fertility [21, 22, 32].

Maintenance of normoglycemia in diabetics is imperative and has been achieved by orthodox and traditional therapeutic approaches. Many rural dwellers have opted for the traditional remedies with years of proven efficacy, good availability and affordability. *Cleome rutidosperma* DC is a medicinal plant traditionally used for the management of diabetes in Southern Nigeria. It is a terrestrial low-growing annular, erect or tufted herb, up to 70—100 cm tall with white or brown taproots [26]. *C. rutidosperma* is found in waste grassy grounds and has trifoliate leaves and small, violet-blue flowers. It is native to West Africa rainforest, from Guinea to Nigeria, Zaire and Angola, but naturalized in various parts of tropical America and Southeast Asia [35]. Pharmacological properties of the plants reported include: analgesic, antipyretic, anti-inflammatory, diuretic and antioxidant activities [10, 11].

Despite proven therapeutic efficacy however, many medicinal plants have toxic potentials yet to be verified and these are important for drug safety and development. There is a sparsity of information on the reproductive toxicities that may accompany the management of diabetes with *C. rutidosperma*. Male infertility is one of the complications associated with diabetes. The management of diabetes should therefore consider the effect of treatment on the reproductive system, especially in males.

This study was designed to evaluate the reproductive toxicity potentials of the management of diabetes with *C. rutidosperma* leaves using changes in spermatozoa morphology, characteristics and Sertoli cell histopathology in male Wistar rats as indicators of toxicity.

## MATERIALS AND METHOD

### Plant preparation

Fresh leaves of *Cleome rutidosperma* were harvested from Ughelli, Delta State. It was identified and a voucher specimen deposited at the Department of Botany, University of Ibadan (Voucher-Number UIH-22548). The leaves were air dried, pulverized and macerated in methanol (96 %) for 72 hours. The decanted filtrate was concentrated using a rotary evaporator and the extract obtained was stored at 4 °C. The extraction was carried out according to the method described by Oridupa and Saba [28]. Fresh extract was reconstituted daily for dosing.

### Experimental animals

Thirty male Wistar rats (140—180 g) were obtained from and housed at the Experimental Animal House, Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Nigeria. They were fed with commercially available pelletized rat ration and allowed access to clean water ad libitum. Only normoglycaemic rats were included in the study. The fasting blood glucose concentration was determined in overnight fasted rats using a glucometer (Accu-Chek®Active, Roche Diagnostic, Germany). The study was conducted in accordance with the regulation of the Animal Care Use and Research Ethics Committee (ACUREC), University of Ibadan (UI-ACUREC/App/01/2017/005).

The rats were randomly distributed into six groups. Group 1 served as control normoglycaemic rats. Diabetes was induced in groups 2—6 of rats with alloxan monohydrate (100 $mg.kg^{-1}$; intraperitoneal; Sigma-Aldrich). Group 2 was administered Glibenclamide (0.07 $mg.kg^{-1}$; oral). Groups 3, 4 and 5 were orally administered *C. rutidosperma* extract (100 $mg.kg^{-1}$, 200 $mg.kg^{-1}$ or 400 $mg.kg^{-1}$), while Group 6 rats were untreated diabetics throughout the study. Minute quantities of blood was withdrawn from the tail vein to determine the blood glucose levels throughout the course of the study. The antidiabetic study was conducted according to the methods described by Folasire et al. [14].

### Sample collection and analysis

All rats were sacrificed by cervical dislocation on day 15. Orchidectomy was performed by pre-scrotal or midline incision according to the method described by

Oyeyemi and Ubiogoro [29]. The *tunica vaginalis* was excised to expose the testicles which were milked out of the incision site. The spermatic cord was ligated, excised and sperm samples were collected from the caudal epididymis. The sperm morphology was determined from about 400 spermatozoa in smears stained (Wells and Awa stains). The sperm characteristics (sperm volume, motility, count and percentage livability) were also determined.

Routine histopathology was carried out according to the method described by Bancroft and Gamble [7]. The testes of the rats were also harvested and preserved in Bouin solution, followed by dehydration with grades of ethanol (70, 80, 90, 95 and 100 %). The samples were cleared in two changes of xylene, impregnated with 2 changes of molten paraffin wax and blocked out. The samples were cut, about 5—6 μm thickness, using a rotary microtome and mounted on glass slides. The slides were stained with haematoxylin and eosin stain, and examined under the light microscope (Olympus CH, Japan). Photomicrographs were taken with an Amscope camera fitted on an Accu-scope microscope and assessed with the aid of ToupView software.

**Statistical Analy sis**

The data obtained were reported as means ± SD and analyzed using ANOVA with Tukey Kramer multiple comparison test using GraphPad Prism 5.0 (GraphPad Software, San Diego) and differences considered significant at $P < 0.05$.

## RESULTS

Elevated blood glucose levels significantly decreased in the extract treated rats, especi ally in rats administered 200 $mg.kg^{-1}$ extract compared to untreated diabetic rats and comparable to glibenclamide-treated rats (Table 1). The andrology profiles showed significant ($P < 0.05$) differences between normoglycaemic and diabetic rats.

Rudimentary tails (primary abnormality) observed were significantly increased in the diabetic untreated rats (3.21 ± 0.16 cells) compared to normoglycaemic rats (2.0 ± 0.41 cells) and the diabetic treated groups. Secondary sperm abnormalities seen included normal heads without tails, normal tails without heads, bent tails, curved tails, curved mid-pieces, bent mid-pieces and looped tails. There were significant ($P < 0.05$) increases in the observed normal heads without tails, curved tails, curved mid pieces and bent mid pieces of diabetic untreated rats compared to normoglycaemic rats. There were significant ($P < 0.05$) increases in the total abnormal sperm count of all groups when compared with normoglycaemic rats. However, extract-treated rats at 200 $mgkg^{-1}$ (47.80 ± 1.36 cells) showed a significant ($P < 0.05$) decline in total sperm abnormalities compared with normoglycaemic (53.55 ± 2.71cells) and diabetic controls (63.90 ± 1.46 cells) (Table 2).

The sperm motility and count of the diabetic control was significantly ($P < 0.05$) lower than in normoglycaemic control. However, there were increases in the extract-treated rats, especially those administered 200 $mg.kg^{-1}$, compared to normoglycaemic controls. The live-dead ratio and sperm volume of extract treated diabetic rats were similar to that of normoglycaemic control and significantly higher than untreated diabetic rats (Table 3).

The histopathology showed normoglycaemic rats had no observable lesions. Diabetic untreated rats showed spermatogenic arrest and degeneration of the Sertoli cells. Extract-treated rats at 100 $mg.kg^{-1}$ had ectasia of tubular lumen and hypospermia, 200 $mg.kg^{-1}$ showed tubular atrophy with sparing of pachytene spermatocytes, while ectasia of tubular lumen and hypospermia was observed in 400 $mg.kg^{-1}$ treated rats. Diabetic rats administered glibenclamide showed no observable lesion (Fig. 1).

## DISCUSSION

*Diabetes mellitus* is a metabolic derangement of glucose clinically characterized by hyperglycaemia. Prolonged cellular exposure to hyperglycemia is the primary factor implicated in most diabetic complications [34]. One of these complications in males include disruption of normal reproductive functions clinically presented as infertility and erectile disorders. Male infertility may be due to defects in sperm atozoa morphology and characteristics [22, 29]. The main focus of diabetes therapy is regulation of blood glucose levels aimed at reversal of the hyperglycaemic state and eventually the complications [14]. In this study, a significant decline in blood glucose levels was observed in *Cleome rutidosperma*-treated diabetic rats throughout the course of the experiment. Also, significant reversal of derangements in sperm morphology and characteristics

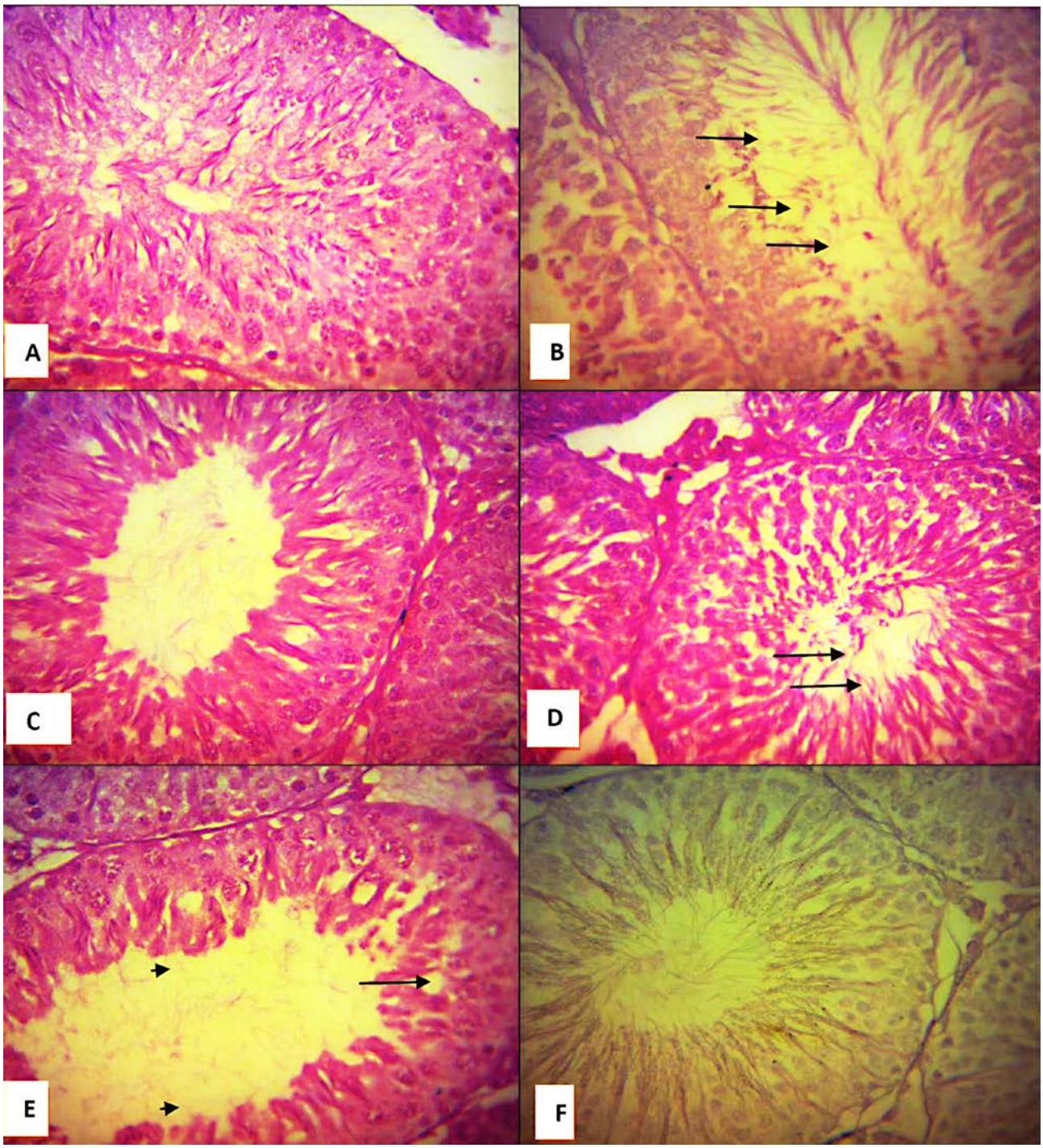


**Fig. 1. Photomicrograph of diabetic Wistar rat testis administered with *Cleome rutidosperma* leaf extract**

**A** (Control)—no observable lesion; **B** (Untreated Diabetic)—few immature stages found at the margin of the tubular lumen; **C** (Diabetic treated with 100 mg.kg$^{-1}$ *C. rutidosperma*)—no observable lesion; **D** (Diabetic treated with 200 mg kg$^{-1}$ *C. rutidosperma*)—immature stages found in the tubular lumen; **E** (Diabetic treated with 400 mg.kg$^{-1}$ *C. rutidosperma*)—relative widening of the seminiferous lumen (arrowheads), with interstitial oedema (arrows); **F** (Diabetic treated with Glibenclamide)—no observable lesion. H&E ×400

**Table 1. Blood glucose levels (mg.dl$^{-1}$) of alloxan-induced diabetic rats administered with methanol extract of *Cleome rutidosperma* leaf observed within 14 days of administration**

| Group | Day 1 | Day 3 | Day 5 | Day 7 | Day 10 | Day 14 |
|---|---|---|---|---|---|---|
| **Control** | 67.25±5.36 | 84.25±5.66 | 97.75±4.66 | 85.00±5.72 | 94.25±3.75 | 99.50±6.70 |
| **Diab Control** | 426.00±9.96 | 235.00±13.30 | 320.33±10.51 | 422.33±10.91 | 397.33±8.97 | 326.67±9.30 |
| **Diab + 100 mg.kg$^{-1}$** | 466.33±8.70 | 281.00±9.90 | 192.67±14.73 | 191.67±8.60 | 195.33±15.60 | 179.67±10.73 |
| **Diab + 200 mg.kg$^{-1}$** | 271.60±11.02 | 236.40±14.72 | 72.20±6.18* | 69.20±7.81* | 137.80±7.15 | 70.60±10.66* |
| **Diab + 400 mg.kg$^{-1}$** | 480.00±10.02 | 258.40±8.51 | 270.80±19.32 | 373.00±8.90 | 143.00±12.07 | 189.80±11.86 |
| **Diab + Gliben** | 252.25±14.56 | 234.50±13.97 | 194.50±7.75 | 253.75±7.20 | 284.25±10.56 | 156.00±10.80 |

*—Significantly lower ($P < 0.05$) blood glucose level compared to control in the same column; Diab—Diabetic rats; Gliben—Glibenclamide

**Table 2. Spermatozoa abnormalities observed in alloxan-induced diabetic rats administered with methanol extract of *Cleome rutidosperma* leaf for 14 days**

| Group | Control | Diab Control | Diab + 100 mg.kg$^{-1}$ | Diab + 200 mg.kg$^{-1}$ | Diab + 400 mg.kg$^{-1}$ | Diab + Gliben |
|---|---|---|---|---|---|---|
| **Rud tail** | 2.0±0.41 | 3.21±0.16$^{a}$ | 2.0±0.58$^{b}$ | 2.0±0.45$^{b}$ | 2.0±0.45$^{b}$ | 2.0±0.41$^{b}$ |
| **NHWT** | 4.75±0.63 | 6.0±0.34$^{a}$ | 4.67±0.88$^{b}$ | 4.60±0.51$^{b}$ | 4.60±0.68$^{b}$ | 4.75±0.63$^{b}$ |
| **NTWH** | 4.25±0.48 | 5.60±0.23$^{b}$ | 4.67±0.88$^{b}$ | 4.60±0.51$^{b}$ | 4.60±0.60$^{b}$ | 5.0±0.41$^{a, b}$ |
| **Bent tail** | 10.3±1.31 | 10.9±0.82 | 10.33±0.88 | 8.20±0.49$^{a, b}$ | 11.0±0.45 | 10.0±0.41 |
| **Curved tail** | 9.2.0±0.58 | 11.98±0.45$^{a}$ | 11.67±0.67$^{a}$ | 8.60±0.40$^{a, b}$ | 11.20±0.73$^{a, b}$ | 10.50±0.29$^{a, b}$ |
| **Curved MP** | 10.5±0.65 | 11.91±0.68$^{a}$ | 11.67±1.20$^{a}$ | 9.0±0.32$^{b}$ | 11.0±0.70$^{a, b}$ | 11.75±0.75$^{a}$ |
| **Bent MP** | 10.3±0.85 | 11.1±0.28$^{a}$ | 12.0±0.58$^{a, b}$ | 9.0±0.45$^{a, b}$ | 10.80±0.37$^{b}$ | 10.25±0.25$^{b}$ |
| **Looped tail** | 2.25±0.48 | 3.20±0.37 | 2.0±0.58 | 1.80±0.37 | 2.20±0.37 | 2.25±10.48 |
| **TASp count** | 53.55±2.71 | 63.90±1.46$^{a}$ | 59.01±2.08$^{a, b}$ | 47.80±1.36$^{a, b}$ | 57.40±1.75$^{a, b}$ | 56.50±1.66$^{a, b}$ |
| **TSp count** | 407.50±3.23 | 410.0±3.12 | 410.0±2.89 | 406±2.92$^{b}$ | 403.0±2.0$^{a, b}$ | 407.50±3.2 |
| **% Sp Ab** | 13.14 % | 15.59 %$^{a}$ | 14.39 % | 11.77 %$^{a,b}$ | 14.24 %$^{a}$ | 13.87 % |

Values differ significantly ($P < 0.05$) from normoglycaemic controla or diabetic control$^{b}$;
Diab3—Diabetes; Gliben—Glibenclamide; Rud tail—Rudimentary tail; NHWT—normal head without tail; NTWH—normal tails without head; Curved MP—curved mid-piece; Bent MP—bent mid-piece; TA Sp count—total abnormal sperm count; TSp count—total sperm count; % Sp Ab—% sperm abnormality

**Table 3. Sperm characteristics of alloxan-induced diabetic rats administered with methanol extract of *Cleome rutidosperma* leaf for 14 days**

| Group | Motility [%] | Live/Dead [%] | Volume [cm$^3$] | Count [%] |
|---|---|---|---|---|
| **Control** | 72.50±5.00 | 96.50±1.73 | 5.18±0.05 | 100.0±12.75 |
| **Diab Control** | 57.50±3.54$^{a}$ | 79.00±1.41$^{a}$ | 5.07±0.01$^{a}$ | 97.00±1.40 |
| **Diab+100 mg.kg$^{-1}$** | 73.33±5.77$^{b}$ | 96.00±1.73$^{b}$ | 5.13±0.06 | 116.00±7.20$^{a, b}$ |
| **Diab+200 mg.kg$^{-1}$** | 89.00±5.48$^{a, b}$ | 96.80±1.64$^{b}$ | 5.18±0.04$^{b}$ | 133.20±11.30$^{a, b}$ |
| **Diab+400 mg.kg$^{-1}$** | 68.00±4.47$^{b}$ | 96.80±1.64$^{b}$ | 5.18±0.04$^{b}$ | 106.00±17.01 |
| **Diab+Gliben** | 75.00±12.91b | 96.50±1.73b | 5.18±0.05b | 103.00±12.91 |

Values differ significantly ($P < 0.05$) from normoglycaemic controla or diabetic controlb;
Diab—Diabetes; Gliben—Glibenclamide

associated with *diabetes mellitus* was observed in extract-treated diabetic rats especially at 200 mg.kg$^{-1}$, suggesting the extract has spermatogenic properties.

The increased incidence of sperm cell abnormality in untreated diabetic rats showed evidence of altered spermatogenesis and maturation which can be caused by decreased testosterone bioavailability and also deficient epididymal secretions [33]. The study by Kamaruzaman et al. [17] concluded that Gynura procumbens significantly influenced sperm maturation in amelioration of diabetic male infertility by upregulation of related proteins. La Vignera et al. [19] also mentioned that diabetes decreased the serum testosterone levels in association with a steroidogenetic defect in Leydig cells and increased oxidative stress by hyperglycemia which caused sperm nuclear and mitochondrial DNA damages. These researchers also related spermatogenesis derangement and germ cell apoptosis in type 1 diabetes to local autoimmune damage, while impaired sperm parameters and decrease testosterone serum levels in type 2 diabetics was associated with insulin resistance, obesity, and other related comorbidities [15, 19]. Studies by previous researches have reported medicinal plants such as Teucrium polium, Eryngium caucasicum and Zingiber officinalis, which reversed the decline in testosterone levels and attributed this effect to the ability of the plants to inhibit oxidative stress generated by the hyperglycemic state [2, 8, 31].

Various mechanisms may explain the sperm cell damage observed in patients with diabetes. These include: endocrine disorders, neuropathy, and increased oxidative stress [3, 25]. Insulin is the primary hormone responsible for cellular glucose metabolism including in spermatozoa where the glycolytic pathway for energy generation is a major mechanism employed for sperm motility [13]. Deficiency or insensitivity to insulin which is typical of diabetes also alters this mechanism in sperm cells [6]. The neurohormonal regulation of male reproduction is also altered in diabetes, as well as increased generation of free radical oxygen species [24]. The male reproductive organ is highly vascularized which predisposes the testes to substantial volume of hyperglycemic blood, thus increased exposure to reactive oxygen species, advanced glycation end products, amongst other toxicants [9].

Furthermore, diabetes-induced damage to Sertoli cells may be implicated in the production of defective spermatozoa as the Sertoli cells are responsible for spermatogenesis and part of sperm cell maturation [12, 30]. This study showed a reversal of Sertoli cell vacuolation and denser germinal centers in *C. rutidosperma*-treated diabetic rats. This was clinically demonstrated by higher sperm counts and a lower incidence of abnormal sperm cells. This correlates with the report of Alves et al. [4] that damaged Sertoli cells leads to the production of defective sperm cells which are unable to fertilize a mature ovum. *C. rutidosperma* also induced a significant increase in sperm motility which is important in the movement of the sperm cells through the female genital tract for fertilization. Similar reversal of spermatogenic damage in diabetes have been reported for *Alpinia galanga, Citrullus vulgaris* and *Xanthosoma sagittifolium*, amongst other medicinal plants [18, 23, 27].

## CONCLUSIONS

In conclusion, the male reproductive complications associated with diabetes were reversed by treatment with *Cleome rutidosperma* leaves. The production of viable and motile spermatozoa increased. This study demonstrated that *C. rutidosperma*, especially at 200 mg.kg$^{-1}$ did not further progress infertility in the diabetic rats treated, but reversed diabetes-induced damages in the sperm cell morphology, characteristics and testicular tissue. These findings are relevant in males within the reproductive age and particularly animals intended for breeding.

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# RELATIONSHIPS BETWEEN THE SPREAD OF PATHOGENS AND THE MIGRATORY CONNECTIVITY OF EUROPEAN WILD BIRDS

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## ABSTRACT

**Among emerging infectious diseases, 75 % are zoonotic. Migratory birds are important to public health because they carry emerging zoonotic pathogens or infected arthropod vectors. Disease is an important factor in the evolution of avian migrations and patterns of migratory connectivity. Research suggests that pathogen densities and diseases may influence the evolution of migratory behaviour. During the annual life cycle, European migratory birds spend: 2—4 months at the breeding locality, approximately 6 months on the wintering grounds, and several months (3 and more) on migration routes. There are many factors which determine when and where an outbreak of a disease may occur. Therefore, a complete understanding of the avian migratory systems has a high priority in the prevention of future outbreaks.**

**Key words: birds; climate change; infectious diseases, migratory connectivity**

## INTRODUCTION

The research on wildlife diseases has significantly increased across Europe due to the growing concern of emerging and re-emerging pathogens. This interest has also concentrated on investigations into the risk to human and domesticated animal health.

The aim of this paper was to provide a reference text on links between migration of European wild birds and infectious diseases.

## AVIAN MIGRATION

Migration is defined as a large-scale return movement of a population, which occurs each year between regular breeding and wintering areas [16]. Avian migrations occurs primarily in response to seasonal changes in food resources. Food abundance varies with the climate. Within the sphere of migration, there is a whole range of movements from long distance trans-continental or oceanic migration to short distance wanderings [6].

The European territory is included in the Palearctic-Afrotropical migration system [14]. The Palearctic-Afrotropical migration system consists of three migration corridors: East Atlantic, Black Sea/Mediterranean (Central Europe included), and West Asia/West African corridor [1]. European breeding birds migrate mainly through the Euro-African flyway. It was assumed that 1.52—2.91 billion of European passerine and non-passerine birds cross the Sahara desert each autumn [8].

According to the length of the migratory movement or the proportion of species population involved in migration, we can distinguish 3 main categories of avian migrants. The first category are referred to as the partial migrants which form populations consisting of 2 groups of individuals. The first group migrate periodically and the second group consists of non-migratory specimens, which usually reside on the particular area during the whole annual life cycle [24]. Which portions of population migrates and which portion does not migrate is controlled by variable factors that are often unknown. Migration can be initiated by behavioural interactions between dominant and subdominant individuals under intraspecific pressure, which forces them to move away from the breeding grounds [24]. Portions of partial migratory population can differ genetically [19]. Or, partial migration can be initiated by a combination of environmental factors in coincidence with social interactions [7].

The second category of migrants are the short-distance migrants who migrate to specific nonbreeding grounds which are situated not farther than 2000 kilometres from the breeding localities. And the third category are the long-distance migrants which move usually farther than 2000 kilometres from the particular breeding range [20].

## IMPACT OF GLOBAL ENVIRONMENTAL CHANGES ON AVIAN MIGRATION

There are many reports which demonstrate that global climatic changes affect populations of migratory birds [22]. Climate can affect birds directly, or indirectly. Different species vary in their ability to compensate for the influences of weather [15]. In migratory species, the climate change has shifted migration schedules in Europe [29].

Spring migration is generally considered more important for birds than autumn migration. Spring migration determines the arrival at breeding grounds, which is critically important for mating territory choice [11]. It was assumed that birds migrate earlier in spring in correlation with warming. In general, long-distance migrants lag behind short-distance migrants in terms of their response on short-term changing climatic conditions on breeding grounds [28].

One of the strongest signals that climate change is affecting avian life histories can be seen in recent shifts in the timing of migration and breeding in many bird species, potentially altering survival of species and populations [3, 5]. Birds can respond to changing climate in four ways:

1. They could adapt facultatively, adjusting their behaviour within its pre-existing limits to cope with changing conditions.
2. Birds could adapt by evolutionary change which would entail genetic modification under the action of natural selection.
3. Birds could change their distributions and migrations so as to remain within regions of a favourable climate.
4. They could respond insufficiently in any of these ways, and so decline to extinction in at least some parts of their range [17].

It is likely that man-made changes to the environment will contribute to changes in the characteristics and patterns of long distance migration. Continued loss and fragmentation of important habitats at stopover sites along major migration corridors will create bottlenecks, resulting in increased displacement and mixing of bird species [21]. These changes will affect also the ecology of avian pathogens [21].

## MIGRATORY CONNECTIVITY

During their annual life cycle, European migratory birds spend 2—4 months at the breeding locality, approximately 6 months on the wintering grounds and several months (3 and more) on migration routes.

Movements through a diverse mosaic of biotopes and environmental conditions create a complex of ecological interactions between migrating birds and numerous factors of the environment, biotic and abiotic. These situations can result in exposure to many pathogens, non-native for European ecosystems [26].

Knowledge about migratory connectivity, the degree

to which individuals from the same breeding site migrate to the same wintering site, is essential to understand the processes affecting populations of migrants throughout their annual cycle [25]. Consequently, it is crucial to know where birds travel and winter and to what degree individuals from a certain population are using the same migration route or migrate to the same wintering site, i.e. how and to what extent breeding and non-breeding populations are connected [26, 27].

When this connectivity is strong, individuals from one breeding site migrate mainly to one wintering site [27].

## MIGRATORY CONNECTIVITY AND INFECTIOUS DISEASES

Among emerging infectious diseases, 75 % are zoonotic; originating principally from wildlife [2]. Migratory birds are important to public health because they carry emerging zoonotic pathogens, either as a reservoir host or by dispersing infected arthropod vectors. In addition, bird migration provides a mechanism for the establishment of new endemic foci of disease at great distances from where an infection was acquired [21].

Understanding migratory connectivity for a population can give insight into adaptive behaviours that reduce pathogens and disease. Understanding the biology and conducting effective conservation of migratory species requires knowledge of migratory connectivity, the geographic linkage of individuals, or populations between phases of the annual cycle [13].

Quantifying the degree to which individuals move from a breeding range to the same nonbreeding region is necessary for understanding how epizootiological events during one phase of the annual cycle influence subsequent phases [12, 23, 26].

## THE ROLE OF EUROPEAN WILD BIRDS IN THE SPREAD OF PARTICULAR ZOONOSES

Birds are susceptible to fewer zoonotic agents than mammals, reflecting the evolutionary distance between birds and humans [4]. Despite their lower susceptibility, birds participate effectively in the transmission and spread of zoonoses, even over great distances, by acting as natural hosts, reservoirs and amplifying or liaison hosts for zoonotic agents [10]. The stress of migration can lead to immunosuppression and increased disease susceptibility, as well as reactivation of latent infections [21].

A notable example of HPAI (H5N1) risk is when migrating birds from Asia and northern Europe congregate in sub-Saharan Africa. Migratory birds also interact with populations of domestic and free living sedentary birds at stopover sites or at the end of their journey. This makes concentration points along migration corridors (such as the Straits of Gibraltar, Messina, Bosphorus and Sinai Peninsula) especially important.

Apart from acting as a reservoir of zoonotic agents, migratory birds can be infested with arthropod vectors, which in turn transmit pathogens that can be carried by birds over long distances and may be responsible for diseases of major importance. Borreliosis, or Lyme disease, is the most prevalent vector-borne zoonosis in humans in the Northern Hemisphere, which, a few decades ago, occurred on only an occasional and localised basis [9, 18].

Another prominent epidemiological role of birds (especially wild ones) is to act as liaison hosts, or even amplifying hosts, for some arboviruses (arthropod-borne viruses). They include arboviruses caused by flaviviruses antigenically related to Japanese encephalitis, which often cause acute clinical symptoms (encephalitis) in horses and humans [21].

## CONCLUSIONS

There are many factors that determine when and where a disease outbreak will occur; one of these factors is wildlife as a reservoir and vectors for the pathogen. Therefore, a complete understanding of the world's migratory systems is a high priority in the prevention of future outbreaks. The Palearctic-Afrotropical migratory system and its connection with pathogens is both broad and complex. The number of birds involved and the vast area that the migration encompasses, ensures us that additional research is needed.

As the current extent and understanding of migratory birds are limited, tracking is necessary in order to better understand the migration routes, population dynamics, site fidelity, migration timing, origin of pathogens, etc. The surveillance of pathogens in wildlife is in the interest of both veterinary and human medicine.

There have been several epidemics in the past. And wildlife including birds has helped fuel these epidemics by both being reservoirs for the pathogens and by actively spreading the disease. The best way to control and prevent further epidemic outbreaks is to increase our understanding of the migratory systems of birds and the ways of transmission of diseases, in addition to the surveillance of wildlife.


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# PREVALENCE AND CO-INFECTION OF *BARUSCAPILLARIA GENUS* (NEMATODA, CAPILLARIIDAE) IN DOMESTIC GEESE IN UKRAINE

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## ABSTRACT

**New data about the fauna of nematodes of *Baruscapillaria* genus have been obtained which show that they parasitize domestic geese in the Poltava region of the Ukraine. It has been established that the species composition of *Capillariidae* is represented by two species—*B. anseris* (M a d s e n, 1945, M o r a v e c, 1982) and *B. obsignata* (M a d s e n, 1945, M o r a v e c, 1982). For the first time in the Ukraine, parasitisation with non-specific geese species of *Capillariidae* family, *B. obsignata*, have been substantiated. It was found that capillariosis of geese was more frequent in the co-invasions of the birds' digestive channel; the prevalence of invasion was 41.97 %. According to the results of helminthological dissection and identification of isolated pathogens, 40 varieties of co-invasions were found, where nematodes of the *Baruscapillaria* genus were combined with cestodes of two species: *Drepanidotaenia lanceolata* (B l o c h, 1782) and *Tschertkovilepis setigera* (F r o e h l i c h, 1789), as well as with nematodes of four other species: *Amidostomum anseris* (Z e d e r, 1800), *Trichostrongylus tenuis* (M e h l i s, 1846), *Heterakis gallinarum* (S c h r a n k, 1788) and *Heterakis dispar* (S c h r a n k, 1790). Most often, we recorded capillariosis as a part of two- (prevalence—17.75 %) and three-component (11.75 %) co-invasions, and the main coexisting helminths of *Capillariidae* from *Baruscapillaria* genus were nematodes *A. anseris* (prevalence 22.78 %) and *H. dispar* (14.15 %).**

**Key words: *Anser domesticus*; associative course; capillariosis; co-invasions prevalence**

## INTRODUCTION

Geese breeding is a promising and in-demand poultry industry worldwide, particularly in the Ukraine. This is due to the fact that geese, unlike other farmed poultry, are the least demanding concerning growing and confining conditions. These birds are characterized by their precocity, as well as providing a wide range of products for the food, perfume, pharmaceutical and light industries [4, 6, 7, 11].

One of the main reasons restricting the development of the geese breeding is the problem of helminthoses of the digestive canal, in particular capillariidoses, which cause significant economic loses to the farms. Due to the parasite invasions, young geese are lagging behind in growth and development, their preservation is reduced, as well as the productivity and breeding value of adult birds [3, 5, 8, 12].

Scientists have pointed out the widespread prevalence of geese capillariosis in different countries of the world, where invasion occurs mainly as part of the associated parasitic diseases of the digestive tract of birds. In Japan, in 15 species of birds of the *Anseriformes* order, three species of *Capillariidae* parasites have been identified: *Eucoleus contortus*, *Capillaria (C.) anatis*, *Baruscapillaria (B.) mergi*. The prevalence of invasion ranged from 8.89 to 100 %, and the intensity (according to the results of post-mortem autopsy) from 1 to 55 specimens of helminths per bird [18]. After studying the faeces of grey wild geese in Austria, scientists found that the prevalence of capillariosis invasion was low compared to other helminthoses and protozoan parasitosis and reached only 0.2 % [15].

At the same time, in the Czech Republic, two species of *Capillariidae* have been identified on broiler geese farms: *C. caudinflata* (prevalence 0.2 %) and *C. obsignata* (7.7 %). Moreover, *Capillariidae* in birds have been parasitized in associations with: *Notocotylus attenuatus, Apatemon gracilis, Cotyrulus cornutus, Hypoderaeum conoideum, Retinometra longicirrosa, Drepanidotaenia lanceolata, Sobolevicanthus fragilis, Diorchis stefanskii, Microsomacanthus microsoma, Dilepis undula, Amidostomum anseris, Trichostrongylus tenuis, Ganguleterakis dispar* and *Heterakis gallinarum*. The authors diagnosed two-component (in 29.7 % of poultry), three-component (7.8 %) and four-component (1 %) invasions [2]. In Germany, researchers have found that in grey geese, the causative agent of capillariosis was associated with nematodes of *Amidostomum* and *Trichostrongylus* genus, as well as the protozoa of *Eimeria* genus [16]. Therefore, the study of *Capillariidae* fauna which parasitize domestic geese in Ukraine, as well as the peculiarities of these nematodes' parasitizing the body of birds is a relevant area of research.

## MATERIALS AND METHODS

This research was conducted during 2016—2018 in the laboratory of the Department of Parasitology and Veterinary-Sanitary Examination at the Poltava State Agrarian Academy and under the conditions of 5 poultry enterprises and 132 small private farms in the Poltava region of the Ukraine (Velykobahachanskyi, Hadiatskyi, Hlobynskyi, Dykanskyi, Zinkivskyi, Kotelevskyi, Lubenskyi, Lokhvytskyi, Myrhorodskyi, Shyshatskyi districts).

In order to determine the species composition of pathogens, we performed autopsies of geese's digestive organs canals. The helminths were collected by the method of complete helminthological dissection of birds' digestive organs canals [13]. The collected helminths were fixed in 70 % ethyl alcohol. For the differentiation of nematodes, they were preliminarily placed in lactophenol, and the cestodes were stained with acetocarmine. A total of 417 organs of the digestive tracts (glandular and muscle parts of the stomachs, small intestine, large intestine, oesophagus, ingluvies) of geese were examined; large Gray, Danish Legart, Gorkovskaya, Mirgorodska, as well as mixed breeds.

The occurrence of helminth agents in the geese was indicated by the prevalence rate (%). The identification of helminths' species was carried out by a qualifier [10, 14]. Microphotographs were obtained by means of a digital camera and a MikroMed 5Mpix (China) microscope using a ×10, ×40 lens and a ×10 photo eyepiece.

## RESULTS

According to the results of this parasitological investigation, it was found that capillariosis was a common nematode invasion of geese in the Ukraine. The average prevalence of capillariosis invasion of geese under the conditions of poultry farms in the Poltava region reached 57.79 % with an invasion intensity of 39.55 ± 2.27 specimens per bird. The indicators of the prevalence of the geese nematodes of *Ba-*

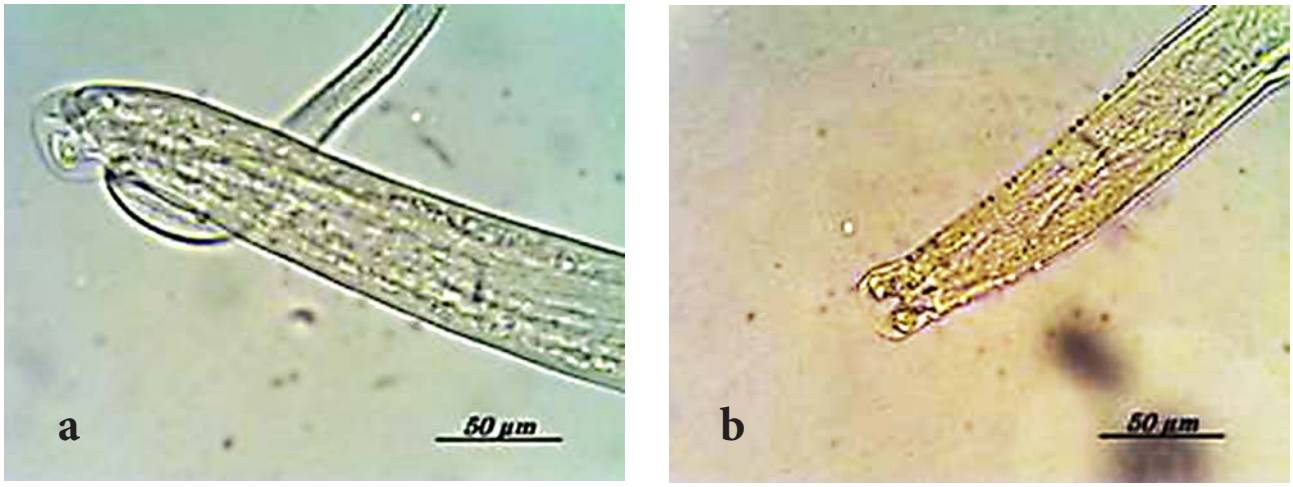


**Fig. 1. Caudal end of ♂ *Baruscapillaria* genus nematodes':**
**a**—*B. anseris*; **b**—*B. obsignata*

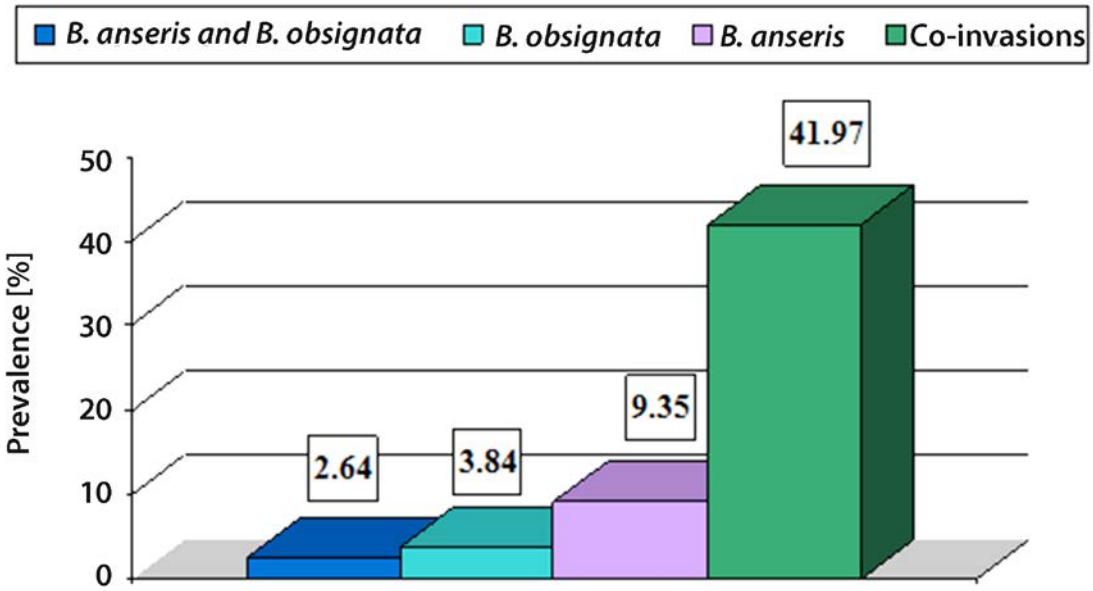


**Fig. 2. Indicators of the prevalence [%] of *Baruscapillaria* genus in the composition of mono- and co-invasions**

*ruscapillaria* genus (*Nematoda*, *Capillariidae*) in different areas showed slight fluctuations and ranged from 51.22 % (Dykanskyi district) to 68.98 % (Shyshatskyi district). It was also observed that *Capillariidae* fauna was represented by two species—*B. anseris* (M a d s e n, 1945, M o r a v e c, 1982) (Fig. 1a) and *B. obsignata* (M a d s e n, 1945, M o r a v e c, 1982) (Fig. 1b). The predominant species was *B. anseris* with the average prevalence in geese reaching 46.76 %, and the intensity of 38.3 specimens per bird. At the same time, the number of *B. obsignata* nematodes was much lower than that of *B. anseris*, with a prevalence of 23.98 % and the intensity of 15.7 specimens per bird.

It was found that the geese's capillariosis often had an associative course of a co-invasion of the geese's digestive canal (prevalence 41.97 %). Mono-invasion capillariosis was diagnosed less frequently (15.83 %); of this 2.64 % of the birds showed simultaneous parasitisation of capillariid nematodes of both species (*B. anseris* and *B. obsignata*) while 9.35 % of the birds were parasitized only with *B. anseris* and 3.84 % only with *B. obsignata* (Fig. 2).

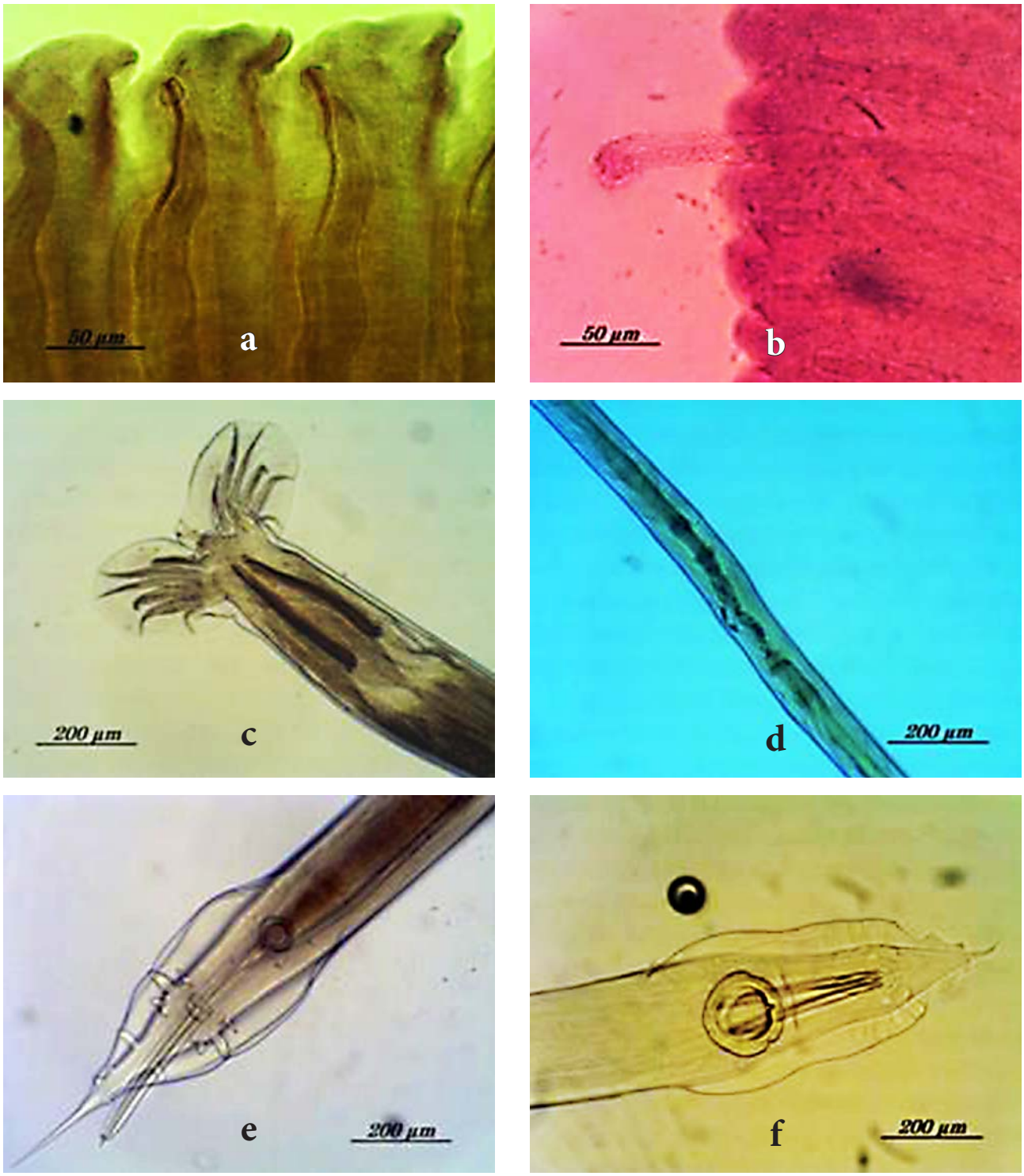


**Fig. 3. Species composition of coexisting nematodes of Baruscapillaria genus in geese:**
**a**—cirrus bursa in hermaphroditic proglottids of *D. lanceolata*; **b**—outward cirrus in hermaphroditic proglottid of *T. setigera*; **c**—caudal end of ♂ *A. anseris*; **d**—vulvae area of ♀ *T. tenuis*; **e**—caudal end of ♂ *H. gallinarum*; **f**—caudal end of ♂ *H. dispar*

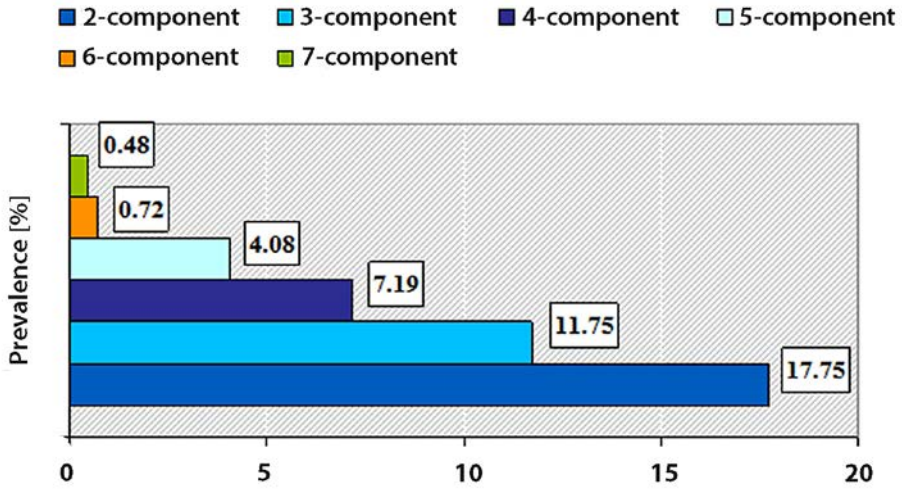


**Fig. 4. Prevalence indicators of various components of co-invasions in geese during parasitisation with *Baruscapillaria* genus's nematodes**

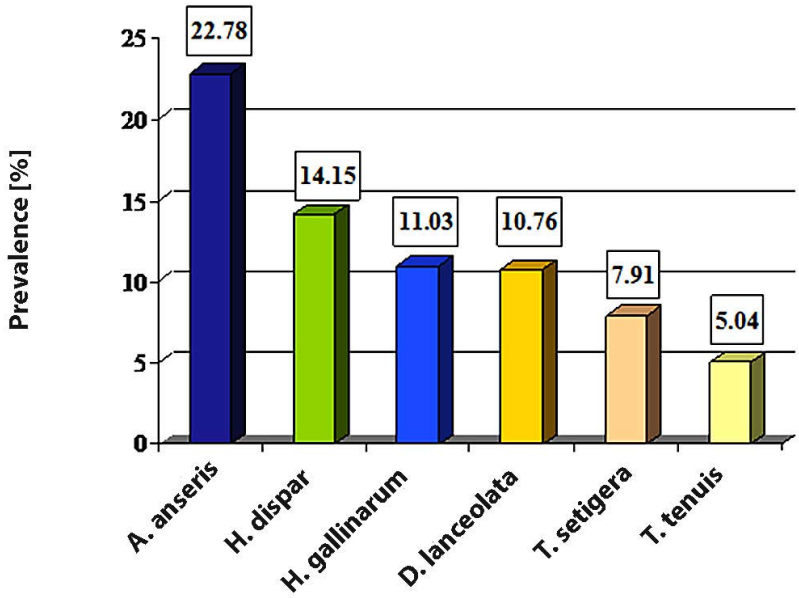


**Fig. 5. Species composition of cohabitants of *Baruscapillaria* genus's nematodes in geese's digestive canal co-invasions**

In general, 40 varieties of co-invasions were found, where nematodes of the *Baruscapillaria* genus were combined with cestodes of two species: *Drepanidotaenia lanceolata* (B l o c h, 1782) (Fig. 3a) and *Tschertkovilepis setigera* (F r o e h l i c h, 1789) (Fig. 3b), as well as with nematodes of four species: *Amidostomum anseris* (Z e d e r, 1800) (Fig. 3c), *Trichostrongylus tenuis* (M e h l i s, 1846) (Fig. 3d), *Heterakis gallinarum* (S c h r a n k, 1788) (Fig. 3e) and *Heterakis dispar* (S c h r a n k, 1790) (Fig. 3f).

The prevalence of geese's capillariosis in combination with several species of helminths is presented in Fig. 4. As can be seen, the most common were 2- and 3-component co-invasions.

Among the two-component co-invasions, the association of *Baruscapillaria* genus's nematodes and *A. anseris* (5.52 %) were most commonly diagnosed. Such associations of capillariid were less common: with *H. dispar* (3.59 %), *T. setigera* (2.88 %), *H. gallinarum* (2.16 %), *T. tenuis* (1.92 %), *D. lanceolata* (1.68 %).

Among three-component co-invasions, there were established associations of capillariid with: *A. anseris, T. tenuis, H. gallinarum, H. dispar, D. lanceolata, T. setigera* in different combinations. The prevalence ranged from 0.24 to 1.92 %.

Four-component co-invasions involved the following associations: *B. anseris, B. obsignata, A. anseris* and *T. setigera* (1.92 %); *B. anseris, A. anseris, H. dispar* and *D. lanceolata* (1.44 %); *B. anseris, B. obsignata, H. gallinarum* and *D. lanceolata* (1.19 %); *B. anseris, B. obsignata, A. anseris* and *D. lanceolata* (0.96 %); *B. anseris, B. obsignata, A. anseris* and *H. gallinarum* (0.96 %); *B. anseris, B. obsignata, A. anseris* and *H. dispar* (0.24 %); *B. anseris, B. obsignata, H. dispar* and *H. gallinarum* (0.24 %); B. *anseris, A. anseris, H. dispar* and *T. setigera* (0.24 %).

Five-component co-invasions were represented by four varieties of associations: *B. anseris, B. obsignata, H. dispar, A. anseris* and *D. lanceolata* (1.92 %); *B. anseris, A. anseris, H. dispar, H. gallinarum* and *T. setigera* (1.68 %); *B. anseris, B. obsignata, H. dispar, H. gallinarum* and *D. lanceolata* (0.24 %), as well as *B. obsignata, A. anseris, H. dispar, H. gallinarum* and *T. setigera* (0.24 %).

Six- and seven-component co-invasions consisted of: B. anseris, B. obsignata, A. anseris, H. dispar, H. gallinarum and D. lanceolata (0.72 %), B. anseris, B. obsignata, *A. anseris, H. dispar, H. gallinarum, T. tenuis* and *D. lanceolata* (0.24 %), as well as *B. anseris, B. obsignata, A. anseris, H. dispar, T. tenuis, D. lanceolata* and *T. setigera* (0.24 %).

It was determined that the main cohabitants of *Baruscapillaria* genus's nematodes in geese's bodies were nematodes *A. anseris* (prevalence 22.78 %) and *H. dispar* (14.15 %). A smaller percentage was determined for capillariid associations with *H. gallinarum, D. lanceolata, T. setigera* and *T. tenuis* (Fig. 5).

It was observed that capillariosis caused by *Baruscapillaria* genus's nematodes was a common invasion of domestic geese in Ukraine; it mainly occurs as a part of co-invasions together with pathogens of cestodoses and nematodoses of the birds' intestinal canal.

## DISCUSSION

Studies have shown that nematodes of *Baruscapillaria* genus are common parasites of domestic geese in Ukraine. The avian affection rate averaged 57.79 % and capillariid fauna was represented by two species: *B. anseris* and *B. obsignata*. Significant distributions of capillariid parasites among domestic and wild waterfowl birds in Poland, Japan, Nigeria, India have been confirmed by a number of authors [1, 5, 8, 9].

We also found that capillariosis pathogens of birds occurs more frequently in the form of co-invasions (prevalence 41.97 %) together with cestodes (*Drepanidotaenia lanceolata, Tschertkovilepis setigera*) and nematodes (*Amidostomum anseris, Trichostrongylus tenuis, Heterakis gallinarum, Heterakis dispar*) of geese's digestive tract. In total, 40 varieties of co-invasions were isolated, where nematodes of *Baruscapillaria* genus were present in association with two to seven helminths. Moreover, with the increase in the number of parasites in associations, the prevalence was gradually reduced from 17.75 % (two-component) to 0.48 % (seven-component). In our opinion, this is due to the antagonism of the individual parasites and the heavy strain on the organism of birds that can lead to their death. Similar data indicating a decrease in prevalence with an increasing number of parasites in co-invasion were reported [2].

It has been shown that nematodes of *Baruscapillaria* genus most often coexist with *A. anseris* and *H. dispar*. We rarely diagnosed capillariid associations with *H. gallinarum, D. lanceolata, T. setigera* and *T. tenuis*. Scientists attributed this dependence to the fact that helminth associations that are localized in different organs (muscular stomach, small and large intestines) do not compete with each other and are therefore more frequently observed. At the same time, when locating nematodes of different species in one organ (small intestine), their antagonism was noted, which was manifested by a decrease in the number of parasites [17].

The data obtained make it possible to increase the effectiveness of planning measures for the control and prevention of capillariosis in domestic geese, taking into account the peculiarities of parasitisation with nematodes from the Baruscapillaria genus.

## CONCLUSIONS

Two species from the genus *Cappillaria* were isolated from domestic geese (*Anser anser dom.*) in Ukraine: *Baruscapillaria anseris* (prevalence 46.76 %) and *Baruscapillaria*

*obsignata* (23.98 %). According to the results of helminthological dissections, the average prevalence of *Baruscapillaria* genus nematodes was 28.72 %. The features of *Cappillaria nematodes* in geese are characterized mainly by the formation of two- (prevalence 17.75 %) and three-component (11.75 %) parasitic associations, where *Baruscapillaria* genus nematodes coexist with cestodes: *D. lanceolata* and *T. setigera*, as well as nematodes: *A. anseris, T. tenuis, H. gallinarum* and *H. dispar*. And most often, capillariid helminths parasitize as co-invasions together with *A. anseris and H. dispar*; the prevalence reaching 22.78 % and 14.15 %, respectively.

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# *IN VITRO* STUDY OF IMMUNE PROPERTIES OF NEW LACTOBACILLI ISOLATES FROM PHEASANT GUT

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## ABSTRACT

**The goal of this paper was to study the effect of *Lactobacillus reuteri* B1/1, B2/1 and B6/1 on the relative expression of selected interleukins (IL-1β, IL-15), macrophage inflammatory protein (MIP-1β), and the relative percentage of T lymphocyte subpopulations in peripheral mononuclear blood cells (PMBCs). The mRNA expression levels of interleukins and MIP-1β of PMBCs were evaluated at 24 h and 48 h post inoculation using the quantitative real-time polymerase chain reaction (qRT-PCR). The percentage of T lymphocyte subpopulations in PMBCs was determined by flow cytometry. The group that was administered *L. reuteri* B1/1 had the most significant stimulation of the expression of pro-inflammatory interleukins and MIP-1β, in particular after 24 h. Similarly, we observed a rise in the relative percentage of T cells including CD3+, CD4+ and CD8+ lymphocytes in the groups with *L. reuteri* B1/1 and *L. reuteri* B2/1. Overall, *L. reuteri* B1/1 and *L. reuteri* B2/1 showed a promising stimulatory effect on the relative expression of pro-inflammatory interleukins, MIP-1β and percentage of T cell subpopulations *in vitro*. On the flip side, *L. reuteri* B6/1 did not induce the expression of the IL-1β gene.**

**Key words: *Campylobacter*; cytokine; lactobacili; peripheral mononuclear blood cells**

## INTRODUCTION

Poultry meat can become contaminated with *Campylobacter* spp. during slaughter and may be passed on to humans through consumption and handling of contaminated poultry products. Importantly, campylobacteriosis is the most commonly reported zoonosis and chicken meat is considered the main source of this infection [16]. Moreover, the broiler immune system is usually inefficiently activated by *Campylobacter jejuni*. Colonization and the expression of relevant immune proteins is suppressed [2].

Unquestionably, cytokines and chemokines are key regulators of innate immunity via the development of in-

flammatory responses to *Campylobacter* infection. Differentiated T helper cells rapidly secrete particular cytokines upon antigen challenge [18]. The use of new probiotic preparations could be a promising form for the prevention and treatment of *Campylobacter* infections in poultry by the modulation of cytokine production and thus an immune answer as well as by inhibition of the pathogen.

In recent years, great attention has been focused on the use of probiotic lactobacilli derived from natural sources to improve human and animal health. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer health benefits on the host" [11].

*Lactobacillus* is the largest genus of lactic acid bacteria (LAB), which includes 183 species. They are Gram-positive bacteria that ferment glucose, produce lactic acid and other substances. Moreover, lactobacilli are the dominant bacteria in the animal gastrointestinal system. They play an important role mainly in the maintenance and recovery of gut health [37, 39]. In chickens treated with various *Lactobacillus* species it has been observed that they modulate many aspects of the immune response to pathogens [17].

The development of each new probiotic preparation needs research into the selected strain to be used, how does it affects the host organism, mechanism of action, safety and genetic stability must be documented [14]. Many other factors have to be tested, including the survival in gastric and intestinal fluids, the capability to adhere to the intestinal surface, and the sensitivity to antibiotics during *in vitro* experiments [9]. In terms of protection against pathogenic organisms, it is necessary to test the immunomodulatory effect and antimicrobial activities of newly isolated potential probiotic strains.

Among other things, one of the selection criteria for safety is that the potential probiotic strain should demonstrate ATB (Antibiotic)-sensitive based on the EFSA (European Food Safety Association) requirement [10] and does not contain transferable (acquired) ATB resistance genes located for example on conjugated plasmids or transposons. Probiotic microorganisms should not increase the existing risks of antibiotic resistance. If the lactobacilli live in an environment that is regularly exposed to ATB, such as the intestines of poultry kept in intensive farm conditions, acquired resistance may also develop. Therefore, we decided to isolate potential probiotic strains from wild birds—pheasants, where a low incidence of resistant isolates might be expected.

For the possible use of new, potentially probiotic lactobacilli, isolated from pheasant gut designed for the prevention or treatment of campylobacteriosis in chicken, we focused on the study of the effect of *L. reuteri* B1/1, B2/1 and B6/1. Specifically, we focused on the relative gene- expression of selected pro-inflammatory interleukins, MIP-1β, the relative percentage of T lymphocyte subpopulations in the PMBCs of chickens. Moreover, we tested antimicrobial activities of *L. reuteri* isolates against selected pathogens.

## MATERIALS AND METHODS

### Cultivation and isolation of peripheral mononuclear blood cells

The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol number 836/17-221 and the animals were handled in a humane manner.

Peripheral mononuclear blood cells (PMBC) were obtained from the peripheral blood of clinically healthy poultry reared under standard conditions by puncture of vena cutanea ulnaris using Heparin (10—20 $U.ml^{-1}$ PBS, Zentiva, Czech Republic) as an anticoagulant. The blood was diluted in a ratio of 1 : 2 with PBS, transferred and layered into Leucosep tubes (Greiner bio-one, DE) containing Histopaque-1077 (Sigma-Aldrich, UK). The isolation of the PMBC was done by centrifugation (19 000 × g, 40 min, 20 °C; Hettich Rotina 420R Centrifuge, DJB Labcare, UK). The mononuclear cells collected from the gradient interface were washed two times with PBS (16 000 × g, 5 min) [3]. Trypan blue was used to determine cell viability and a Bürker chamber to count the total number of cells diluted in Türk solution. Isolated PMBCs were placed on a 12-well cultivation plate (Orange Scientific, BE) with concentration $1 \times 10^7$ $cells.ml^{-1}$ and cultured overnight (39 °C, 5 % $CO_2$) in RPMI 1640 medium enriched with 10-mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Lonza, BE) and 10 % foetal bovine serum (Lonza, BE). Each *Lactobacillus* isolate at a concentration of $1 \times 10^9$ $CFU.ml^{-1}$ in 200 µl PBS was added to the individual PMBCs cultures with cultivation lasting 24 and 48 h. The cultivation (culture medium, growth conditions) of the new lactobacilli isolates were performed as described previously [32].

**Bacterial strains**

The strains of lactobacilli used in the present study were isolated from the gut contents of healthy pheasants. In a previous study, the strains were identified by matrix-assisted laser desorption/ionization—time of flight mass spectrometry (MALDI-TOF MS) as *Lactobacillus reuteri* B6/1, B2/1 and B1/1. The strains were characterized by the production of exopolysaccharides [31].

**Homogenization and isolation of the total RNA from PMBCs**

The PMBCs were collected from each well by pipetting and then centrifuged for 1 min at 8000 × g. The cell lysate was homogenised two times for 45 s at 2700 rpm using 1.0 mm zirconia/silica beads (BioSpec Products, USA) and 1 ml of TRI Reagent (Sigma-Aldrich, Germany) in a Mag-Na Lyser instrument (Roche, UK). In the separation phase, 50 µl of 4-bromanisole (Molecular Research Center, USA) was added. For the purification of total RNA from the cell lysate, the RNAeasy mini kit (Qiagen, UK) was used. The purity and concentration of the total RNA was determined on a NanoPhotometer (Implen, Germany), and 1 µg of the total RNA was reverse transcribed by using Maxima first strand cDNA synthesis kit (Thermofisher, USA) and oligo-dT primers. The resulting cDNA was 10× diluted in DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template for qRT-PCR or stored at −20 °C.

**Quantitative Real-time PCR**

The mRNA levels of interleukins and MIP-1β were determined. The mRNA relative expression of a reference gene, coding for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used for data normalization. The specific primer sequences used for qPCR are listed in Table 1. All primer sets allowed DNA amplification efficiencies between 94—100 %. Amplification and detection of specific products were performed via the CFX 96 RT system (Bio-Rad, USA). The cycling conditions included an initial denaturation: at 95 °C for 15 min and 38 cycles; denaturation 95 °C for 20 s, annealing 60 °C for 30 s and final elongation 72 °C for 30 s. A melting curve ranging from 50—95 °C, with readings taken every 0.5 °C, was obtained for each individual qRT-PCR plate. Each sample was subjected to quantitative real-time PCR in triplicate, and mean values of triplicates were used for subsequent analysis. We confirmed that the efficacy of amplification of each gene (including GAPDH) was essentially 100 % in the exponential phase of the reaction, where the cycle of quantification (Cq) was calculated. The Cq values of the genes studied were normalized to an average Cq value of the reference gene (ΔCq), and the relative expression of each gene was computed mathematically as 2–ΔCq.

**Table 1. List of primers used in qRT-PCR for IL-1β, IL-15 and MIP-1β mRNA detection in PMBC culture**

| Primer | Sequences 5′–3′ | References |
|---|---|---|
| IL-15 For | TGGAGCTGATCAAGACATCTG | [26] |
| IL-15 Rev | CATTACAGGTTCCTGGCATTC | |
| IL-1β For | GAAGTGCTTCGTGCTGGAGT | [5] |
| IL-1β Rev | ACTGGCATCTGCCCAGTTC | |
| MIP1-β For | GGCAGACTACTACGAGACCAACAG | [36] |
| MIP1-β Rev | ACGGCCCTTCCTGGTGAT | |
| UB For | GGGATGCAGATCTTCGTGAAA | [7] |
| UB Rev | CTTGCCAGCAAAGATCAACCTT | |

**Flow cytometry procedure**

The direct immunofluorescent method and double immunostaining labelled isolated lymphocytes with mouse anti-chicken monoclonal antibodies (SouthernBiotech, USA) in arrangement CD3PE/CD4FITC, CD3PE/CD8 FITC (T-cells) were used. Polyclonal goat-anti mouse FITC-conjugated immunoglobulin F(ab')2 fragment (Dako, Denmark) was used at a working dilution of 1 : 50 with PBS as the control antibody. Fifty µL of cellular suspension ($1.10^7$ lymphocytes in PBS) and 2 µl of specific or control MoAbs were mixed and incubated in the dark at 22 °C for 15 min. After washing in 0.5 ml PBS and re-suspended in 0.2 ml of PBS with 0.1 % paraformaldehyde, the cells were measured by FACScan (Becton Dickinson, Germany) with a 15 mV argon ion laser. The fluorescence data was collected and analysed on at least 10,000 lymphocytes using the Becton Dickinson Cell Quest programme (Germany). The results were expressed as the relative percentage of the lymphocyte subpopulation that was positive for a specific MoAb. Samples were measured in triplets before cultivation, 24 h and 48 h post inoculation (p. i.) in the following arrangement: control (C) and lactobacilli strains marked B1/1, B1/2 and B1/6.

**Determination of antagonism *in vitro***

The method to test the antagonistic activity of *Lactobacillus reuteri* B1/1, B2/1, B6/1 was performed according to J a c o b s e n et al. [23] with some modifications. Sterile discs with a diameter of 6 mm (BBL, Cockeysville, USA) were placed on the surface of 20 ml of peptone-yeast extract-glucose (PYG) agar in Petri dishes. The composition of PYG agar was as follows: peptone for bacteriology 5 g; enzymatic casein hydrolysate 5 g; yeast extract 10 g; glucose 10 g; and agar 18 g.1000 $ml^{-1}$ distilled water (pH 6.9). The discs were inoculated with 10 µl of the night cultures of lactobacilli ($1 \times 10^8$ $CFU.ml^{-1}$) in de Man-Rogosa-Sharpe broth (MRS; Carl Roth GmbH + CO. KG, Karlsruhe, Germany) and the plates were then cultivated anaerobically (Gas Pak Plus, BBL Microbiology systems, Cockeysville, Maryland, USA) at 37 °C for 48 hours. After incubation, paper discs were removed and the plates were overlaid with 3 ml of 0.7 % PYG agar, inoculated with 0.3 ml of the night culture of a respective indicator strain and incubated aerobically at 37 °C for 24 hours. The following indicator strains were used: *Escherichia coli* 0149 F4 (Research Institute of Veterinary Medicine in Brno, CR); *Staphylococcus aureus* CCM 4223; *Salmonella Typhimurium* CCM 7205 (Czech Collection of Microorganisms in Brno, CR) and *Bacillus cereus* (isolate obtained from the Laboratory of gnotobiology, UVMP in Košice, SR). Two controls were used: blank disc and discs inoculated with 10 µl of sterile MRS medium. The results are presented as the arithmetic means of three measurements (mm) ± SD.

**Statistical analysis**

The one way ANOVA with Tukey post-test by Minitab 16 software was used (SC&C Partner, Brno, Czech Republic). The difference between the mean values for various treatment groups were considered statistically significant at $P < 0.05$, $P < 0.01$, $P < 0.001$. The values were expressed as means or median ± standard deviation (SD).

## RESULTS

**Relative expression of cytokines by quantitative real-time PCR**

The relative expression for IL-1β gene (Fig. 1a) was upregulated in the B1/1 group compared to the control ($P < 0.001$) and B2/1 ($P < 0.01$) 24 h p. i. After 48 h p. i., the relative expression of IL-1β was upregulated in the same group (B1/1) compared to other groups ($P < 0.001$). Interestingly, group B6/1 did not exhibit gene expression for IL-1β.

Similarly, the relative expression for IL-15 gene (Fig. 1b) was upregulated in both the B1/1 and B2/1 groups compared to the control and B6/1 ($P < 0.001$) group, as well as in the B1/1 group compared to the B2/1 group ($P < 0.01$) 24 h p. i. In the second sampling, the upregulation of the

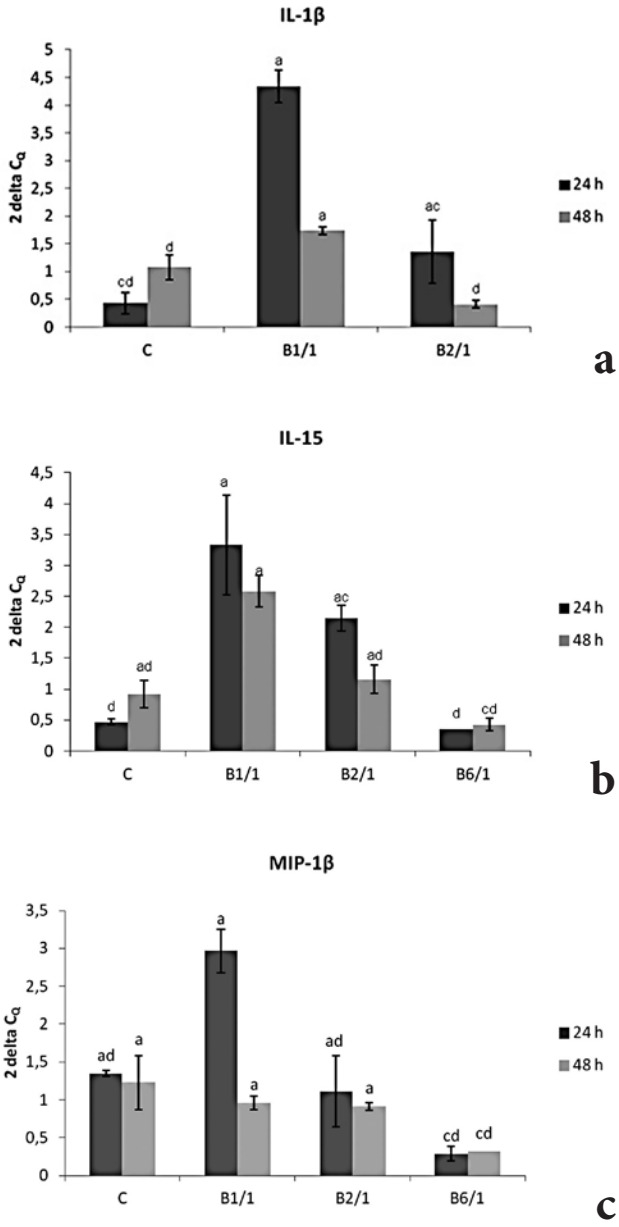


**Fig. 1. Relative expression level of a) IL-1β; b) IL-15; and c) MIP-1β in PMBC culture. Results at each time point are the median of 2–ΔCq. Means with different superscripts are significantly different acP < 0.01; adP < 0.001**

relative expression for IL-15 was recorded mainly in the B1/1 group compared to the other groups ($P < 0.001$).

A significant increase in the relative expression for MIP-1β (Fig. 1c) was observed in the B1/1 group compared to the other groups ($P < 0.001$) 24 h p. i. In contrast, during the second sampling (48 h p. i.) the relative expression of MIP-1β was downregulated in all treated groups compared to the control ($P < 0.001$).

**Immunophenotyping of lymphocytes**

The relative percentages of CD3+, CD4+ and CD8+ cells are presented in Fig 2. In the first sampling (24 h p. i.) we noted significantly higher proportions of T cells including CD3+, CD4+ and CD8+ lymphocytes in lactobacilli B1/1 and B2/1 groups in comparison with the control ($P < 0.001$). The highest relative percentage was observed in the B2/1 group, and it was significantly higher compared to the lowest values, which were observed in the B6/1 group. Forty eight hours post inoculation, the T cells showed an increase only in the B2/1 group of lactobacilli compared to the control and all other groups. This increase was observed in CD3+ and CD8+ ($P < 0.001$), as well as CD4+ ($P < 0.01$) subpopulations.

**Antimicrobial activities of lactobacilli**

The highest inhibitory activity against *S. aureus* CCM4223 was shown by *L. reuteri* B6/1 and B1/1 compared to *L. reuteri* B2/1 ($P < 0.01$). On the other hand, the efficiency of inhibitory activity against *S. Typhimurium* was the highest in *L. reuteri* B2/1 compared to other lactobacilli strains ($P < 0.001$). Antibacterial activity against *E. coli* O149F4 was the highest in *L. reuteri* B1/1 compared to other lactobacilli strains ($P < 0.05$) (Table 2).

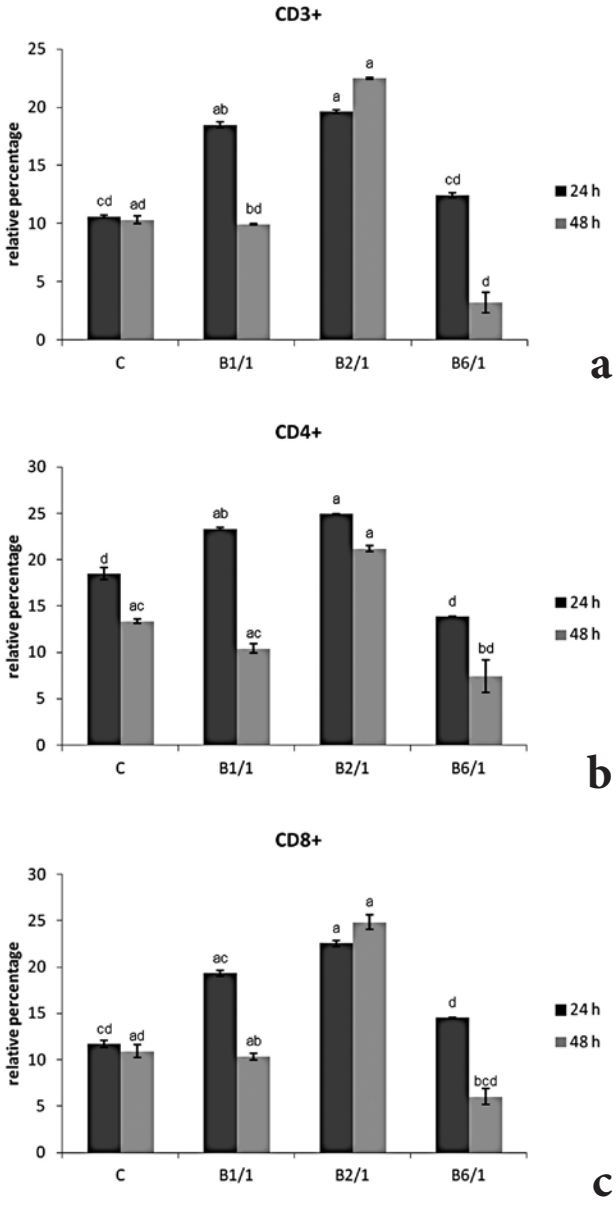


**Fig. 2. Relative percentage of the determined lymphocyte' subpopulations a) CD3+; b) CD4+; and c) CD8+. Means with different superscripts are significantly different abP < 0.05; acP < 0.01; adP < 0.001**

**Table 2. Antimicrobial activities of lactobacilli towards indicator bacteria. The results are presented as the arithmetic means of three measurements (mm) ± SD, Means with different superscripts are significantly different abP < 0.05; acP < 0.01; adP < 0.001**

| *Lactobacilli* | Indicator strains | | | | |
|---|---|---|---|---|---|
| | ***S. aureus* CCM 4223** | ***B. cereus*** | ***S. Typhimurium* CCM 7205** | ***E. coli* O149 F4** | ***pH after* 48 h incubation** |
| ***L. reuteri* B1/1** | 13.33±0.58a | 34.00±0.00 | 25.00±1.00d | 40.33±2.08a | 3.46±0.05 |
| ***L. reuteri* B2/1** | 9.00±1.73c | 34.67±2.08 | 54.33±0.58a | 32.00±1.00b | 3.52±0.06 |
| ***L. reuteri* B6/1** | 15.33±0.58a | 34.67±1.15 | 31.00±1.00d | 28.00±3.61c | 3.69±0.02 |

## DISCUSSION

This study was mainly focused on the evaluation of the immune properties and antimicrobial activities of new lactobacilli isolates from pheasant guts.

The most significant stimulating effect on the expression of all pro-inflammatory interleukins and MIP-1β was found in the group that was administered *L. reuteri* B1/1 mainly 24 h p.i. Similarly, H o f f m a n n et al. [19] reported that *L. reuteri* 100-23 significantly induced the expression of IL-1, IL-6 and MIP-2 in intestinal epithelial cells of mice. Interestingly, *L. reuteri* B6/1 did not induce the expression of the IL-1β gene in PMBCs. For the initiation of inflammation IL-1β represents a key cytokine. Moreover, IL-1β plays an important role in the regulation of MIP-1β expression and thereby contributing to the maintenance of inflammation [38]. Likewise IL-15 induces the expression of chemokines and their receptors on T-cells. IL-15 improves the proliferation of cytotoxic and helper T cells, and directs heterophils and NK cells to the sites of inflammation [34].

We observed a rise in the relative percentage of T cells including CD3+, CD4+ and CD8+ lymphocytes in the groups with *L. reuteri* B2/1 and *L. reuteri* B1/1 as compared to the control 24 h p.i., and this indicates the ability of new lactobacilli isolates to stimulate the required cellular immune response. It is known, that the individual strains of *Lactobacillus* spp. are able to induce the production of Th1 proinflammatory cytokines [21], while other strains induce the production of Th2 regulatory anti-inflammatory cytokines [30]. In agreement with a previous statement, in subsequent studies it would be appropriate to confirm this effect in *in vivo* conditions.

L i n et al. [27] demonstrated that *Lactobacillus reuteri* 6475 suppressed the activity of AP-1 transcript factor, which regulates the expression of pro-inflammatory cytokines in response to TLR activation. In another study, *L. reuteri* GMNL-263 reduced serum MCP-1, TNF, and IL-6 levels in mice fed with a high fat diet [20]. Taken together, the studies indicate that several strains of *Lactobacillus* can keep the balance in Th polarization (Th1/Th2), thereby creating better conditions for inflammation control [22].

Conversely, no stimulatory effect of *Lactobacillus reuteri* B6/1 on the relative expression of cytokines, chemokine and percentage of T cell subpopulations were observed. Several authors noticed, that certain isolates of *Lactobacillus reuteri*, affected neither: colonic microbial composition in piglets [28], IgA concentration and cytokine levels in saliva [4, 24] nor the incidence of nosocomial diarrhoea, including rotavirus infection [35]. We assumed that the final effect of the *L. reuteri* strain that was used in this study is also dependent on the real concentration of antimicrobial substances. In any case, the production of antimicrobial substances is essentially involved in the antimicrobial activity of lactobacilli; but their concentration could be different among certain isolates [15].

The antimicrobial activity is one of the most important criteria for selecting a probiotic strain against potentially harmful pathogens. *Lactobacillus* strains are unique in their production of antimicrobials substances against pathogens. Their antimicrobial activity is mainly due to the production of hydrogen peroxide, organic acids, especially lactic acid and acetic acid, and bacteriocins [33, 25].

According to our results, the tested lactobacilli strains inhibited the growth of both gram-positive and gram-negative indicator bacteria probably by reducing the pH of the environment as a result of organic acid production. After 48 hours of lactobacilli incubation, the pH ranged from 3.46—3.78 which is unsuitable for most pathogenic bacteria. In addition, the lipophilic and undissociated form of lactic acid and acetic acid allows these molecules to exhibit antibacterial character through the penetration of the bacterial membrane. Undissociated acid fractions diffuse passively across the membrane and subsequently ionize depending on intracellular pH, causing the cytoplasm to acidify and form inhibitors [29]. We noted a difference in the size of the inhibitory zones among the individual strains. Gram-positive Bacillus cereus was more sensitive than *S. aureus* CCM 4223. The sensitivity of *E. coli* O149 F4 and *S. Typhimurium* CCM 7205 to the lactobacilli tested was approximately the same. Likewise, B i l k o v á et al. [1] referred to *S. aureus* as the bacteria that are most resistant to *L. reuteri*, *L. murinus* and *L. mucosae probiotics* compare to *Yersinia enterocolitica* and *Listeria monocytogenes*, which were sensitive to the antimicrobial activity of lactobacilli. G e o r g i e v a et al. [13] tested the inhibitory activity of the 23 lactobacilli strains by the disc-diffusion method, which demonstrated their antimicrobial activity against pathogenic strains of *S. aureus*, *Bacillus cereus* and *E. coli*, with the majority of strains recording a correlation between the acidification intensity (pH) and the inhibitory zone size. D e A n g e l i s et al. [6] confirmed antimicro-

bial activity against *S. aureus* and *E. coli* in *L. plantarum* and *L. reuteri* (isolated from swine feces). The antimicrobial activity of *L. rhamnosus* against *S. Typhimurium* was demonstrated as a result of lactic acid accumulation [8] and pH reduction [12].

## CONCLUSIONS

In summary, *L. reuteri* B1/1 and *L. reuteri* B2/1 showed promising stimulatory effects on the relative expression of pro-inflammatory interleukins, MIP-1β and the percentage of T cell subpopulations *in vitro*. On the flip side, *L. reuteri* B6/1 suppressed the relative expression of selected interleukins and MIP-1β and did not affect the percentage of T cells. Overall, selected lactobacilli strains inhibited the growth of both gram-positive and gram-negative indicator bacteria. These conclusions show the importance of individual testing of each probiotic strain for its various effects on the immune status of the host, mainly for their wide uses throughout the fermented dairy, food, and meat processing industries. In addition, it is necessary to confirm their properties also *in vivo* conditions, because they may not be the same. Based on the results, *L. reuteri* B1/1 was selected for a subsequent *in vivo* experiment in broiler chickens infected with *Campylobacter jejuni* CCM6189, which is currently in progress.

## ACKNOWLEDGEMENTS

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# SEROLOGICAL SURVEY AND ASSOCIATED RISK FACTORS ON *TOXOPLASMA GONDII* INFECTION IN GOATS IN MILA DISTRICT, ALGERIA

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## ABSTRACT

***Toxoplasma gondii* is a protozoan parasite prevalent in humans and other animals worldwide having medical and veterinary importance on account of reproductive failure causing significant socioeconomic losses. The aims of this study were to estimate the seroprevalence of *T. gondii* infection in goats, determined the possible risk factors associated, and evaluate the performances of the latex agglutination test (LAT) to anti-*T. gondii* antibodies screening using the indirect Enzyme-linked immunosorbent assay as a reference test (iELISA). A total of 184 serum samples from goats reared on 25 farms in Mila district from North-Eastern Algeria were collected and tested for anti-*T. gondii* IgG antibodies using two commercial serological tests (ELISA and LAT). A seroprevalence rate of 71.73 % and 63.58 % was obtained by both ELISA and LAT tests, respectively. The analysis of some factors thought to be related to the onset of this infection such as age, sex, management system, locality and presence of cats showed no significant relationship ($P > 0.05$); these factors did not seem to affect the frequency of the infection. The seropositivity level of *T. gondii* was significantly higher in aborted goats ($P = 0.007$), which suggested that they may play a significant role in pregnancy failure. In the concordance evaluation between the two serological tests (ELISA and LAT), the Cohen's Kappa value was calculated and the results showed a K of 0.519 ($p = 0.000$) belonging to the range of 0.41—0.60 indicating just average agreement. The results of the Mc Nemar test showed that both tests gave significantly different results and seropositivity values ($P < 0.05$). The high prevalence observed in this study indicated a widespread exposure to *T. gondii* from goats and the potential risk of *T. gondii* infection for humans in North-Eastern Algeria. These results elucidate**

**the challenges of applying serology to estimate goat exposure to *T. gondii*. The choice between the two serological tests will depend on their performances, as well as the availability of the equipment, laboratory conditions and the number of samples to be tested.**

**Key words: Algeria; ELISA; goat; LAT; performances; risk factors; *Toxoplasma gondii***

## INTRODUCTION

Toxoplasmosis is a cosmopolitan zoonotic disease caused by a protozoan parasite, *Toxoplasma gondii*. It is of economic importance for both veterinary and human medicine [34]. The infection of sheep and goats causes significant losses in reproduction, but also has a major impact on public health, as the consumption of infected products such as undercooked meat and unpasteurized milk facilitates its zoonotic transmission [11]. Fetal mortality rates including goat abortion in the affected flocks can reach 50 % and in subclinical cases, losses are low [55].

Goats are economically important animals in many countries and constitute one of the main sources of meat and milk for Islamic populations and villagers [53]. For example, villagers' consumption of unpasteurized goat milk due to their cultural traditions and dietary habits is an important source of *T. gondii* infection [40]. Seroprevalence studies have been conducted on different animal species in different parts of the world [11, 38]. There are very few reports on the prevalence of toxoplasmosis in goats in different parts of Algeria.

The importance of *T. gondii* disease for public health and the lack of epidemiological data in humans and domestic animals in Algeria led us to conduct this cross-sectional study on goat farms. This study allows us to estimate the prevalence of this infection and to know the main risk factors associated.

Also, the evaluation of serological tests becomes important in order to ascertain the most sensitive and specific tests in epidemiological studies to use in different animal species [51, 72]. In this regard, the objective was also to evaluate the performance of two serological tests (ELISA and LAT) in the detection of anti-*T. gondii* antibodies in goats.

## MATERIALS AND METHODS

### Study area and the environment

This study was carried out in the province of Mila, in North-Eastern Algeria, from January to April 2017. Mila lies inland about 82 km from the Mediterranean coast. The wilayate (district) is characterized by a varied relief and presents two large distinct zones; to the north, mountains, and hills: M'sid, Aicha, Zouagha and El-Halfa, and to the south, the plains and highlands with an area of 3481 km2.

Our study was conducted in two municipalities in the North of this wilayate; the municipality of Terrai Bainen and that of Zeghaia. The elevation was 185 to 1190 m. The north of this region was home to three large cork oak forests, to the west, there was a large forest of exploitation, and between the two towns is the Beni-Haroun Dam; the largest water dam at the national level which supplies a large part of Eastern Algeria with drinking and irrigation water.

The region has a Mediterranean climate with hot, dry summers and cold, wet winters. The climate is humid in the North, subhumid to semi-arid in the center and semi-arid in the South. The rainfall varies between 600 and 900 mm in the North of the wilayate (920 mm on the mount of Msid Aicha), between 400 and 600 in the centre and less than 400 mm in the South. In the summer, the temperature varies between 25 and 40 °C and the average winter temperatures range from 0 to 12 °C [60].

The breeding season usually starts in July, when daylight begins to decline. The rearing mode is usually semi-intensive or extensive. All herds included in this study grazed during the spring season until the end of December with variations depending on the climatic conditions; these goats feed almost exclusively outside the barn without supplementation. In the winter, goats were housed and fed with straw, barley, and wheat bran. The association between goat and sheep farming is very common in this region.

### Study plan and the target population

A cross-sectional study was conducted. According to the data from the Algerian Ministry of Agriculture, there were 8652 herds and about 34 000 goat heads at Mila during the study period. An appropriate number of goats aged 4 to 96 months were sampled by a simple random sampling method.

Also, the number of goats to be taken from each farm

was defined based on the total number of animals. The important thing was to have a representative sample of at least 10 % of all individuals in each farm visited.

The required sample size was calculated according to the following formula with an expected prevalence of 10 % and a 95 % confidence interval [70]:

$$N = [Z2 \times P(1 - P)]/d2$$

Where;

- N is the number of samples to be collected in the study,
- Z is the value of the normal distribution for the confidence interval of 95 % [Z2 = (1.96)2],
- P is the expected prevalence,
- d is the absolute error of 10 %.

A minimum of 100 samples was required. To increase the power of the statistical analysis, the sample size was multiplied by 2. Twenty-five herds were randomly selected and the herd sizes ranged from 5 to 120 heads.

At the individual level, the sample size was determined for each flock to detect the existence of the disease. The calculations were performed according to the formula commonly used in veterinary epidemiological surveys by Thrusfield [72]:

$$n = [1 - (1š - p)1/d] \times [N - (d/2)] + 1$$

Where:

- n is the size of the sample in each flock,
- p is the detection probability of least one seropositive goat,
- N is the size of the flock,
- d is the number of seropositive goats in the herd.

The probability of detecting at least one seropositive goat in a flock was 95 % (p = 0.95), while the number of seropositive goats in each flock (d) was calculated assuming that the herd was equal to 10 %. Finally, 10 to 30 blood samples were collected from each flock, making a total of 210 goats.

**Collection of epidemiological data**

A pretested structured questionnaire was administered to each farmer under the supervision of the principal investigator before taking any blood samples. The measurable data on herd size, sex, age, breed, history of abortion, presence of cats and management system had been collected previously. The farms sampled consisted mainly of goats of local breeds (100 %). The sample (184 goats tested serologically) included 129 females (70.10 %) and 55 males (29.89 %). The animals were divided into three age groups: ≤ 12 months (24.45 %), 12 to 36 months (29.34 %) and more than 36 months (46.19 %). Of the females selected, 73 (56.58 %) had a history of abortion.

**Collection of the blood samples**

The blood (5 ml) was drawn from the jugular vein of selected goats using disposable needles and plain vacutainer tubes and transported to the laboratory on ice. Serum samples were harvested by centrifugation at 2,000 × g for 10 min, then stored in labeled Eppendorf tubes at −20 °C until testing.

**Laboratory analysis**

The detection of anti-*T. gondii* antibodies was conducted in the Microbiology Laboratory of the National Veterinary School of Algiers (ENSV). After the thawing of the sera at room temperature, it proceeded to the serological analyzes of the 184 sera using two commercial serological kits: the ELISA ID Screen® Toxoplasmosis Indirect Multi-species (IDvet, Grabels, France) and the LAT Toxo-Latex® (SPINRER EACT, S. A. Ctra Santa Coloma, Spain) according to the manufacturer's protocol.

**ELISA (Enzyme Linked Immunosorbent Assay)**

The IgG antibodies against *T. gondii* in the sera were tested by the ELISA test following the instructions of the ID Screen® Toxoplasmosis Indirect Multi-species kit manufacturer. In this procedure, a volume of 10 µl of the test samples was applied to the wells of the 96-well ELISA plate except for 4 wells reserved for the positive and negative controls. Finally, the optical densities at 450 nm were recorded under the ELISA reader.

The ELISA test detects IgG antibodies against the *T. gondii* P30 antigen. For the interpretation of the results, the percent S/P (S: Sample, P: Positive control) was calculated with the following formula:

$$S/P\ \% = (OD \text{ of the sample}/OD \text{ of the positive control}) \times 100$$

where OD is the optical density.

If S/P is less than or equal to 40 %, the result is considered negative; if S/P is between 40 % and 50 %, the result is considered doubtful; if S/P is greater than or equal to 50 % and less than 200 %, the result is considered positive; if S/P is greater than or equal to 200 %, the result was strongly positive. The test was validated if the mean value of the OD of the positive control was greater than 0.350 (OD > 0.350) and the ratio of the OD of the positive controls (DOcp) to the mean of the OD of the negative controls (DOcn) was greater than 3.5.

**LAT (Latex Agglutination Test)**

The TOXO-Latex reagent is a suspension of polystyrene latex particles coated with *Toxoplasma gondii* soluble antigen. The latex particles allow visual observation of the antigen-antibody reaction. If the reaction occurs, the latex suspension changes and clear agglutination becomes evident due to the presence of antibodies at a concentration greater than 4 $IU.ml^{-1}$. The reagents are checked until they reached room temperature, then shaken gently to disperse the latex particles. Fifty µl of the diluted serum (1 : 10) were pipetted into a circle of the plastic slide, 25 µl of latex beads coated with *T. gondii* soluble antigen (TSA) were added, mixed and shaken for 4 minutes at room temperature using a rotator.

A positive result is presented with a formation of an opaque circular veil whose diameter is equal to or greater than half the diameter of the well. This formation of the veil is explained by the agglutination which must be interpreted as a presence of anti-*T. gondii* IgG. The agglutination model was read in comparison with the positive control (provided in the commercial kit). The data thus collected were analyzed statistically.

**Statistical analysis**

Data from the field and laboratory surveys were captured and coded with Microsoft Excel® 2010 and analyzed using SPSS 20 for Windows software (SPSS Inc., Chicago, IL, USA). Descriptive statistics were used to summarize the data. The questionnaire survey information was used to define the explanatory variables to be tested in a logistic regression model for any association assessment between goat serological status (dependent variable) and risk factors (independent variables).

Taking into account the ELISA results, these factors were analyzed using a univariate model, so the odds ratio (OR) and 95 % confidence intervals (95 % CI) were used to quantify the association between these risk factors and *T. gondii* infection. Differences in the prevalence of *T. gondii* with different variables such as sex, age, management status, presence of cats, history of abortion and locality were analyzed using a Chi-square test. The differences were considered statistically significant and highly significant at $P < 0.05$ and $P < 0.01$, respectively.

The seroprevalence results obtained by the ELISA and LAT tests were compared using the chi-square test and their concordance was determined by calculating the Kappa coefficient. Using ELISA as a reference test, the sensitivity (Se), the specificity (Sp), the positive predictive value (PPV) and the negative predictive value (NPV) of the LAT test were calculated and interpreted. The agreement with kappa values of 0.00 to 0.20 was considered light, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 substantial and 0.81 to 1.00 almost perfect [45].

## RESULTS

**Seroprevalence in goats**

Our investigation showed that specific anti-*T. gondii* IgG antibodies were detected in the sera regardless of the technique applied. An overall prevalence of *T. gondii* of 71.73 % (95 % CI; 64—79.4 %) and 63.58 % (95 % CI; 56.43—70.20 %) were detected by ELISA and LAT tests, respectively, from the sera of 184 goats. Of the 132 ELISA positive sera, 27 (20.45 %) were negative at a dilution of 1 : 10 in the LAT assay. The results of the serological analysis are summarized in Table 1.

**Seroprevalence at the flock level**

Of the 25 flocks of goats, 24 (96 %) (95 % CI, 83.30—99.92 %) and 25 (100 %) (95 % CI; 78.58—99.21 %) tested positive with ELISA and LAT, respectively, so they contained at least one seropositive goat. On seropositive farms, the seroprevalence of *T. gondii* varied from 0 % to 100 %. Using the indirect ELISA test, each farm housed at least one seropositive animal. In six (06) herds (25 %), all of the animals sampled were positive for *T. gondii*. On the remaining farms, different proportions of animals reacted positively; the majority (29.16 %) being seropositive from 60 to 66.66 %.

**Risk factors for *T. Gondii* infection**

Data collected from farms using a questionnaire were analyzed to identify the potential risk factors. The seroprevalence of *T. gondii* in male and female goats was 69.1 % and 72.9 %, respectively (Table 1), suggesting that the seroprevalence in of the female goats were slightly higher than that in males, but the difference was not statistically significant (P > 0.05). According to the age, the prevalence values were 68.9 % in kids, 70.4 % in young goats, and 74.1 % in adults; these values did not differ significantly.

The results showed that no statistically significant difference was observed between the prevalence of *T. gondii* infection and the different factors, such as sex, age, location, rearing method and presence of domestic cats (P > 0.05); the history of abortion was significantly associated with seropositivity to *T. gondii* (Table 1).

**Comparison of seroprevalence results of LAT and ELISA tests**

Overall, the 184 serum samples were tested by both the iELISA (1/100 dilution) and LAT (1 : 10 dilution) kits for comparison. The agreement between the two methods (ELISA and LAT) was determined by the use of the Cohen Kappa test which showed a K coefficient of 0.519 (P = 0.000) belonging to the range 0.41—0.60, which corresponded to an average agreement between these two techniques (Table 2).

A full agreement between the two tests was obtained on

**Table 1. Analysis of factors likely to influence the risk of *T. gondii* infection taking into account the results obtained by the ELISA test.**

| Variables | Number sampled | Number positive [%] | 95 % CI on prevalence | Odds Ratio [OR] | 95 % CI on OR | P-value |
|---|---|---|---|---|---|---|
| **Age** | | | | | | |
| ≤ 12 months | 45 | 31 (68.9) | 54.34—80.47 | 0.93 | 0.39—2.2 | 0.792 |
| 12—36 months | 54 | 38 (70.4) | 57.17—80.86 | | | |
| > 36 months | 85 | 63 (74.1) | 63.91—82.24 | | | |
| **Sex** | | | | | | |
| Male | 55 | 38 (69.1) | 55.97—79.72 | 0.83 | 0.42—1.66 | 0.602 |
| Female | 129 | 94 (72.9) | 64.62—-79.8 | | | |
| **Management system** | | | | | | |
| Extensive | 19 | 12 (63.2) | 41.04—80.85 | 0.64 | 0.24—1.74 | 0.383 |
| Semi-intensive | 165 | 120 (72.7) | 65.48—78.95 | | | |
| **Presence of cats** | | | | | | |
| Yes | 145 | 107 (73.8) | 65.5—82.1 | 1.58 | 0.74—3.34 | 0.235 |
| No | 39 | 25 (64.1) | 54.3—82.9 | | | |
| **Town** | | | | | | |
| Zéghaia | 101 | 70 (69.3) | 58.5—80.1 | 0.76 | 0.4—1.47 | 0.419 |
| Terrai Bainen | 83 | 62 (74.7) | 63.9—85.5 | | | |
| **Abortion** | | | | | | |
| Yes | 73 | 60 (82.2) | 71.88—89.29 | 2.99 | 1.34—6.68 | 0.007* |
| No | 56 | 34 (60.7) | 47.63—72.42 | | | |
| **Total** | **184** | | | | | |

OR—Odds Ratio; CI—Confidence Interval at 95 %; *—significant; P-value is significant at P ≤ 0.05

**Table 2. Comparison of the LAT technique with the indirect ELISA as a reference test in the detection of *T. gondii* infection in goats**

| *Toxoplasma gondii* | ELISA indirect | | LAT | |
|---|---|---|---|---|
| | | | (+) | (-) |
| Goats | (+) | 132 | 105 | 27 |
| | (-) | 52 | 12 | 40 |
| | **Total** | **184** | **117** | **67** |
| **Intrinsic values** | Sp = 76.92 %<br>Se = 79.54 %<br>K = 0.51 | | Mc Nemar test (P < 0.05)<br>Ra = 78.80 % | |

Se—sensitivity; Sp—specificity; Ra—relative accuracy; K—Kappa value

105 (79.54 %) positive samples and 40 (76.92 %) negative samples. However, only 27 and 12 sera were positive for ELISA and LAT respectively.

The comparison of the results of the two tests by the calculation of the Mc Nemar test value indicated that the seropositivity differed significantly between the two tests (P = 0.024). Considering the ELISA test as a reference test, the sensitivity, specificity and positive and negative predictive values of the LAT test were calculated as follows: 79.54 %, 76.92 %, 89.74 %, and 59.70 %, respectively.

## DISCUSSION

Toxoplasmosis has a very wide geographic distribution and is considered to be one of the most common parasitic infections of man and other warm blooded animals. Clinical symptoms of toxoplasmosis are not specific. Therefore, the evaluation of serological tests becomes important in order to use sensitive and specific tests in serological surveys [51, 72]. The prevalence of Toxoplasmosis across the world is variable, with prevalence rates from 0 to 100 percent in different countries [54, 68], depending upon their customs, traditions, lifestyles of the inhabitants, weather conditions, age of the animals and husbandry practices [64]. Apart from this, the prevalence rate may also be associated with the presence of cats that excrete oocysts, which after sporulation become infectious to man and other animals [20]. *T. gondii* infections are widespread in some food animals, especially chickens, camels, pigs, sheep, and goats which represent the most consumed animal species in Africa for their meat, and there is a wide disparity between the levels of infection in different animal species [19].

We have hereby reported for the first time, the evidence for *T. gondii* infection and its seroprevalence, risk factors and the serological performance of two different tests in the humid agro-ecological area of Algerian goats. The overall prevalence of 71.73 % and 63.58 % of *T. gondii* antibodies by the ELISA and LAT tests were detected respectively. The results of our study have demonstrated that *T. gondii* infections are widely present in goats in the northeastern area of Algeria. This might indicate actively circulating, recently acquired or recrudescence of previously acquired *T. gondii* infections in goats due to climatic stress, malnutrition and prevalent diseases like brucellosis which reduce the animal's resistance [55]. Previously, *T. gondii* infections in goats were also reported from the central parts of the country [17] and neighboring countries like Tunisia and Libya [6, 44]. However, the high prevalence in goats of this study might be a function of the cumulative effects of the age, related to the absence of regular culling programs [67]. Moreover, the inadequate attention by the government, a local change in landownership and increased farming which necessitate cat keeping to control rodents, might have additionally contributed to the high prevalence.

At the international level, the seroprevalences obtained in this study by both tests were higher than the global average [23, 26, 43]. According to the technique of ELISA, the prevalence rates recorded in our study were high but lower than the level of infections (95.24 %) found in Turkey [10], and in Brazil (92.4 %) [33]. T e s h a l e et al. [69] found an almost similar prevalence (74.8 %) in the goats of Ethiopia.

Thus, it was found to be close to that reported in Zimbabwe (68.59 %) [35]. The prevalence rate in this study regardless of the test applied was higher than those reported by several authors in different areas of the world; prevalence rates of 59.4 % in Giza, Egypt [8], 52 % in Pakistan [66], 51 % in Saudi Arabia [59], 46.0 % in Brazil by the IFAT test [14], 42.8 % in Granada and Carriacou by the MAT test [16], 35 % in southern Tunisia by the MAT test [44], 31 % in Uganda [11], 27.9 % in Satun Province, Thailand [40], 25.4 % in Pakistan [56], and 14.1 % in China [78]. The differences in the prevalence reported by all of these studies could be accounted for based on the host, breed, sex, farm size, environmental and climate conditions, management practices, and density of cats and wild felids [5, 18, 32, 54, 78]. Thus, the variation might be due to the difference in sample size, cut-off values and sensitivity in the serological tests employed [19, 42]. In the study area, goats are one of the most important animals for the production of meat and milk. Attention should be payed to the milk as well as the meat from these animals as they are considered a potential source of human toxoplasmosis especially as the results demonstrated that goats are highly infected [62].

The prevalence of *T. gondii* was 11.64 % (34/184) and 13.35 % (65/184) in males and in females, respectively. The prevalence in females was higher than in males, but the difference was not significant ($P > 0.05$). This supports the findings of previous reports [9, 41, 76]. Some reports indicated that female animals were more infected by *T. gondii* than males [3, 69]. Our results did not coincide with two other studies [12, 24], which indicated that the prevalence of anti-*T. gondii* antibodies was significantly higher in females than in males. This may be explained by the fact that hormonal differences between males and females play an important role in determining the susceptibility to parasitic infection [57]. Thus, the immunity in females may be reduced by various factors such as pregnancy, nutrition, and lactation [49, 50]. Moreover, Zhao et al. [78] indicated that males were more infected than females. On the other hand, Bisson et al. [11] and Cavalcante et al. [15] observed that sex was not a significant factor in determining the exposure to *T. gondii* infection in goats.

It is widely accepted that animals acquire Toxoplasma infections with the acquisition of age through the ingestion of infective oocysts from the environment [28, 29]. The age of animals is considered to be an important factor in determining the prevalence rate of Toxoplasmosis in animals [25]. Although no statistically significant differences were found in this study, this indicated an equal chance of contracting the infectious agent regardless of the age of the animal ($P > 0.05$). Besides, Ahmad et al. [1] and Chikweto et al. [16] reported a significant increase in seroprevalence with age in small ruminants. The high prevalence obtained in the older age groups may be due to the cumulative effect of age, the long exposure period to infectious oocysts in the environment [69], and low immunity following aging of the immune system [57]. Contrary to our findings, several authors indicated that age influences seropositivity in ruminants [3, 46, 73, 74, 77]. Some studies have recorded, consistently with results of our study, that age does not affect the *T. gondii* infection [7, 22, 31, 43].

In Mila Province, goats are raised extensively in large farms or semi-intensively by individual families. The current study found a higher seroprevalence of *T. gondii* in goats in both the extensive and semi-intensive management systems, but these showed a lower prevalence than those in the extensively reared. The difference was not statistically significant ($P > 0.05$). These findings are consistent with those reported by Ahmad et al. [1] and Neto et al. [53] suggesting that extensive management in sheep and goats presents a greater risk of *T. gondii* infections. The increases in the prevalence of toxoplasmosis in extensive and semi-intensive practices have also been found in small ruminants from other countries [48, 73, 75]. However, Younis et al. [77] have shown that seroprevalence has been significantly increased in goats exploited in intensive systems than in extensive and semi-intensive operations, consistently with those recorded by Al-mabruk et al. [3] and Tzanidakis et al. [71]. As compared to extensive or semi-intensive management, animals raised intensively were usually caged and received little chance to ingest contaminated food and water by the oocysts of *T. gondii* excreted by cats [4].

Since animals reared under extensive conditions are pastured in comparatively large grazing areas, it is likely that these animals are exposed to *T. gondii* oocysts at a high level and that the oocysts may be highly dispersed but this would depend on the number of cats.

The ingestion of *T. gondii* oocysts may become more likely because storage of fodder may increase the abundance of other intermediate hosts of *T. gondii* such as rodents on the farm and thus also the abundance of cats because farmers often keep cats to get rid of rodents. More-

over, the use of bulk feed or pasture also posed a threat of getting toxoplasmosis. Both practices render animals to come closer in contact with oocysts shed by wild and domestic felids.

In this study, and as found by Ibrahim et al. [37], a statistically insignificant difference in seroprevalence was observed between the two localities (Terrai Bainen and Zéghaia). It might be linked to uniformity of climate that is mild and moist, providing good conditions for the sporulation of *T. gondii* oocysts. This is consistent with the results of previous studies [1, 58]. The high prevalence in goats is probably due to animal, plant and human pressures on the land. As long as the region receives high rainfall, it is more arable and has more households per unit area. Therefore, it probably has a higher percentage of domestic cats, which increases the risk of environmental contamination by *T. gondii* oocysts. The locality factor and essentially climate factor are judged as factors that predisposes and promotes a high susceptibility to infection with the protozoan *T. gondii*. The high humidity and significant vegetation cover characterizing this area protects oocysts from desiccation and promote their survival and sporulation. Also, the diversity of animal species (herbivore, carnivore, and birds) found in these localities ensures a perfect continuity of the parasite's evolutionary cycle in nature and thus its perennial nature. The influence of the environment and wildlife in the epidemiology of toxoplasmosis has been documented by several authors [20, 30, 68].

Felids, in particular the cat, are the only known animal that can excrete environmentally resistant oocysts, playing an important role in the epidemiology of toxoplasmosis [21]. Previous risk factor studies found the presence of cats on farms as a putative risk factor for the transmission of the *T. gondii* infection and increased seropositivity in small ruminants [47, 48, 53]. Interestingly, variables that addressed the presence of cats were specifically associated with these factors. A reason for "the presence of cats" not being a risk factor in this study may be the presence of large numbers of stray cats all over the countryside. In agreement with these results [37, 63, 65], there is no statistically significant association between the presence of cats and the seroprevalence of *T. gondii* in small ruminants. Cats become infected by consuming their prey, especially small rodents, or even birds whose meat contains bradyzoite cysts [18, 39]. These prey are frequently observed around farms in the study area.

The high seroprevalence observed is evidence of a strong environmental contamination by oocysts, which could be due to several factors in combination; on the one hand, the density of domestic cats in the region, the resistance of oocysts excreted by these cats in the wild in relation to the humid climatic conditions favorable to their survival and their sporulation, and which remain viable in the soil for months or years, and the long and repeated contact of goats with the parasite in relation to the frequent grazing pattern [38, 68].

*T. gondii* infection in small ruminants is not only relevant due to the zoonotic aspects, but also because it is an important cause of ovine and caprine abortion [13, 21, 39]. In our study, Toxoplasmosis was highly significantly increased in aborted goats 60/73 (82.2 %) compared to those with normal births 34/56 (60.7 %). These results were in agreement with those reported by other researchers [2, 36, 61, 77]. According to many authors, the highest prevalence was reported on farms with epizootic abortions [22, 52]. Contrary to our findings, *T. gondii* seropositivity in goats was not statistically significantly associated with the proportion of abortions relative to the number of animals [71].

The evaluation of serological tests is becoming increasingly important in order to be able to use sensitive and specific tests dedicated to epidemiological investigations. The difference in *T. gondii* antibodies screening in goats, by the use of the two serological tests (ELISA and LAT) in our study, may be due to the type of antigen used and the class of antibodies measured. We compared the efficacy of the LAT with that of ELISA (reference test). The calculation of Cohen's kappa coefficient showed a value of 0.59 indicating an average agreement between the two tests. The LAT test showed specificity and sensitivity of 76.92 % and 79.54 %, respectively.

Younis et al. [77] revealed a sensitivity and specificity of the LAT tested in sheep of 87.5 % and 76.5 %, respectively, compared with biological assays in cats. Figueiredo et al. [29] reported that there is a high positive and significant correlation of the IHAT compared to the ELISA, and ELISA compared to the IFAT. Fereig et al. [27] compared to the efficacy of the TgGRA7-based iELISA with that of LAT (reference test). The recorded kappa values were 0.682 in goats indicating a substantial agreement between the two tests. The choice of test will depend on the availability of the equipment, the laboratory conditions and the number of samples to be tested.

## CONCLUSIONS

In summary, the results of our study, for the first time, in the northeastern of Algeria, confirmed that *T. gondii* is endemic and that the infection is widely distributed in goats in the area. Extensive management was associated with higher odds ratios for being seropositive for *T. gondii* in goats. Humans can become infected by *T. gondii* through the ingestion of oocyst-contaminated food, water, or undercooked meat. The enhanced prevalence of *T. gondii* antibodies in goats suggested a high contamination of the environment by *T. gondii* oocysts. This indicated that the consumption of raw meat and milk of those animals in Mila province may represent a potential risk factor for human infections.

However, to better understand the nature and significance of these interactions, additional clinical data are needed as well as the isolation and molecular characterization of this etiological agent. Such data are essential to elucidate the relative importance of the various sources of infection for humans and goats and to achieve measures to prevent the infection. As a result, further work is needed to assess whether the soil and water on goat farms or in other regions of Algeria are contaminated by *T. gondii* oocysts.

Our data demonstrated that the serological assays studied in the present investigation can be very useful. Since the utilization of reliable methods for determining the prevalence of *T. gondii* infection in goats is available, it will allow adequate management and control of this infection and associated pregnancy failures.

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# MICROBIOLOGICAL QUALITY OF SLOVAK TRADITIONAL CHEESE

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## ABSTRACT

**The aim of this study was to investigate the microbiological quality of traditional Slovak "bryndza" cheese made in Slovakia. Besides the common pathogenic bacteria, we focused on the analyses of verocytotoxigenic *Escherichia coli* (VTEC), the occurrence of which has been analysed only occasionally in a few products. As we chose food of the highest risk which contained raw milk, we expected several positive findings. The presence of *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter* spp. was not confirmed. The enumeration of *Staphylococcus aureus* was more successful. In the case of VTEC stx and eae screening, the presence of genes producing verocytotoxins vtx1, vtx2 and the gene encoding virulent factor intimin—eae in nine samples by molecular-biological methods were revealed. Only one isolate, which carried genes vtx1 a vtx2 and did not belong to these serogroups: O157, O111, O26, O103, O145, or O104, was detected by confirmation assays.**

**Key words: microbiology; pathogenic bacteria; raw milk; traditional Slovak dairy products**

## INTRODUCTION

In Slovakia, "bryndza" belongs to one of the traditional products the consumption of which is common and profitable from different points of view. Many scientific studies [1, 3, 19] state its positive influence because of the original microflora the composition of which is unique. After the proper technological procedures, "bryndza" flora can even act against many pathogenic bacteria [4]. It belongs to the much-sought-for favourite products and as a traditional food, it is prepared for tourists, mainly in the Slovak regions of Liptov and Orava.

However, based on its composition (it is made either from sheep only products—100 % sheep unpasteurized cheese, or from raw sheep cheese and pasteurized cow cheese 1 : 1), it can be considered as one of the foods with the highest risk. For this reason it has been advised that

pregnant women, small children or immunosuppressive patients avoid these kinds of products [11].

The Slovak State Veterinary and Food Administration and other related bodies (district organisations, veterinary and food institutes) pay special attention to the official control of this traditional product. Every year many samples taken from the producers or from retail outlets are analysed to decrease the risk to the consumers who buy the food that poses potential risk. Even the producers themselves pay attention to guarantee the safety of their products within their own controls.

The aim of this study was to investigate the microbiological quality of traditional Slovak "bryndza" cheese made in Slovakia. In addition to the common pathogenic bacteria, we focused on verocytotoxigenic *Escherichia coli* (VTEC), the occurrence of which has been analysed only occasionally in a few products.

## MATERIALS AND METHODS

In our study we analysed the "bryndza" cheese produced from either untreated sheep cheese (21 samples) or the mixture (1 : 1) of raw sheep and cow cheese (4 samples). All samples were analysed in accordance with the legislative requirements [6] and were made only by the Slovak producers from different geographical areas (Fig. 1). Three samples were taken at the retail level, and the remaining ones were provided by the producers. Every producer provided only one sample per production so that the variability of product types were as wide as possible. The samples did not reflect the total amount of production.

The samples were analysed for the presence of pathogenic bacteria, such as *Salmonella* spp. [17], *Listeria monocytogenes* [16] and coagulase positive staphylococci (CPS) [12] and only three samples were analysed for the occurrence of *Campylobacter jejuni* [15]. In the case of exceeding a count of $10^5$ CFU.g$^{-1}$ for CPS, staphylococcal enterotoxin was detected (using the VIDAS equipment). All samples were analysed in parallel for the presence of verotoxigenic *Escherichia coli* [14]. Similar results from these kinds of samples have not yet been obtained in Slovakia because the official controls of bryndza were conducted only occasionally in relation to human cases.

If negative results were obtained at screening, the procedure was terminated. In the case of positive findings, the pooled colonies [22] were further analysed by conventional PCR and the serotypes were identified.

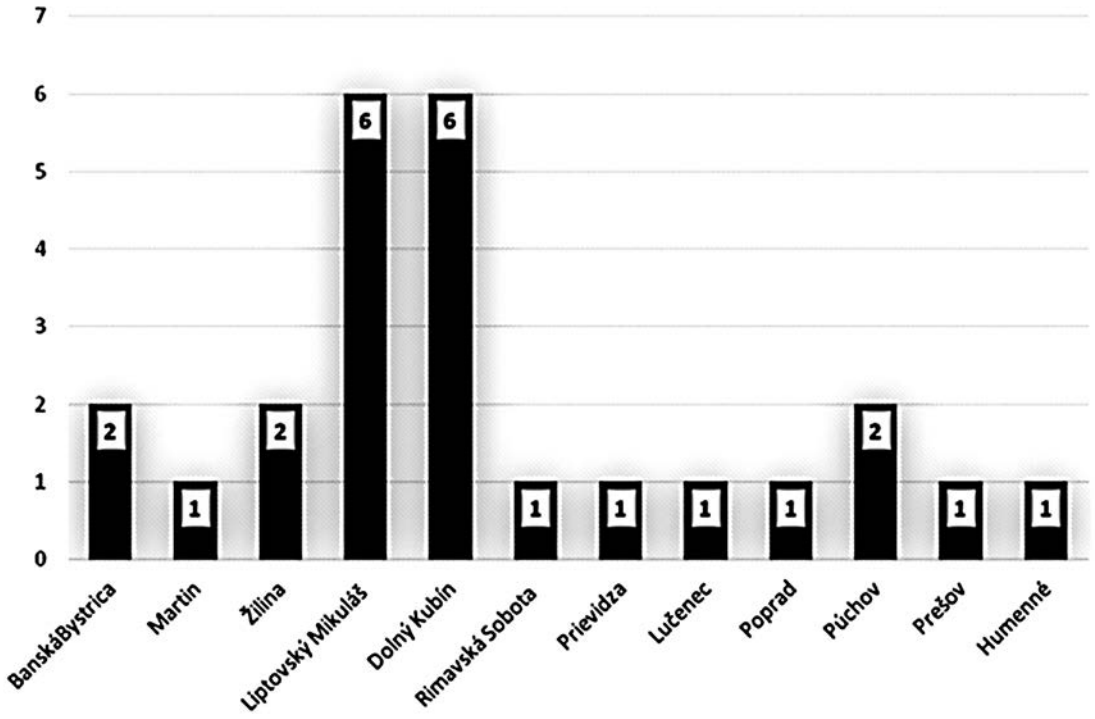


**Fig. 1. Number of producers involved according to the regional distribution**

The methodological procedure for the detection of the presence of verocytotoxin-producing *E. coli* (VTEC) was based on CEN ISO/TS 13136:2012 [14] and consisted of several steps. At first, incubation of the test portion was performed in a non-selective liquid nutrient medium—buffered peptone water (BPW) at 37 ± 1 °C for 18—24 hours. In the second step, 1 ml of microbial enrichment was transferred to an Eppendorf tube for isolation of the DNA [13]. The isolated DNA was subjected to a real-time PCR assay for the detection of virulence genes vtx1, vtx2 and eae in screening. The real-time PCR reaction was performed using Taq Man Universal PCR Master Mix (Applied Biosystems, Warrington, UK) in a thermocycler 7500 Real-time PCR System (Life Technologies, Applied Biosystems, USA). The results of the screening determined the next step of examination. The analyses of the negative samples with an absence of vtx genes were stopped. In the case of the presumptive detection of VTEC, the samples were subjected to the procedure for the determination of genes associated with serogroups. The positive colonies were analysed from pools by conventional PCR reaction [22] and then the serotype was characterised using real-time PCR.

## RESULTS

The absence of pathogenic bacteria indicated a high quality of the traditional unpasteurized products. Pathogenic bacteria (*Salmonella* spp. and *Listeria monocytogenes*) were not detected in any of the 25 samples. In the previous years, there was a monitoring programme conducted by the Veterinary and Food Institute (VFI) in Dolný Kubín that focused on *Campylobacter* spp. in the same kind of products [5]. It showed 3.41 % occurrence of *Campylobacter* spp. in unpasteurized dairy products. In our study, only three samples were analysed for *Campylobacter* spp. because the conditions to which the other samples were exposed were not suitable for this analysis. None of the 3 samples were positive for *Campylobacter* spp.

Based on the previous laboratory analyses of similar food matrix performed at our institute, high counts of CPS were expected. Increased levels were expected in correlation with summer temperature, poor storage conditions or problematic transport. Despite that, the results were better than expected (Tab. 1).

Cheese samples that according to the Commission Regulation 2073/2005 [6] exceeded the legal limit of 104 CFU.g-1 represented 4/25 (16 %). They were considered not to be in compliance with 2073/2005 and not suitable for human consumption. From these four samples there were two with counts exceeding 105 CFU.g-1. They were examined for the presence of staphylococcal enterotoxin and showed negative results.

The first screening of VTEC conducted in our study revealed the presumptive detection in nine samples (39 %) and also presumptive detection of enteropathogenic *E. coli* (EPEC) in three samples. EPEC generated the attaching and effacing lesions and carried the eae gene. Fig. 2 shows our results of VTEC screening.

The majority of the samples with the presumptive detection of VTEC belonged to a subset of enterohaemorrhagic *E. coli* (EHEC). This subset was characterized by the presence of vtx and eae genes and in this screening, six samples belonged to the EHEC group. In one case, the presumptive detection of vtx2 and eae was positive. In five samples the screening revealed the presence of both vero-

**Table 1. CPS counts detected in samples (CFU.g$^{-1}$)**

| Sample No. | Count | Sample No. | Count | Sample No. | Count | Sample No. | Count | Sample No. | Count |
|---|---|---|---|---|---|---|---|---|---|
| 1 | $1.4 \times 10^2$ | 6 | $1.0 \times 10^4$ | 11 | $< 1.0 \times 10^1$ | 16 | $1.3 \times 10^3$ | 21 | **$3.2 \times 10^5$** |
| 2 | $5.0 \times 10^3$ | 7 | $< 1.0 \times 10^1$ | 12 | $< 1.0 \times 10^1$ | 17 | $< 1.0 \times 10^1$ | 22 | $3.2 \times 10^2$ |
| 3 | $3.2 \times 10^2$ | 8 | $< 1.0 \times 10^1$ | 13 | $5.5 \times 10^2$ | 18 | $8.0 \times 10^3$ | 23 | $< 1.0 \times 10^1$ |
| 4 | $2.5 \times 10^2$ | 9. | $8.2 \times 10^3$ | 14. | **$1.5 \times 10^5$** | 19. | $3.0 \times 10^3$ | 24. | $3.0 \times 10^2$ |
| 5 | $< 1.0 \times 10^1$ | 10. | $4.0 \times 10^3$ | 15. | $8.0 \times 10^4$ | 20. | $8.2 \times 10^2$ | 25. | $9.1 \times 10^3$ |

Samples in bold were analysed for the presence of staphylococcal enterotoxins

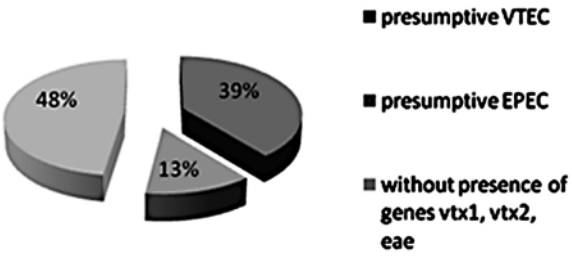


**Fig. 2. Results of PCR screening of cheese samples in liquid nutrient medium for VTEC detection (VTEC—erocytotoxigenic *E. coli*; EPEC —enteropathogenic *E. coli*)**

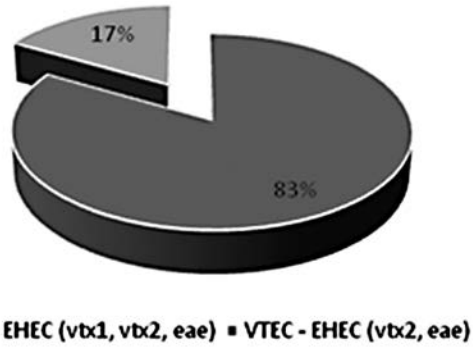


**Fig. 3. Percentage of identified genes of virulence in EHEC isolates, obtained from PCR screening of liquid nutrient medium with cheese samples for VTEC detection (VTEC-EHEC—verocytotoxigenic *E. coli* belonging to the subset of enterohaemorrhagic *E. coli*)**

cytotoxin genes vtx1 and vtx2 and intimin-coding gene eae (Fig. 3). These findings mean that in the test portion the presumptive detection indicated the presence of VTEC responsible for the attaching and effacing lesions on the gut mucosa.

The examination of pools from isolated colonies was used for the confirmation of the presumptive detection of genes vtx1, vtx2 and eae in cheese samples. These test assays were based on conventional PCR [22]. The incidence of vtx1 and vtx2 genes were confirmed in the pool made by the colonies. The eae gene encoding the virulent factor intimin was not detected. This is the usual finding. The pool consisted of ten colonies, but every colony could harbour different genes, combination of the genes, only one of them or none. This is the reason why we did not detect an isolate harbouring genes vtx1, vtx2 and also eae, but only the isolate harbouring genes vtx1 and vtx2. The isolated strain of *E. coli* underwent the identification of serogroups, but these did not belong to the highly pathogenic serogroups O157, O111, O26, O103, O145, or the serogroup O104.

## DISCUSSION

Cheese is generally considered a safe and nutritious food, but foodborne illnesses linked to cheese consumption have occurred in many countries. Many of these foodborne outbreaks were caused by the contamination with *Staphylococcus aureus. S. aureus* infections have been linked to the use of unpasteurized milk or to the contamination due to improper handling [8]. In our study we did not detect the staphylococcal enterotoxin. In the past 10 years there was only one sample of raw cheese positive for staphylococcal enterotoxin, type C.

The majority of the human outbreaks due to the consumption of raw milk cheeses were caused by *Salmonella* spp., followed by VTEC. The consumption of contaminated soft and semi-soft cheeses is often implicated in outbreaks with VTEC, especially when they are made from raw cow or goat milk [25]. In Slovakia, *Salmonella* spp. occurs usually in fresh poultry meat. In the dairy products investigated in 2014, two *Salmonella* serotypes were detected in positive samples: *Salmonella enterica* subsp. enterica serovar *Enteritidis* and *Salmonella enterica* subsp. enterica serovar *Montevideo*. These samples were of the same product type—traditional Slovak cheese made from raw sheep milk.

The most common pathogenic bacterium in Slovakia seems to be *Listeria monocytogenes*. This pathogen occurs not only in cheeses made from raw milk but also in those made from pasteurized milk (sheep, goat or cow, or in combination) as a consequence of failure in the pasteurization process [18]. This problem is linked to the handling and manipulation with the final product after heat treatment; although Ortenzi et al. [21] reported that *Listeria* counts decreased during the proper ripening and storage.

The most important results seem to be those obtained by the VTEC analysis. Baylis [2] mentioned that the prevalence of VTEC in products made from raw milk was very low. The outbreaks were caused mainly by enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC). However, VTEC was also responsible for some cases leading to the development of haemolytic uremic syndrome (HUS) in humans. The contamination of raw milk with VTEC is a significant problem not only for fresh cheese

curd, but also for hard ripened cheeses, because of the ability of VTEC to survive during the production procedure—periods of maturation. In 2002, during an outbreak in Canada, two strains of *E. coli* O157:H7 were isolated from unpasteurized Gouda cheese. One isolate was found in a 104 day old cheese [7].

Our results are comparable with those reported by authors from other countries. In Turkey, 2 % of Van herby cheese samples were positive for *E. coli* O157:H7 [24]. A study conducted in Italy revealed contamination in 7 % of the examined samples, but only three strains were detected. One isolate which originated from mozzarella cheese and two isolates from raw milk were positive for stx2 genes. Serotypes of these *E. coli* strains were not determined [20]. The results from Iran showed contamination of the traditional cheese by vtx2 and vtx1 in 120 (2.5 %) of the samples. In one of the cheese samples, the vtx1 gene was detected. Also, vtx2 gene was confirmed, in two of cheese samples [23]. According to EFSA data, thirty-six cheeses were positive for VTEC in 2016. Only one of these samples was E. coli O157 that originated from raw cow's milk. The majority of isolates belonged to the serogroup O26 [10]. Ten years ago, six *E. coli* O157 were found in cheeses. One strain was isolated in Slovakia from forty samples (2.5 %). Five Belgian strains were collected from soft and semi-soft cheeses made from unpasteurized cow's milk. Both of the countries tested samples only for the presence of serotype O157 [9] but no virulence genes were analysed at that time.

## CONCLUSIONS

The analysis of Slovak traditional bryndza cheese demonstrated the need for continuous monitoring of production procedures. Because of specific characteristics and the consumer's popularity of this cheese, systematic monitoring of its microbiological safety appears necessary. It is known that its composition and the original microflora show antimicrobial effects on the pathogenic bacteria. In our case, the most probable bacteria to survive in such a matrix seem to be coagulase-positive staphylococci.

With respect to VTEC results, they were the first obtained from the Slovak traditional food. Not only pathogenic *E. coli* were detected but also the presence of genes, associated with the production of verocytotoxins vtx1, vtx2 and eae, were investigated. In our study that included 25 samples, one VTEC isolate was detected (4 %). The screening revealed the presumptive detection of vtx1, vtx2 and eae genes in 56 % and the presumptive detection of vtx2 and eae genes in 11 %. From the point of view of food safety, this contamination would represent the highest risk to consumer's health. The contamination of cheeses by *E. coli* is not unique. Many findings have been described in Europe and also throughout the world. The practice of good manufacturing procedures is one of the most important measures in order to ensuring the production of safe foods. It is the right way to avoid or minimise the detrimental effects of pathogenic bacteria.

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# MORPHOMETRIC CHARACTERISTICS OF THE EXTREME EASTERN ALGERIAN DOMESTIC DONKEY (*EQUUS ASINUS*)

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## ABSTRACT

**The aim of this study was to define the morphometric variability of the extreme Eastern Algerian donkeys. The study was carried out from June to December 2018 in El-Tarf, Souk-Ahras, and Tebessa provinces. The study population involved 65 individuals (32 jacks and 33 jennies) between the ages of 3 and 16 years old. In total, 13 body measurements were used, and 5 zootechnical indexes were calculated. The quantitative and qualitative characteristics were studied in order to establish an ethnic and functional classification of this particular subspecies. The qualitative data demonstrated that the coat colour was variable. Bay and greyish colours were dominant with a respective rate of 61.5 % and 38.5 %, respectively. The head, the nose, as well as the eyes contour colours were mainly grey with 52.3 %, 58.5 % and 50.8 % respectively. The partial absence of the coat particularities was observed. The population presented a significate relationship between the quantitative characters ($P < 0.05$). The donkeys were longilinear, of a rectilinear profile, compact with massive trends. They had a hypermetric format. The animals are good for meat production. The General Linear Model (GLM) showed that the body measurements were variable by sex and body mass. The principal component analysis (PCA), the multiple correspondence analyses (MCA) and the ascending hierarchical classification (AHC) revealed that the population was composed of 2 clusters representing 4 animal classes. This study was the first report on the phenotypic characterization of donkeys in the extreme Eastern Algerian area, based on corporal measurements. The results indicated the existence of heterogeneity and suggested the possibilities of genetic improvement within the species.**

**Key words: Algeria; Body measurement; donkey; ethnology; improvement; phenotype**

## INTRODUCTION

Donkeys were domesticated in Africa since 5 000 years BCE [2, 24]. They were used to satisfy human needs in

transport and work, to allow the movement of people and goods, and to influence the organization of the first cities and pastoral societies [7, 42]. Their numbers decreased with the advent of motor vehicles.

The first genetic studies on mitochondrial DNA revealed that the African Wild Ass was the common ancestor of the present domestic donkey. Two different populations were described: *Equus africanus* and *Equus africanus somaliensis* [2, 19, 51]. However, the Maghreb domestic donkey has not yet been genetically identified; hypotheses suggest the possibility of belonging to the Atlantic donkey [36].

In Algeria, few studies have described the composition of the donkey population from the colonial era [5, 40, 46] to the present [1, 27]. The lack of data on the Domestic Animal Diversity Information System (DAD-IS) about the species in Algeria reflects the neglect and disinterest leading to the reduction of its population from 315 000 in 1961 to 136 000 in 2013 [37]. This study was conducted to contribute to a better understanding of the autochthonous donkey population in Algeria which may influence its preservation and genetic improvement. It may also serve as a reference for other work involving the Algerian and the Maghreb asinus species.

## MATERIALS AND METHODS

### Study area

This study was conducted from June to December 2018 in 3 wilaya (provinces) in the extreme East of Algeria: El-Tarf (36° 46' 02" N, 8° 18' 50" E), Souk-Ahras (36° 17' 15" N, 7° 57' 15" E) and Tebessa (35° 24' 00" N, 8° 07' 00" E) (Fig. 1).

### Ethical approval

Given the passive nature and the lack of harm to the animal's health and welfare, no ethical approval was required for this study.

### Studied animals

A morphometric description was carried out on 65 donkeys (32 jacks and 33 jennies) between the ages of 3 and 16 years old. The donkeys were divided into 3 age groups: 3—6 years (young); 6—9 years (adult); and >9 years old. The age of the animals was estimated by the observation of the dentition [25, 38]. The body condition scoring was based on the rating grid cited by Pearson and Ouassat [35], Vall et al. [47] and Svendsen [45]. The animals were used in traction work in the area of the study.

### Data collection

Data collection was derived from the approach developed by Lauvergne [27] and adapted by FAO [14] for qualitative and quantitative characteristics.

### Qualitative traits studied

Coat colour with its particularities, muzzle, head, mucous membranes, and eye border colours were described by direct observation of the animals in daylight [8, 10].


**Fig. 1. Study area**

**Body measurements and zootechnic indexes**

The body measurements were taken by the same operator in the morning. Height, width, and length measures were taken using a measuring rule. A measuring tape was used for the diameter and perimeter measurements. In total, thirteen (13) quantitative characteristics were measured for each animal. These concerned: the Withers Height (WH) measured from the highest point at the withers to the ground [29]; the Heart Girth (HG), the circumference measurement taken around the chest just behind the paws and behind the withers [16]; the Body Length (BL), the distance between the tip of the shoulder and ischium [16]; the Back Length (BkL), from the base of the withers to the base of the tail [16], the Cannon Circumference (CC), a metric tape was placed perpendicular to the axis of the cannon, four fingers below the lower part of the "Knee" joint [3]; the Cannon Length (CL), measured between the metacarpal IV head and the distal end of the metacarpal [3]; the Neck Length (NL), measured between the cranial edge of the atlas wings and the apex of the scapula [3]; the left and right ear length (LEL-REL); the Rump Width (RW), the distance between the ilia [3]; the Rump Height (RH), the distance between the rump and the ground [3] ; the diameter between the Ribs (DR); and the Head length (CLH), which was measured on the midline between the top of the occipital region and the tip of the nose [3] (Fig. 2).

From the linear measurements, 5 corporal indexes developed in horses and adapted for donkeys were calculated [8, 10, 15]; they were defined in the following formulas. The Profile Index (PI) = WH/BL and the Body Index (BI) = BL/HG made possible the distinction between brevilinear, mediolinear and longilinear conformations. The animals were small (< 1) or medium (= 1); or fit for work traction. They could also have long conformation (> 1); good animals for speed [30, 31]. The Metacarpothoracic index (MTI) = CC/CW allowed defining three animal types: hypermetric, eumetric, and ellipometric animals [6]. The Compactness Index (CI) = BW/WH [4, 12] and the Height in Front of- Behind HFBI = WH/RH [13, 28]. The (CI) defined animals with or without weight overload; FBH ≤ 1: straight back (no overload) or HFBI > 1: the anterior region was higher than the posterior (overload). The body weight (BW) of each animal was calculated according to two validated formulas: BW1 = HG2.826/4434.7 [11] and BW2 = (HG2.575 × HW 0.240)/3968 [4].

**Statistical analysis**

Normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test) were checked for all the variables. Variables without parametric test requirements were log-transformed to meet the assumptions of the analysis.

Principal component analysis (PCA) collapsed the 18 inter-correlated variables into independent vectors. The test reduced the dimensionality to a small number of representative and uncorrelated variables. Also, it prevented multi-collinearity.

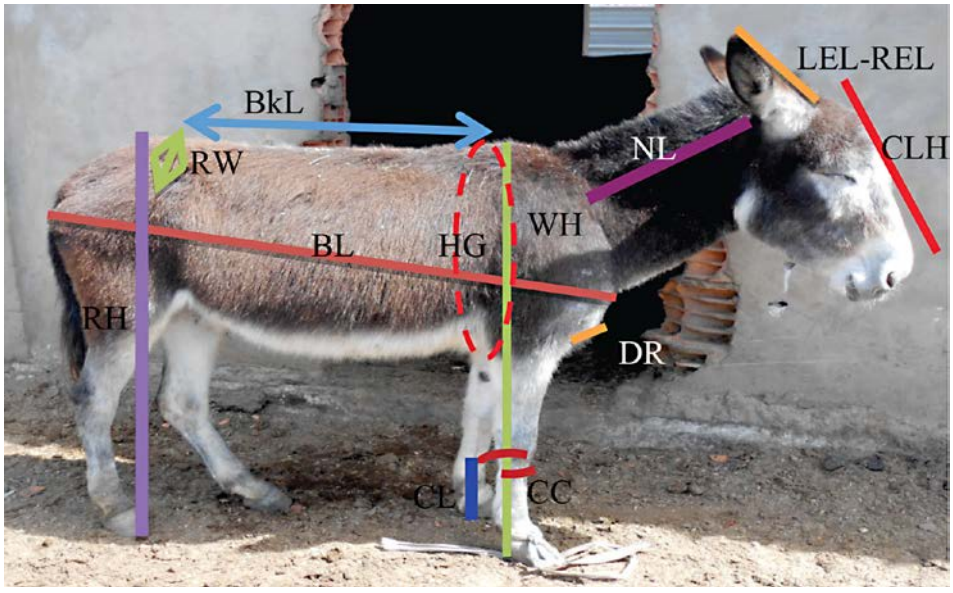


**Fig. 2. Body measurements**

Multivariate analysis of variance (MANOVA) with the General Linear Model (GLM) procedure was conducted. It assessed the variability of the defined independent vectors by sex, age, body mass, and their interactions. When significant overall F-test values were identified in each MANOVA, we compared the two post-hoc procedures, Scheffé method for univariate F [18].

Factorial analysis (AFC) was used for the qualitative variables. That permitted the differentiation of the donkeys and the construction of a typology, which consisted of identifying individuals who were relatively similar to each other to present common qualitative characteristics. Finally, to obtain the optimal number of groups, an ascending hierarchical classification (CHA) was used. These tests were processed by the SPSS (version 17) and the R software.

## RESULTS

### Qualitative traits studied

For the donkeys studied, two main coat colours were observed: the bay colour (61.5 %) with its shades (light, dark and burnt) and the grey colour (38.5 %) with its shades (light and dark). The head, muzzle and eye contour colours were predominantly grey with 52.3 %, 58.5 %, and 50.8 %, respectively. Data analysis revealed a partial absence of the coat particularities, especially the back stripes and the zebra marks (100 %).

### Quantitative traits studied

The donkeys had dolichocephalic head, length was greater than width (CLH = 40.79 ± 4.65 cm) with longer ears (REL = 26.06 ± 2.23 cm, LEL = 25.88 ± 2.05 cm) (Table 1).

**Table 1. Descriptive analysis of the body measurements in the extreme Eastern Algerian donkeys**

| Traits | Min | Max | Mean ± SD (n = 65) | SE | Var. |
|---|---|---|---|---|---|
| **HG** [cm] | 101.00 | 131.00 | 114.94 ± 6.78 a | 0.06 | 0.25 |
| **BL** [cm] | 103.00 | 135.00 | 115.76 ± 7.23 a | 0.11 | 0.75 |
| **HW** [cm] | 94.00 | 132.00 | 110.15 ± 8.49 a | 0.07 | 0.28 |
| **NL** [cm] | 26.00 | 52.00 | 39.62 ± 5.38 a | 0.84 | 45.96 |
| **REL** [cm] | 21.00 | 32.00 | 26.06 ± 2.23 a | 0.90 | 52.27 |
| **LEL** [cm] | 22.00 | 32.50 | 25.88 ± 2.05 a | 1.05 | 72.03 |
| **CLH**[cm] | 24.00 | 50.00 | 40.79 ± 4.65 a | 0.67 | 28.91 |
| **CL** [cm] | 10.00 | 22.00 | 14.07 ± 2.28 b | 0.28 | 4.97 |
| **CC** [cm] | 11.00 | 17.00 | 13.93 ± 1.33 C | 0.25 | 4.20 |
| **BkL** [cm] | 51.00 | 107.00 | 69.95 ± 10.11 b | 0.58 | 21.62 |
| **RW** [cm] | 27.50 | 40.00 | 32.76 ± 3.03 e | 0.28 | 5.21 |
| **DR** [cm] | 19.00 | 33.00 | 25.93 ± 2.99 e | 0.17 | 1.77 |
| **RH** [cm] | 96.00 | 137.00 | 114.02 ± 9.25 a | 1.25 | 102.17 |
| **BW1** [kg] | 104.08 | 217.04 | 151.30 ± 25.45 a | 0.38 | 9.17 |
| **BW2** [kg] | 108.95 | 227.33 | 158.83 ± 26.77 a | 0.37 | 8.92 |

Values with different alphabet letters across the row for each parameter were significantly different at $P < 0.05$. (HG) Heart Girth, (BL) Body Length, Withers Height (WH), (NL) Neck Length, (REL) Right Ears Length, (LEL) Left Ear length, (CLH) Head length, (CL) Cannon Length, (CC) Cannon Circumference, (BkL) Back Length, (RW) Rump Width, (DR) Diameter between the Ribs, (RH) Rump Height, (BW 1) Body Weight 1, (BW 2) Body Weight 2

**Table 2. Calculated zootechnical indexes**

| Indices | Min | Max | Mean ± SD (n = 65) | SE | Var. |
|---|---|---|---|---|---|
| **CI** | 0.95 | 1.18 | 1.05 ± 0.05 | 0.01 | 0.00 |
| **PI** | 0.84 | 2.48 | 0.98 ± 0.20 | 0.02 | 0.04 |
| **BI** | 0.37 | 1.10 | 1.00 ± 0.09 | 0.01 | 0.01 |
| **MTI** | 0.10 | 0.15 | 0.12 ± 0.01 | 0.00 | 0.00 |
| **HFBI** | 0.89 | 1.03 | 0.97 ± 0.03 | 0.00 | 0.00 |

(PI) Profile Index, (BI) Body Index, (MTI) Metacarpo-thoracic index
(CI) Compactness Index, (HFBI) Height in Front of- Behind

**Table 3. Body measurements according to the factors (sex and BCS)**

| Traits | According to sex | | P | According to BCS | | P |
|---|---|---|---|---|---|---|
| | **Male** n = 32 | **Fem ale** n = 33 | | **BCS = 3** n = 43 | **BCS = 4** n = 22 | |
| **HG** [cm] | 116.85 ± 7.18 | 112.97 ± 5.82 | * | 113.44 ± 4.66 | 117.52 ± 6.33 | * |
| **BL** [cm] | 116.85 ± 8.81 | 114.64 ± 5.02 | Ns | 115.82 ± 6.93 | 116.86 ± 6.94 | Ns |
| **HW** [cm] | 112.67 ± 9.90 | 107.55 ± 5.81 | * | 109.70 ± 4.59 | 111.55 ± 7.45 | * |
| **NL** [cm] | 40.45 ± 6.33 | 38.75 ± 4.09 | Ns | 36.61 ± 5.27 | 41.75 ± 4.31 | |
| **REL** [cm] | 26.58 ± 2.36 | 25.53 ± 1.99 | ns | 25.38 ± 1.14 | 26.73 ± 2.10 | ns |
| **LEL** [cm] | 26.36 ± 2.15 | 25.38 ± 1.84 | ns | 26.12 ± 1.22 | 26.52 ± 1.92 | ns |
| **CLH** [cm] | 41.33 ± 5.64 | 40.23 ± 3.33 | ns | 38.94 ± 7.73 | 41.86 ± 3.62 | ns |
| **CL** [cm] | 14.85 ± 2.16 | 13.27 ± 2.15 | ** | 14.46 ± 1.80 | 14.25 ± 2.54 | ns |
| **CC** [cm] | 14.38 ± 1.32 | 13.47 ± 1.20 | ** | 14.87 ± 1.44 | 14.09 ± 0.85 | ns |
| **BkL** [cm] | 72.21 ± 10.12 | 67.63 ± 9.70 | ns | 72.02 ± 11.74 | 71.18 ± 4.19 | ns |
| **RW** [cm] | 32.70 ± 3.38 | 32.83 ± 2.67 | ns | 34.07 ± 3.84 | 32.73 ± 3.44 | ns |
| **DR** [cm] | 26.00 ± 2.81 | 25.86 ± 3.21 | ns | 26.80 ± 3.52 | 26.36 ± 3.26 | ns |
| **RH** [cm] | 117.27 ± 9.70 | 110.67 ± 7.52 | ** | 112.88 ± 8.74 | 116.48 ± 9.66 | ns |
| **BW1** [kg] | 158.60 ± 26.79 | 143.78 ± 21.94 | * | 145.23 ±16.99 | 160.85 ± 25.04 | * |
| **BW2** [kg] | 166.75 ± 28.61 | 150.66 ± 22.34 | * | 152.82 ± 17.57 | 168.42 ± 26.18 | * |

(HG) Heart Girth, (BL) Body Length, Withers Height (WH), (NL) Neck Length, (REL) Right Ears Length, (LEL) Left Ear length, (CLH) Head length, (CL) Cannon Length, (CC) Cannon Circumference, (BkL) Back Length, (RW) Rump Width, (DR) Diameter between the Ribs, (RH) Rump Height, (BW 1) Body Weight 1, (BW 2) Body Weight 2; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: $P > 0.05$.

The body length (BL) was measured from the tip of the shoulder to the tip of the ischium. It was 115.76 ± 7.23 cm. The withers height was HW = 110.15 ± 8.49 cm and the rump height (RH) was 114.02 ± 9.25 cm with the highest variation (102.17 %) compared to the other measurements (Table 1). The HG was 114.94 ± 6.78 cm (Table 1). The BW average was 158.83 ± 26.77 kg, it varied between 108.95 kg and 227.33 kg. Our results showed a highly significant correlation between HG and BW ($P < 0.01$). There was a significant correlation between the quantitative characteristics ($P < 0.05$).

Both the Profile and the Body indexes ($PI \approx 1$ and $BI \geq 0.90$) allowed us to organize the population as longilinear shaped, whereas the pelvic index ($HFBI \leq 1$) indi-

**Table 4. Body measurements in the extreme Eastern Algerian donkeys according to age**

| Traits | 3 ans6 ans | 6 ans—9 ans | > 9 ans | P |
|---|---|---|---|---|
| | n = 28 | n = 16 | n = 21 | |
| **HG** [cm] | 116.46 ± 7.56 | 111.69 ± 5.38 | 114.94 ± 6.78 | ns |
| **BL** [cm] | 116.79 ± 7.89 | 113.81± 6.51 | 115.76 ± 7.23 | ns |
| **HW** [cm] | 110.54 ± 8.58 | 108.13 ± 8.14 | 110.15 ± 8.49 | ns |
| **NL** [cm] | 38.89 ± 6.46 | 40.56 ± 4.59 | 39.62 ± 5.38 | ns |
| **REL** [cm] | 26.50 ± 2.17 | 25.78 ± 2.25 | 26.06 ± 2.23 | ns |
| **LEL** [cm] | 26.20 ± 2.17 | 25.81 ± 1.56 | 25.88 ± 2.05 | ns |
| **CLH** [cm] | 41.18 ± 5.86 | 40.38 ± 2.55 | 40.79 ± 4.65 | * |
| **CL**[ cm] | 14.77 ± 2.30 | 12.66 ± 1.60 | 14.07 ± 2.28 | ns |
| **CC** [cm] | 14.18 ± 1.21 | 13.44 ± 1.17 | 13.93 ± 1.33 | ns |
| **BkL** [cm] | 70.64 ± 9.59 | 67.13 ± 6.44 | 69.95 ± 10.11 | ns |
| **RW** [cm] | 33.20 ± 2.90 | 32.13 ± 3.48 | 32.76 ± 3.03 | ns |
| **DR** [cm] | 26.21 ± 2.77 | 25.06 ± 3.73 | 25.93 ± 2.99 | ns |
| **RH** [cm] | 115.89 ± 9.44 | 110.81 ± 8.74 | 114.02 ± 9.25 | ns |
| **BW1** [kg] | 157.30 ± 28.93 | 139.07 ± 19.00 | 151.30 ± 25.45 | ns |
| **BW2** [kg] | 164.70 ± 30.17 | 146.43 ± 20.50 | 158.83 ±26.77 | ns |

(HG) Heart Girth, (BL) Body Length, Withers Height (WH), (NL) Neck Length, (REL) Right Ears Length
(LEL) Left Ear length, (CLH) Head length, (CL) Cannon Length, (CC) Cannon Circumference, (BkL) Back Length
(RW) Rump Width, (DR) Diameter between the Ribs, (RH) Rump Height, (BW 1) Body Weight 1
(BW 2) Body Weight 2; * P < 0.05; ** P < 0.01; *** P < 0.001; ns: P > 0.05

cated that it was a rectilinear population. The HW was less important than the RH.

According to the dactylo-thoracic index, the donkeys were hypermetric (MTI > 0.1). They were compact with heavy tendencies. They could not even bear loads of their own weight (CI > 1). The results indicated that the body mass of the donkeys was greater than their size; they were overweight (Tables 1 and 2).

The multivariate analysis showed that the morphometry of the donkeys was variable by both sexes ($\lambda = 0.68$, $F = 3.75$, $p = 0.004$) and body status (BCS) ($\lambda = 0.80$, $F = 1.98$, $p = 0.004$). On the contrary, the variables were not affected by age ($\lambda = 0.75$, $F = 1.23$, $p = 0.27$). The interactions (sex × age), (sex × BCS), (BCS × age) and (sex × age × BCS) did not have any effects on the variation of the different measures performed. The interactions expressed the respective statistical values ($\lambda = 0.90$, $F = 0.44$, $p = 0.94$), ($\lambda = 0.97$, $F = 0.23$, $p = 0.97$), ($\lambda = 0.79$, $F = 1.02$, $p = 0.43$) and ($\lambda = 0.89$, $F = 0.47$, $p = 0.93$).

According to sex and BCS; Silhouette measurements (HG, BL, HW, NL, REL, LEL, CLH and RH, BW1 and BW2) were positively correlated with each other. There were highly significant differences between the two sexes ($F = 9.91$, $p = 0.003$) and BCS ($F = 7.54$, $p = 0.008$). Males expressed higher body measured values than females. They were generally heavier than females (Table 3).

Body measurements and zootechnical indexes related to the skeleton (Cl, BKL), proportions (PI, BI), format (CC, MTI), width (RW, DR), and corpulence (CI, HFBI) were not affected by sex, BCS or by the interaction between those factors ($P > 0.05$).

According to age, animals aged between 6 and 9 years old expressed lesser length and width than other animals with a lower HG and HW and a shorter head (CLH) (Table 4). According to the statistical study, age and its interactions with the other factors of variations (sex × age), (BCS × age) and (sex × age × BCS) didn't have any effect on the difference of the morphometry or the zootechnical indexes ($P > 0.05$).

### Genetic variability of the population

The CPA performed on the body measurements yielded a cumulative effect on the first two axes expressing a total inertia rate of 63.73 %. The statistical analysis revealed that those two axes presented respective rates of 52.19 % and 11.54 % of the total inertia, which is statistically insufficient to explain the information (Table 5).

To find more meaningful representation, a hierarchical analysis (AFCM) was indispensable. It made possible the distinction between two clusters explaining 92.46 % of the information with a rate of 86.80 % on the first dimension and 5.66 % on the second one. The hierarchical clustering on the factor map illustrated the relationship between the clusters (Fig. 3). It appeared that, while confirming the structure population, the phylogenetic tree had to establish the relationships between the genetic types. The extreme Eastern Algerian donkey population was structured about four genetic types: A, B, C and D. A and B were closer together while C and D were distant. The results revealed some heterogeneity in the population.

## DISCUSSION

### Animal's description

In mammalian species, their hair coat properties reflect their health and welfare directly [33]. Despite its great phylogenetic proximity, the asine species have very different phenotypes from those of the horse [28]. Their diversity may be due to the migration of donkeys throughout the African continent [23]. The Extreme Eastern Algerian donkeys have diversified coat colour with a predominance of bay and grey colours (61.5 % and 38.5 % respectively). The results were correlated with the description given by A y a d et al. [1] for the donkeys in the Kabylie area (Centre of Algeria) where the animals were mostly bay (46 %) and grey (19 %). L a b b a c i et al. [26] reported a dominant brown coat colour of the donkeys of Tlemcen (West of Algeria) with a rate of 65.6 %. Our population was compared to the description of the African donkey *Equus asinus* nubicus (*Equus asinus africanus*) [39]. In addition, our observations were comparable to those obtained in the Bulgar-

**Table 5. Eigen values (Total Variance explained)**

| Component | Initial Eigen values | | | Extraction of the sums of the squares | | |
|---|---|---|---|---|---|---|
| | Total | % of variance | % cumul | Total | % of variance | % cumul |
| **1** | 7.829 | 52.19 | 52.19 | 4.97 | 33.11 | 33.11 |
| **2** | 1.731 | 11.54 | 63.73 | 4.59 | 30.62 | 63.73 |

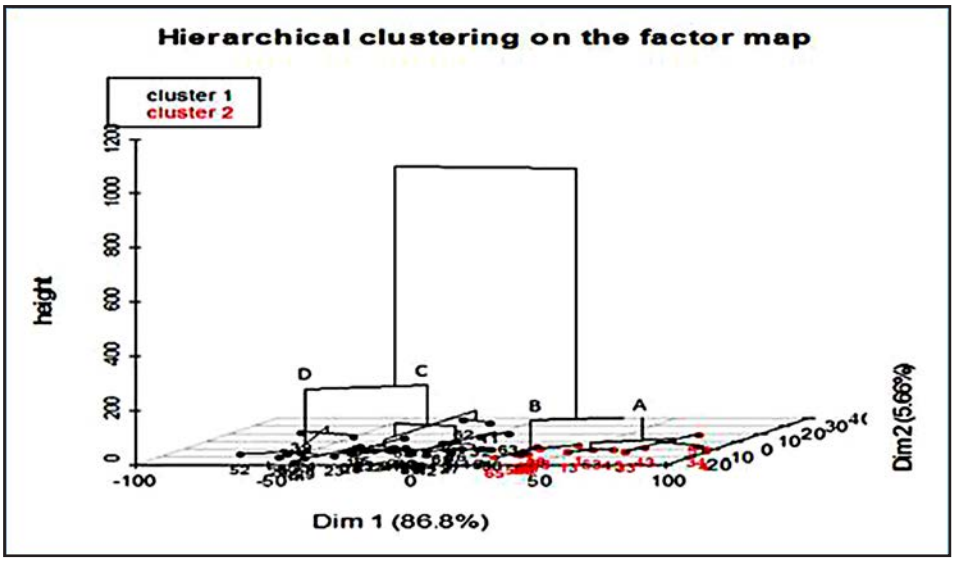


**Fig. 3. Hierarchical clustering on the factor map of the extreme Eastern Algerian donkey population**

ian donkey population [48]. Yilmaz and Ertuğrul [49, 50] reported mostly grey coloured donkeys in Turkey with a rate of 31.4 %. In addition, Gupta et al. [17] described a greyish colour dominance in the donkeys of the South-West region of Bihar (India).

**Body measurements and zootechnical indexes**

Cephalic measurements have been used to define the origins of species. Also, they have been used as the criteria for the characterization and the distinction between breeds [20]. The heart girth (HG) is the most commonly used parameter for the determination of the format of the animals [34, 35]. It reflects the development of the chest, and it's covering muscles [31]. The HG has also been used in the estimation of the animal's live weight (BW) for its lower variations. The circumference and length of the trunk may increase when the animal has a distended abdomen due to gestation or overfeeding [9].

The overall results yielded by this study were found to be closer to the donkeys described in the Kabylie area (Algeria) [1], Chad [8] and Turkey [50] (Table 6). However, they were higher than those raised in Mali and Niger [32] (Table 6). The averages obtained were, on the other hand, less important than those reported for the Tlemcen region (West of Algeria) donkeys [26], the Spanish Catalan donkeys [15] (Table 6), as well as those obtained in Catalan and Poitevine donkeys raised in Morocco [4]. Catalan donkeys were introduced in Algeria between the end of the 19th century and the beginning of the 20th century. Bulgarian donkeys were also relatively large compared to those described in our study [37, 48].

The significant correlations obtained between the quantitative characters ($P < 0.05$) for the population were also reported for the Turkish donkeys [49]. There were no significant differences recorded ($P > 0.05$) by Kefena et al. [23] and Defeu et al. [10] for eastern Ethiopia and

**Table 6. Comparative analysis of the exterior measurements with the results of other authors**

| Study area | North- East of Algeria | Kabylie (center) Algeria | Tlemcen (West) Algeria | Niger | Mali | Tchad | Spain | Turkey (South- East) |
|---|---|---|---|---|---|---|---|---|
| n | 65 | 126 | 61 | 281 | 292 | 408 | 98 | 194 |
| **Authors** | Current study | [1] | [26] | [32] | [32] | [8] | [15] | [50] |
| **HG** [cm] | 114.94±6.78 | 118.5±7.5 | 124.26±7.03 | 107.9±5.25 | 105.1±8.32 | 113.33±0.25 | 157.0±6.65 | 113.5±0.4 |
| **BL** [cm] | 115.76±7.23 | 110.1±5.9 | 157.26±12.88 | 100.3±5.89 | 104.8±7.81 | 109.32 ±0.30 | – | 105.2±0.5 |
| **HW** [cm] | 110.15±8.49 | 106.9 ±5.4 | 116.16±7.23 | 100.4±3.85 | 98.1±5.13 | 102.35±0.23 | 142.20±6.89 | 102.3±0.5 |
| **NL** [cm] | 39.62±5.38 | 46±4.7 | – | 35.1±4.41 | 28.4±2.19 | 46.18±0.19 | – | – |
| **REL** [cm] | 26.06±2.23 | 24.4±1.8 | 30.15±2.19 | 28.2±1.87 | 24.1±1.21 | 24.92±0.06 | 32.45±2.23 | 21.9±0.1 |
| **LEL** [cm] | 25.88±2.05 | 24.4±1.8 | 30.15±2.19 | 28.6±1.74 | 24.2±1.21 | 24.92±0.06 | 32.45±2.23 | 21.9±0.1 |
| **CLH** [cm] | 40.79±4.65 | 48.5±3.3 | – | – | – | 43.72±0.16 | 61.24±2.4 | 48.7±0.2 |
| **CL** [cm] | 14.07±2,28 | 21.07±1.72 | – | – | – | 12.56±0.04 | – | – |
| **CC** [cm] | 13.93±1.33 | 14.7±1.1 | 17.50±1.86 | – | – | 17.17±1.05 | 19.64±1.35 | 13.6±0.0 |
| **BkL** [cm] | 69.95±10.11 | 63.2±2.5 | – | – | – | – | – | – |
| **RW** [cm] | 32.76±3.03 | 32.4±1.6 | 37.15±3.21 | – | – | 36.60±0.25 | 42.28±2.26 | – |
| **DR** [cm] | 25.93±2.99 | 49.2 ± 1.94 | – | – | – | 18.36± 0.06 | – | – |
| **RH** [cm] | 114.02±9.25 | 109.6±4.8 | – | – | – | 106.18±0.41 | 141.80±7.73 | – |

(HG) Heart Girth, (BL) Body Length, Withers Height (WH), (NL) Neck Length, (REL) Right Ears Length, (LEL) Left Ear length, (CLH) Head length (CL) Cannon Length, (CC) Cannon Circumference, (BkL) Back Length, (RW) Rump Width, (DR) Diameter between the Ribs, (RH) Rump Height

the upland of Cameroon donkey's population's characters, consecutively.

The males registered higher body measurement values than females. This variation may be due to physiological differences and activity [22, 43]. The results were consistent with those obtained by K a b o r e [22], R o a m b a [41], J o h n [21] and N i n i n a h a z w e et al. [32].

The zootechnical indexes have been used to describe the structural characteristics, type, and performance of the animals [11, 12]. In our study, they were calculated and analysed to make ethnologic classifications for the extreme eastern Algerian donkeys.

The animals are longilinear, rectilinear, hypermetric and overweight. The body mass was greater than their size [30]. Those results suggested that the population was suitable for meat production. That was in concordance with the description of D e f e u et al. [10] for the uplands of north-western Cameroon donkeys.

**Genetic variability of the population**

The statistical analysis and the phylogenetic tree establish the relationships between 4 heterogeneous genetic types. D e f e u et al. [10] reported 3 heterogeneous genetic types in the uplands of north-western Cameroon donkeys. However, P a p a and K u m e [36] identified 2 genetic types in Albania. The heterogeneity may be due to the genetic stirring between different populations due to the movement of asine populations. This study area was an informal trade crossroads between Algeria and Tunisia, where the donkey was used as the most appropriate means of transportation. R o a m b a [42], K a b o r e [22] and S t a n i š i ć [44] found that the asine population of Senegal, Burkina Faso, and Balkan were homogeneous, respectively.

## CONCLUSIONS

This is the first report on the phenotypic characterization in donkeys in the extreme Eastern Algerian area, based on corporal measurements. The results indicated that heterogeneity exists and suggest that the possibilities of genetic improvement within the species may be possible. However, the molecular characterization would better identify donkey breeds in Algeria.


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# DOGS HEALTH RELATED TO EAR CROPPING

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## ABSTRACT

**Ear cropping is a controversial subject with both supporters as well as detractors. Some consider that the procedure is cruel and unnecessary, while others consider it to be routine and harmless. The cropping of the ears of dogs for cosmetic reasons has no medical merit. It is a cosmetic, surgical procedure performed to achieve a specific appearance that matches the desired image and sometimes the standard of certain breeds of dogs. Cosmetic surgery offers the dog the desired look, but it is not a necessity. This procedure must be performed by a licensed veterinarian. Those interventions cause pain and anxiety and, like all surgical procedures, are associated with the risk of anaesthesia problems, blood loss and infection. There is only a thin line between what is convenient for the animal and what is good. Almost every community has legislation on animal welfare which includes amputation issues. If something is considered normal in some countries, this does not necessarily mean that it is also moral. The lack of legal consequences may lead some owners to produce certain breeds of dogs to their liking, but they should not be allowed to cripple their dog for their own personal benefit. Amputation can only be performed legally by a veterinarian and a new problem arises. This is because there are veterinarians who refuse to practice that kind of intervention. Some of them may think that since the legislation is unclear, they might be charged with a crime.**

**Key words: cruelty; health problems; legislation; prohibited procedures**

## INTRODUCTION

Although controversial, dog "cosmetic surgery" continues to be performed and has reached epidemic proportions [14]. These procedures fall into different categories, from unethical disputes to undoubtedly criminal actions because they are mostly carried out solely to alter the physical appearance of a dog. Originally, these interventions were mainly performed for practical purposes to prevent injuries to working dogs, but the actual reason was mostly

cosmetic [14]. Ear cropping in the past had been practiced mainly to prevent ear damage during fighting and hunting [11]. Today the question of dog cosmetic surgery illustrates the delicate balance between animal welfare arguments and some "traditional" practices. Such a conflict depends in part on the absence of specific legislation which may lack clear details on these issues [14].

It has been demonstrated and discussed that ear cropping and tail docking from an aesthetic point of view will not easily find scientific justification unless it mediates a pathological condition that requires its use [6]. These procedures are usually done for cosmetic reasons or "tradition", or because owners want their dogs to have the characteristic traits of certain breeds. Such traditional aesthetic appearance is ultimately guaranteed by trimming [16]. The ear and tail cut are basically mutilations. However, although some serious infections have been associated with the presence of hanging ears, there is no evidence that ear cropping has any meaning as a prevention method [6]. In general, cutting or not cutting ears or tails is subjected to a fashion trend and it seems that each contradictory position may persist over time. The greatest contemporary ideas have been justified by aesthetics [6]. The position of rejection is also found in Small Animal Surgery, where surgeons believes that these practices are unethical and even illegal [6]. For many years, the practice has deviated from practical interests and has become an aesthetic matter affecting the natural characteristics of the animals [6]. The reasons for these surgeries are out of date. Standards of dog breeds and their "good looks" are currently considered groundless and unethical.

## COUNTRIES OF THE WORLD PERMITTING EAR CROPPING

Today, many countries ban cropping and docking because they consider these practices to be unnecessary, painful, cruel or mutilation. In Europe, the cropping of ears is prohibited in all countries that have ratified the European Convention for the Protection of Pet Animals [5]. Some countries that have not signed that Convention have developed their own laws prohibiting this procedure. For example, the Slovak legislation does not allow ear cropping as a cosmetic procedure. Due to the fact that tail docking and ear cropping can be a painful practices and those are prohibited in many European countries, they should not be carried out solely for cosmetic reasons. Australia and New Zealand have banned ear cropping [14]. However, ear cropping is still permitted in some countries [2] and basically it is permitted upon the choice of the animal's owner. The procedure may only be performed by a veterinarian.

Some countries (Canada, Brazil, Columbia, Russia, and Spain) have not completely banned ear cropping, which means that this procedure is carried out only in certain parts of the country for specific reasons. This type of surgery is completely unlimited in Afghanistan, Argentina, Bolivia, Chile, Egypt, Philippines, India, Indonesia, Costa Rica, Kuwait, Lebanon, Malaysia, Morocco, Mauritius, Mexico, Nepal, Panama, Peru, Sri Lanka, United States, and Taiwan.

## LEGISLATION ON EAR CROPPING IN THE EUROPEAN UNION AND WORLDWIDE

There are a number of limitations affecting these practices around the world. Many countries have adopted legislation that limits these practices and even prohibits them in some cases [9, 11]. Currently, the autonomous community's own legislation prevails. Every European country has laws on animal protections and also they concern the issue of ear cropping and docking as well. Organizations such as the American Veterinary Medical Association and the Canadian Veterinary Medical Association have issued official opinions disagreeing with these cosmetic practices. Generally, cropping and docking are forbidden solely for aesthetic (cosmetic) reasons, but many of them allow cuttings for health reasons. However, the European Union has not developed any legal rules concerning tail docking or ear cropping or other practical surgical procedures. The prohibition of such surgeries exists only in the "European Convention for the Protection of Pet Animals" (ETS No 125) [5] which is the international agreement of the European Council. The European Union Convention on the Protection of Pet Animals prohibits surgery, including docking of tails and ear cropping for non-medical purposes [18].

In Israel, the docking of tails is prohibited for cosmetic purposes, while in Scotland a total ban has been in place on tail docking since 2003. The cropping of dogs' ears is illegal in the UK. The current legislation in England and Wales has incorporated some articles of the European Convention for the Protection of Pet Animals into their

legislation. A total ban is applied in Australia and South Africa. In America, dogs' ear cropping is still being done today, regulated by American Veterinary Medical Association. This procedure is regulated mainly in Northeast America. There are currently nine states that specifically regulate the ear cropping of dogs. Connecticut, Maryland, New Hampshire, New York, and Pennsylvania all prohibit ear cropping. The exception is applied in case of a licensed veterinarian surgery when the dog is under an anaesthetic. Maryland law stipulates that the ear cropping must be appropriate on the animal. Illinois prohibits the torture of animals but makes an exemption for alteration of an animal done under the direction of a licensed veterinarian. Maine prohibits the mutilating of an animal by irreparably damaging parts of the body but makes an exemption for conduct performed by a licensed veterinarian. Massachusetts prohibits ear cropping except when performed by a licensed veterinarian and Washington prohibits ear cropping except when it is considered a customary husbandry practice [19].

The American Kennel Club publicly states that it "recognizes that ear cropping, tail docking, and dewclaw removal, as described in certain breed standards, are acceptable practices integral to defining and preserving the breed character and enhancing good health [1]. However, dogs with natural unmodified ears are not disqualified from entering dog shows [4]. Australia and South Africa have totally banned the procedure which they refer to as archaic, barbaric and useless [6].

In contrast, the American Veterinary Medical Association does not agree with ear cropping and tail docking of dogs when carried out solely for cosmetic purposes and encourages the removal of these procedures according to breed standards [15]. In Canada, ear cropping is prohibited except in two provinces, either by the provincial law or by the competent veterinary associations of the province. Ear cropping is not prohibited in Ontario and New Brunswick, although since 2014 the Canadian Veterinary Medical Association (CVMA) has issued a formal statement of attitude against ear cropping. CVMA does not agree with alteration of any animal by surgical or other invasive method for cosmetic or competitive purposes [4].

The veterinary associations and welfare organizations usually call for this procedure to be prohibited, while the Dog Breed Association and the Kennel Club strongly oppose anti-docking legislation [17]. Most animal welfare groups, as well as many veterinary associations, opposed dog ear cropping and tail docking for cosmetic purposes. Under the Federation Cynologique Internationale (FCI) jurisdiction, only the national club of the country of origin is entitled to change the breed standard.

The Australian Veterinary Association believes that cosmetic tail-docking and ear-cropping of dogs are unnecessary, unjustifiable surgical alterations and are detrimental to the welfare of animals. The American Veterinary Medical Association [15] encourages veterinarians to make dog owners aware of the risks and lack of medical benefits in this regard. Canadian Veterinary Medical Association [4] opposes surgical alteration of any animal for purely cosmetic purposes and recommends that breed associations change their breed standards so that cosmetic procedures are not required. Similarly, the Association of Veterinarians for Animal Rights opposes surgery of various kinds performed to meet 'breed standards' or to correct so-called vices [3]. Procedures such as ear cropping and tail docking in dogs are unacceptable because of the suffering and disfigurement they cause to an animal which are not compensated by any benefits to the animal. If any such procedure can be shown to be necessary for medical or human reasons, then it can be allowed. "Breed standards" in dogs must be altered to allow the animal to be exhibited without surgical mutilations. These laws cannot simply prohibit these practices, and treat them without being surgically mutilated [14]. In other words, there are no objective health reasons for ear cropping. Only the law is strong enough to create an effective ban on such practices.

## EAR CROPPING

Conventional ear cropping is defined as a set of surgical interventions in pets that are not well-founded from a veterinary medical point of view [14]. Ear cropping is a surgical procedure that is performed in some countries in puppies aged 7 to 12 weeks. In large breed puppies it is performed up to six weeks of age and in small breeds up to a maximum of nine weeks under general anaesthesia by surgical intervention [6]. Ear cropping is the surgical removal of the ear-flaps, cutting through the cartilage, skin, blood vessels and very sensitive nerve endings. This procedure must be performed by a veterinarian who already has the necessary experience and skills in that area.

## EAR CROPPING FOR THE HEALTH REASONS

It is possible that ear cropping may alleviate ear infections by increasing the airflow. The erect ear remains drier and it is less likely to develop a health problem. The most common problem is ear inflammation – Otitis externa. For a dog it is a very painful condition. Otitis externa is an inflammation of the auditory canal from the pinna to the tympanic membrane commonly observed in canine patients in small animal veterinary practice [12]. Inflammation can affect any dog, regardless of age or breed, although it is more likely to occur in dogs with long ears such as spaniels or retrievers, or in dogs with very hairy ears. The frequency of diagnosis was significantly higher in dogs with floppy ears, but it was not affected by ear hairiness [13]. These results confirm, at least in part, the findings of Hayes et al. [7] who first documented a significantly higher risk of otitis externa in dogs with hairy, floppy ears. Dogs with otitis externa can easily develop otitis media or, in very severe cases, the inner ear inflammation. Dogs with floppy ears are prone to a higher frequency of middle ear inflammation compared to breeds with erected ears, although the ears were not hairy [13]. The dog's ear canal is considerably longer than its human counterpart, and after extending downward, it makes a sharp turn inward toward the eardrum. The external ear canal is L-shaped, with the L lying on its side. The canal forms an almost 90-degree angle between its two sections: the short, vertical outer section and the longer, horizontal inner section [20]. The ear canal is lined with both apocrine (ceruminous) and sebaceous glands which, in the healthy animal, produce a protective coating of earwax (cerumen). Dogs with long, floppy ears may be at a higher risk of developing otitis media. Breeds with floppy ears are more prone, since air flow is limited and a warm, moist environment builds up, and it is ideal for yeast and bacteria to flourish and spread. Some bacteria (e.g., *Staphylococcus* spp., *Bacillus* spp.) and yeasts may occur in small amount also in healthy dogs [8]. A combination of different factors can lead to the developing of inflammation in dogs.

The shape of the ear also plays an important role. The pinnae of dogs vary greatly in their size and shape. The pinnae of some breeds are short and erect, while other breeds have long, floppy pinnae. Dogs with long floppy ears may be more susceptible to middle ear inflammation due to the auricles keeping warm moist air and foreign bodies in the ear canal, making them more susceptible to secondary factors. The ear flap, have their function; they help prevent water from entering the ear canal, and they can be afflicted with sunburn, frostbite and insect bites, or they can experience various forms of injury or trauma.

An auricular hematoma or othaematoma is a collection of blood underneath the perichondrium of the ear and typically occurs secondary to trauma in dogs. With trauma to the ear, the perichondrium and vasculature are damaged, causing separation from the underlying cartilage and results in a potential space for blood to accumulate. Some studies have reported that animals of different breeds, including urban individuals, with blunt or bowed atria are the individuals with the highest prevalence of otitis media and othaematoma [10]. The causes of external otitis include: parasites (e.g. ear mites), bacterial and fungal infections, allergies and other skin diseases, and tumours of the glands of the canal. Self-trauma to the pinna from scratching at the ear can result in bleeding between the cartilage and the skin of the pinna. Dogs that spend lots of time in the water, also are prone to developing ear infections. Other health problems that may arise are: adverse drug reactions, autoimmune diseases, necrosis (keratinization), excessive cleansing, or hormonal disorders which may lead to an ear environment suitable for the retention of yeast or bacteria. However, although the development of some serious infections may be related to the presence of floppy, hairy ears, there is no evidence that ear cropping is a proper preventive method [6].

## EAR CROPPING AS A SURGICAL PROCEDURE IN DOGS—IS IT NEEDLESS?

Surgery (ear cropping) was recommended in puppies (up to 12 weeks of age), due to less intense feeling of pain and trauma. After 4 months of age, this procedure was already considered painful, even if performed under full anaesthesia. If surgery was carried out after four months, satisfying results were not achieved because auricular cartilage would have already had its shape and definitive position at this age [6]. The ear cartilage becomes stronger in older age and causes pain after surgery. The upper ear part is surgically removed so that the cartilage stands upright. Before the procedure, dogs were given sedatives to relieve

pain. Ear cropping is usually performed under general anaesthesia because the ears are very sensitive to pain and they have many nerve endings.

The ears are disinfected before measuring and indicating which part of the ear has to be cut. The cutting wounds are sutured and cleaned. Surgery lasts 30 to 45 minutes. As with any surgical procedure, post-operative care is required, antibiotics and pain reducing drugs are administered. The post-surgical treatment lasts 10—14 days. The dog's ears are raised up to an upright position for 2—3 months. Several times a day they are disinfected, stuck up and the patient is placed under antibiotic treatment. Puppies who have undergone such a procedure need 6—20 weeks for convalescence. The ears are fortified with straps, bandages, or other aids to support the upright position of the ear. In the case of complications such as e. g. bleeding, swelling, or wound infection, the ears must be re-operated, which could result in the loss of the entire outer ear [6].

The use of new, more modern surgery procedures e. g. laser surgery, nowadays seems to be the best solution for health. Sections are more accurate with less tissue damage, bleeding is minimal to none, the surgical field is clear, allowing the surgeon to perform accurate surgery. The dog can be used relatively soon after the procedure for its normal activities. The laser also removes the nerve endings, thus reducing the pain. Because ear cropping is a surgical procedure and general anaesthesia is required, the procedure is generally carried out by a veterinarian, although it often may be attempted by breeders and dog owners who do it unskilful [14]. After surgery, the ear must be placed in the desired position until the muscles and cartilage have completely recovered to hold this position themselves [6]. Proper postoperative care is very important, but it is not a guarantee that the ears will have the desired shape.

## CONCLUSIONS

Ear cropping for cosmetic purposes is prohibited in most countries nowadays. In the past, this routine practice had its merit, especially in hunting or guard dogs, as the ears were an easy target for wildlife. At present, the indication for ear cropping is only therapeutic, never a cosmetic/aesthetic intervention. Ear cropping surgery is losing popularity in many countries as this procedure is painful and unnecessary. Risks, trauma and pain do not outweigh the value of changing the dog's physical appearance, nor increase its quality of life. Ear cropping is not scientifically justified unless there is a pathology that requires it. Otoplasty can have irreversible consequences on a dog's communication skills. Literally, a dog may be robbed of communication with other dogs and their owners. Dogs are able to express their emotional state, social status or willingness to fight by using their ears. Cropped ears can handicap the dog. The need for a "proper appearance" of a dog will be lost if the breeds standards that are required by the organization are eliminated and owners would not feel the pressure to change the physical appearance of the dog. Most European countries, including all countries that have ratified the European Convention for the Protection of Pet Animals, consider these practices illegal. It has been scientifically substantiated that ear cropping has no effect on improving the health of dogs suffering from external otitis. Advanced medical procedures nowadays increase the likelihood that if the dog suffers an ear inflammation, the veterinarian will not crop the ears for this reason.

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