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Contact: e-mail: folia.veterinaria@uvlf.sk

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REVIEW ARTICLE

MORPHOMETRIC EVALUATION OF THE HIP JOINT IN DOGS

Gabriela Kacková*, Slavomír Horňák, Mária Figurová, Nela Vargová, Veronika Tauberová
Small Animal Clinic, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia

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***Correspondence:** gabriela.kackova@student.uvlf.sk

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Hip dysplasia represents the most prevalent non-traumatic disease leading to lameness in dogs, which subsequently causes secondary joint pathologies such as arthrosis and arthritis. Diagnosis and selection for breeding use radiographic evaluation of the ventrodorsal projection of the hip joint. Early detection and treatment can halt or reverse the disease progression. In veterinary medicine, various radiological measurements are applied to evaluate such anatomical abnormalities. These measurements include the Norberg angle, percentage coverage of the femoral head, and various indices related to the acetabulum and proximal femur. The differences in measurements between breeds are significant and reflect differences in body conformation. Ventrodorsal radiographs are crucial for the diagnosis, screening and monitoring of hip dysplasia. Lack of reference values for morphometric measurements in dogs highlights the need for further research.

Key words: acetabulum; geometry; hip; proximal femur; radiography

INTRODUCTION

The most prevalent non-traumatic disease that causes lameness in dogs is dysplasia of the hip joint. This disease leads to secondary joint disease, arthrosis, and arthritis, and clinical signs such as pain and lameness correspond to this [12]. Many screening procedures use radiographic evaluation of the ventrodorsal projection of the hip joint for selection into breeding. To confirm the joint disease, the Federation Cynologique International (FCI) has given five different grades from A to E, ranging from normal hip joint to severe dysplasia. Even in grade A, some changes can be observed [30]. The effectiveness of early detection can be verified by clinical and radiographic observation. Early identification and appropriate treatment have the potential to halt and even reverse the progression of the disease, thereby altering its pathogenesis [44].

In human pediatric medicine, various radiological measurements play a key role in cases that require surgical intervention. These measurements help human physicians assess the development of the acetabulum and its compatibility with the femoral head. By measuring various dis-

tances and angles on these images, it is possible to gain a detailed view of the development of the acetabulum and its relationship to the femoral head [27, 42]. This approach is not limited to human medicine but is equally applicable to veterinary medicine. While many aspects related to the proximal femur and acetabulum have been investigated in humans, research on these measurements in dogs has focused primarily on the Norberg angle, inclination, and anteversion angle [35]. It has been found that not only breed, but also the sex and body weight of the patient are important factors when measuring acetabular parameters [15].

In dogs, ventrodorsal radiographs of dysplastic hip joints may show abnormalities of the acetabulum, proximal femur, or a combination [22, 31]. Anatomic variations in the angle of inclination, anteversion, and instability or subluxation of the hip joint lead to an increase in the magnitude and changes in the direction of the force exerted on this joint. These changes in biomechanical balance cause increased muscle and ligament tension, as well as increased loading on articular cartilage [45]. This can lead to abnormal growth, reduction of the articular weight-bearing surface, cartilage overload, and the development of osteoarthritis [22, 45]. Nevertheless, the articular surfaces of the hip joint are loaded inhomogeneously. It has been shown that the healthy canine hip joint is physiologically incongruent [21].

Anatomy of the hip joint

The hip joint, also known as the coxofemoral joint is diarthrodial [3] consisting of the femoral head and acetabulum of the pelvis. The normal anatomical relationship between the acetabulum and femoral head provides stability and functionality of the joint. These structures are optimally developed in healthy dogs and provide an even load distribution [18]. The articular surfaces of the femoral head and acetabulum are almost identical in terms of surface area and radius of curvature [3]. The space, which is located between the ilium and the ischium, forms the base of the acetabulum. It begins to ossify 6–8 weeks after birth and facilitates fusion of the pelvic bones at this junction. Occasionally, a smaller *os coxa quartum*, which represents the apophysis, may develop, especially in dogs of larger breeds. The iliac bones fuse with the sacrum and femur and eventually fuse at the pubic symphysis [18]. It is further divided into the ventromedial fossa of the acetabulum, where the ligament of teres, also called the round ligament or ligament of the femoral head, exits. The articular cartilage makes up approximately two-thirds of the entire surface and is located dorsomedially. In animals, the femoral head and neck are inclined to the femoral diaphysis at an angle of 130–145 degrees and, in addition, are rotated anteriorly by 12–40 degrees [3].

Radiographic evaluation

X-rays have been essential in diagnostics of hip dysplasia since 1935 when dysplasia was first reported [5]. Using radiographic evaluation methods, various screening and breeding control programs have been developed to address the widespread occurrence of dysplasia in dogs. There is a need to quickly and accurately identify diseased animals so that selective breeding can be implemented [3]. Radiographs allow detailed visualization of the hip joint, assisting in the assessment of acetabulum development and femoral head position. Different X-ray projections provide different views of the hip joint, allowing for comprehensive evaluation and screening of patients for hip dysplasia. These projections help in identifying abnormalities, treatment planning and monitoring the progression or improvement of the condition [5].

Radiographic images of the pelvis include various positions. The lateral position is used as a routine pelvic image and is also useful for assessing hip luxations [4]. The extended ventrodorsal position is commonly used to evaluate the hip joints, but it is also valuable for visualizing degenerative changes and assessing femoral head overlap [4]. The frog position, a flexed ventrodorsal position, helps measure acetabulum depth, check the fit of the femoral head, and identify minor fractures of the femoral head and neck. The dorsal acetabular rim view position, a dorsoventral position with flexed hips, is used to assess acetabular depth and hip laxity. Lastly, the PennHIP position involves a series of stretched, compressed, and distracted dorsoventral positions using a specific distraction device to evaluate hip laxity. Special PennHIP training and certification are required, and images are submitted for PennHIP evaluation [26].

There is a paucity or absence of published research providing reference values for primary morphometric measurements of the hip joint in dogs that are of biomechanical significance and can be obtained via radiographic examination [22]. Significant differences between dog

breeds have been observed, particularly due to the distinct body structures that characterize each breed [28, 34].

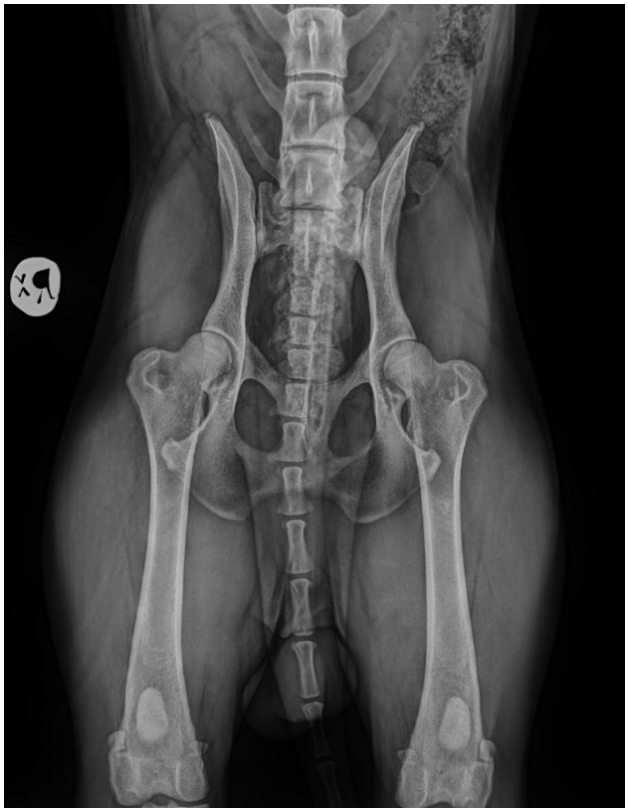

Fig. 1. Extended ventrodorsal position. The dog is reclining in a supine position with its knee joints and hind limbs fully extended, resulting in internal rotation of the knee joints (with the patella in a central position and the femoral condyles of equal size).

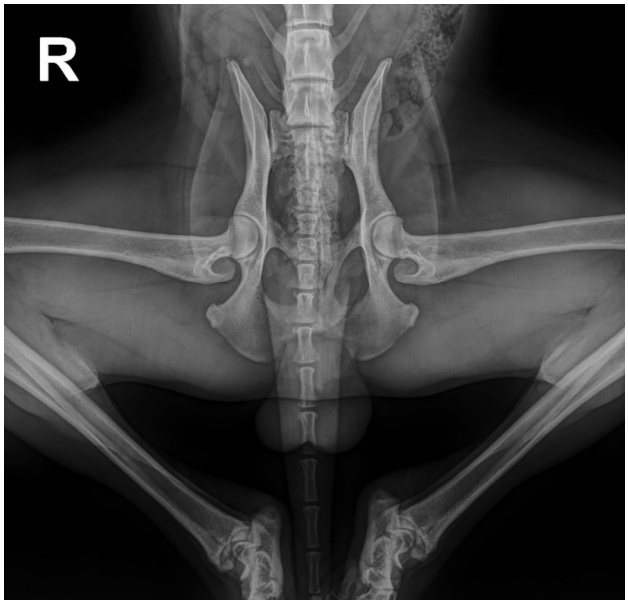

Fig. 2. Frog position. The dog is lying on its back with its hind legs bent outwards; the pelvis should be in a straight position; there should be no strain on the hip joint compared to the traditional ventrodorsal position.

Changes observed in the acetabulum

Acetabular index of depth to width – this measurement assesses the depth of the acetabulum [7]. St. Bernard and Bernese Mountain Dogs had the greatest relative acetabular depth, while Boxers and Labrador Retrievers had the shallowest and most open acetabulum. German Shepherds and Rottweilers had a slightly deeper and less open acetabulum than these breeds [35, 37]. In a study of Maltese and Shih-Tzu dogs, the acetabular index did not depend on any factor - age, sex, and breed [15].

Acetabular width (AW) is the distance measured from the acetabulum's lateral superior margin to the acetabulum's inferior margin [10]. Male Labrador Retriever dogs younger than 6 months of age tend to have higher AW values in cases of bilateral hip dysplasia compared to female dogs [17]. Similarly, these values were also higher in male Sivas Kangal dogs [34]. In addition, AW values were significantly elevated in cases of bilateral hip dysplasia [17]. In the study, AW measurements in Maltese and Shih-Tzu dogs were influenced by breed, sex, and body weight but not age. Male Shih-Tzu dogs showed higher AW values than Maltese [15].

Acetabular angle (AA) measures the inclination of the cranial margin of the acetabulum [25]. AA remains consistent despite pelvic rotation, tilt, and age. In cases of canine hip dysplasia (CHD), AA is significantly increased [17], and dogs that have an acetabular angle index greater than nine degrees should be excluded from breeding groups to limit the number of offspring affected by CHD [25]. Specifically, in cases where CHD was bilateral, AA was significantly higher compared to cases where CHD was unilateral. Furthermore, among dogs with bilateral CHD, males showed significantly higher AA values compared to females [17].

External acetabular angle (EAA) is derived from the horizontal to it´s external angle (HTE) and reflects the orientation of the roof of the acetabulum in the coronal plane. In healthy individuals, the HTE angle is usually 10° or less, but higher angles are common in cases of acetabular dysplasia [34].

The acetabular angle of retrotorsion (AAR) is an indicator of hip abnormality. It is a measurement of the depth of the acetabulum itself by connecting the point where the cranial and dorsal margins of the acetabulum meet with the maximum caudal extension of the acetabular notch. An AAR of 15° to 18° is considered typical for the hip joint.

The range of AAR values in the study for Leonberger's patellofemoral joint was 8° to 23° [8].

Acetabulum head index (AHI) measures the proportion of the femoral head surface that covers the acetabulum. Typical head coverage ranges from 70 % to 90 %, with an average of 90 % [9, 34]. In a group of dogs with a healthy hip joint, the AHI was significantly higher in males [17].

In addition to traditional ventrodorsal (VD) imaging, dorsal acetabular rim imaging of the acetabular rim and weight-bearing region of the acetabulum can also be used to assess the acetabular joint. This view is useful in the radiographic evaluation of hip dysplasia in dogs [23].

Changes observed in the femur

Femoral neck length and width (FNL and FNW) – FNL is determined as the distance between the centre of the femoral head and the centre of the base of the femoral neck, as previously determined to measure the cervicodiaphyseal angle. In an X-ray study on 82 femurs of different breeds of dogs, an average femoral neck length of 18.7 mm was found [28]. Measurements of head and femoral neck dimensions in humans were conducted, resulting in the derivation of the head and neck index. This index evaluates the general deformity of the proximal femur, particularly in relation to avascular necrosis with metaphyseal involvement. The typical value of this index in human anatomy is around 100 mm. A lower quotient indicates a higher degree of deformity compared to the opposite side of the hip joint [34, 42].

The neck-shaft angle (NSA) and the angle of femoral neck anteversion (FAV) are key assessments of the proximal femur. These measurements are extremely important when considering surgical intervention for hip dysplasia. The NSA reflects the relationship between the femoral neck and the shaft, while the FAV indicates the degree of femoral neck rotation. Cervico-diaphyseal angle is often used as a synonym for NSA, which also describes the angle between the axis of the femoral neck and the axis of the femoral diaphysis. In the study by Montavon et al. [24] values of 140.5° to 156.5° were measured. In the a study by Palierne on a diverse sample of dogs, the mean value of this angle was measured to be 140.9°, with a minimum value of 130.0° and a maximum value of 154.0° [28]. In another study by Kara et al. on a sample of healthy femurs of different breeds, the values were 146.24°, with bitches showing a smaller value than dogs [16]. In a sample of 8 dogs of medium to large breeds, the mean value was 129.6° [36]. Computed tomography measurements for medium to large breed dogs showed a mean femoral neck angle (FNA) of 147.50° [1].

Hip axis length (HAL) is the distance measured along the axis of the femoral neck, starting from the base of the greater tuberosity and extending to the inner edge of the pelvic bone. This measurement helps in assessing the geometry of the hip joint [11]. This value has greater sensitivity and specificity than bone mineral density for predicting fracture risk. This means that HAL is a more reliable predictor of fracture risk compared to conventional bone quality measurements [34]. HAL values were significantly higher in CHD, especially in male Sivas Kangal dogs [17, 35].

The mean FSC values of the femoral shaft cortex width were higher in the group of healthy males compared to the female group. However, in the group of dogs diagnosed with hip dysplasia, the trend was reversed with females showing significantly higher FSC values compared to males [17].

Trochanteric width (TW): there is a difference in TW values between males and females depending on their hip health status. Among dogs with healthy hip joints, males had significantly higher TW values compared to females [17, 35]. However, in cases of CHD in dogs, the trend was reversed, with females showing significantly higher TW values compared to males [17].

Femoral Inclination Angle (FIA) is crucial for the load transfer from the femur to the acetabulum. The importance of this angle lies in its role in determining how weight and forces are distributed in the hip joint, which is particularly important in conditions where joint stability is compromised [35]. A modified method based on the symmetrical axis, developed by Rumph and Hathcock, is used to plot the femoral neck and proximal femur axes. One circle is placed exactly at the femoral head. The other three circles are placed in the femoral neck, proximal, and medial femur so that they touch the border of the silhouette. Straight lines are drawn between the centres of these circles to draw the axis of the neck and the axis of the proximal femur [32, 35]. In a study by Sarierler, there were no significant overall differences in the angles of inclination between dysplastic and non-dysplastic dogs, but some breed-specific differences were observed. Specifically, Labrador Retriever and Doberman Pinscher,

Anatolian Carabash, Doberman, Labrador and Sivas Kangal, Irish Setter, Golden Retriever, and German Shepherd showed significant inter-breed differences. In addition, analysis of femoral inclination angles in relation to age and sex revealed no significant differences between males and females or between younger and older dogs [31]. Higher FIA values were observed in congenital hip dysplasia [35]. This was confirmed in a study where the preoperative FIA in dysplastic breeds was 127.6° [29] as well as in a study by Madsen and Svalastoga [20]. The FIA was significantly higher in dogs with a healthy hip joint. In CHD, males had higher FIA values [17]. The association between FIA and femoral neck anteversion was confirmed. Additionally, dogs with a steep inclination angle had significantly greater femoral neck anteversion compared to dogs with a less steep inclination angle [20].

Percentage coverage of the femoral head (PC) is another indicator used to assess the hip joint. The hip joint is considered normal if the acetabulum covers 50 % or more of the femoral head [40]. In a group of dogs with dysplastic hips, the value was 36.9 [29]. PC varies significantly depending on gender and the presence of unilateral hip dysplasia. In dogs with healthy hip joints, males had significantly higher PC values compared to females. Conversely, in cases of unilateral CHD, female dogs showed significantly higher PC values compared to males [17]. PC ranged from 6.5 % to 79.9 % for Labrador Retrievers, from 5.7 % to 79.5 % for Rottweilers, from 8.3 % to 79.3 % for Golden Retrievers, and from 5.4 % to 83.7 % for German Shepherds [40].

Norberg angle (NA)

NA is defined by two straight lines emanating from the center of the femoral head. The first line is tangential to the craniolateral margin of the acetabulum and the second line connects the midpoints of the contralateral femoral heads [23]. NA greater than 105° is usually considered normal and indicates a stable hip joint. Angles less than 105° indicate hip laxity, characteristic of hip dysplasia, when the femoral head is not well seated in the acetabulum, suggesting potential instability or abnormal development of the hip joint [5]. This was confirmed in a study where dysplastic dog breeds had an NA value of 78.9° [29]. A certain NA value (e.g. 105°) may indicate high-quality (tight) hip joints in one breed, but lower-quality (looser) hip joints in another breed. Therefore, it is recommended to evaluate the NA of the hip joints of a particular dog as a percentage rating compared to the NA of other dogs of the same breed. This provides a more accurate picture of the quality of a particular dog's hip joints within its breed [6]. NA ranged between 67.4° and 124.4° for Labrador Retrievers, 59.7° and 128.6° for Rottweilers, 70.2° and 119.4° for Golden Retrievers, and 55.3° and 121.3° for German Shepherds [40]. In a study for the Leonberger breed, a range of NA from 82° to 120° was found [8].

Distraction Index (DI) and Subluxation Index (SI)

Distraction index (DI) and subluxation index (SI) are used to measure hip laxity using ventrodorsal distraction imaging, which is performed when a special distractor is used to separate the femoral head from the acetabulum. DI provides an accurate numerical determination of laxity and is part of the PennHIP method, which is performed under general anesthesia or deep sedation. This test includes three different views of the hip joint: a standard ventrodorsal image to detect osteoarthritis and compression and distraction images to calculate DI [13]. Unlike qualitative assessment methods that use descriptive ratings (e.g., excellent, good, fair), the PennHIP provides quantitative data on joint laxity [38].

A significant advantage of the PennHIP method is that it allows for reliable assessment of young dogs as young as 16 weeks and accurately predicts the risk of developing osteoarthritis later in life [13]. In studies on American Bulldogs, Bernese Mountain Dogs, Newfoundland dogs, and Standard Poodles, DI was the most significant predictor for the development of osteoarthritic changes associated with hip dysplasia, with weight and age of the dogs also playing a significant role [33].

Other studies, such as those of Taroni et al. and Klever et al., revealed that dogs classified as phenotypically normal by the Orthopedic Foundation for Animal (OFA) or FCI may have exhibited significant hip laxity that was detected by distraction radiography. These results suggest that traditional OFA or FCI scoring based on standard ventrodorsal radiographs may underestimate the risk of osteoarthritis [39, 19]. A study found that even phenotypically healthy dogs by OFA may have significant joint laxity as detected by DI, which has been shown to be a more effective tool for identifying hip dysplasia than the NA. Thus, DI may be a valuable adjunct to standard scoring in hip dysplasia screening. These findings high-

light that current methods may slow progress in dysplasia reduction through breeding [39].

CONCLUSIONS

The evaluation of the morphometric parameters of the hip joint in the dog is usually focused on the evaluation of the radiological parameters of the proximal femur and acetabulum. There are many publications that track changes in the acetabulum and proximal femur; however, significant differences in the tracking of these radiological parameters can be observed within individual studies. These differences often result from a variety of methods for assessing and interpreting measured data, underscoring the need for standardized procedures for monitoring these key aspects of the hip joint. For surgeries such as hip replacement, surgeons should consider breed-specific acetabular values to achieve optimal results. Nevertheless, further research is needed to see if there is a correlation between these parameters and various hip disorders, as most of the research done so far has been done on a small number of cases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

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AUTHORS CONTRIBUTIONS

Gabriela Kacková conceptualized the review, conducted the literature search, and drafted the manuscript. Slavomír Horňák proposed the topic and contributed to the critical revision of the manuscript. Mária Figurová acquired and processed the radiographic images used in the review. Nela Vargová assisted with literature selection and manuscript editing. Veronika Tauberová contributed to data interpretation and final manuscript review.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

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ORIGINAL ARTICLE

STUDY OF FELINE LOWER URINARY TRACT DISEASE IN BLIDA, NORTH OF ALGERIA: CLINICAL AND EPIDEMIOLOGIC FEATURES

Asma Dahmani[1]*, Safia Zenia[2]

[1]Institute of Veterinary Sciences, University Blida 1, B.P. 270, Road of Soumaa, 09000, Blida, Algeria; [2]Laboratory Research «Animal Health and Production» Superior National Veterinary School - B.P. 165, Issad Abbas, El Alia. Algiers, Algeria

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***Correspondence:** asmavet42@yahoo.fr

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Feline lower urinary tract disease (FLUTD) is a common problem in cats. The objectives of the present study were to determine the prevalence, clinical signs, causes and the risk factors for FLUTD. From 1514 cats that presented to private veterinary clinic located in the region of Blida, 95 cats (6.27 %) were diagnosed with FLUTD and were included in the study. For each animal, potential risk data were obtained from medical records and cat owner interviews. FLUTD diagnoses were based on physical examinations, urinalyses, ultrasound examinations, and bacterial cultures. The most frequent cause of FLUTD was feline idiopathic cystitis (FIC) (43.15 %), followed by urolithiasis (26.32 %), urinary tract infection (UTI) (21.05 %), urethral plugs (8.42 %) and neoplasia (1.05 %). The most common clinical signs of FLUTD were dysuria (76.84 %), hematuria (60 %), pollakiuria (45.26 %), anuria/oliguria (42.10 %), depression (36.84 %), periuria (34.73 %). In the UTI, the most common bacterial isolate was *Escherichia coli* and the mineral compositions in the analyzed uroliths were mostly struvite crystals. The sex, castration status, breed, living environment, type of food were found to be significantly associated with FLUTD. Male cats ($P < 0.0001$), spayed/neutered cats ($P = 0.00014$), European shorthair breed ($P < 0.0001$), the indoor living cats ($P < 0.00001$), cats having dry food ($P < 0.00001$) were most susceptible to FLUTD. Cats aged over 5 years were the most affected, but no significant difference was found in age ($P = 0.158$). The prevalence of FLUTD was not correlated with the presence of other cats in the household ($P = 0.051$). The present study revealed a non-negligible prevalence of FLUTD in the north of Algeria. Appropriate recommendations need to be introduced to control the disease.

Key words: clinical signs; feline lower urinary tract disease; prevalence; risk factors

INTRODUCTION

FLUTD is a common disease in cats [9]. It is a syndrome that affects the reproductive tract, urinary bladder or urethra [18]. FLUTD is a broad terminology that involves many disorders, including feline idiopathic cystitis (FIC), urethral obstructions, urolithiasis, urinary tract neoplasia, and urinary tract infection (UTI). The most frequent type of FLUTD is FIC. While neoplasias are categorized as being one of the less common causes of FLUTD [5, 8, 9, 12, 18, 20]. Common clinical manifestations of the disease include dysuria, hematuria, stranguria, pollakiuria and periuria [5,8]. The diagnosis is based on the above mentioned symptoms alone, regardless of their cause. Accordingly, urine analyses, radiography and abdominal ultrasonography are used [5] in order to support differential diagnosis of urinary tract diseases [12]. FIC is a diagnosis of exclusion and is generally diagnosed by eliminating other urinary tract disorders such as urolithiasis, bacterial infection, anatomical defects or tumours [12]. The risk factors for FLUTD differ across countries due to geography, season, diets and cats' lifestyle [5]. FLUTD is rare in females and common in male cats, due to the anatomy of the penile urethra [2]. Certain common breeds were reported to experience lower urinary tract disease more frequently, such as Persian, Himalayan, and Russian Blue. Some of these breeds are considered to have a predisposing factor to the formation of uroliths so that urinary tract obstruction occurs. Abyssinian cats were commonly predisposed to bacterial UTI [11]. Castration and spaying are considered risk factors associated with the inhibition of urethral growth, induction of weight gain, and a sedentary lifestyle [16]. Indoor care setting [5], commercial dry food and excess body weight [18] were reported to increase the risk of FLUTD. In Algeria, only one study on prevalence and risk factors of FULTD has been published [19]. The current lack of information about risk factors for FULTD in Algeria cats and the usefulness of this knowledge for designing and implementing FULTD control motivated this study. Therefore, the objectives of the present study were to determine the prevalence, clinical signs, and risk factors for FLUTD in cats in Blida, north of Algeria.

MATERIALS AND METHODS

1. Animal and data collection

A total number of 1514 cats presented to private veterinary clinic located in the region of Blida between June 2023 and June 2024 were reviewed, and 95 cats diagnosed with FLUTD were included in the study. Only patients displaying urinary tract symptoms (hematuria, pollakiuria, periuria, obstruction and pain) were included. Owners reported crying out during urination, an abnormally difficult walk, or startling upon contact with the abdomen as signs of pain in the cats. Demographic data and risk factors were collected through face-to-face questionnaires with the cats' owners. The following data were acquired: the animal's age, breed, sex, body weight, type of food, household and lifestyle, the animal's reproductive status, presence of animals in the household. The types of food included dry and wet food. The lifestyles were indoor housing or outdoor housing. Also, the owners provided information about the duration and persistence of symptoms, urination frequency, and possible stressors.

2. Procedures (methods)

Detailed clinical examination of each patient was carried out. Visual inspection was applied while respiration, pulse rates and rectal temperature were carefully recorded. The studied patients were categorized into FIC, urolithiasis, urethral plug (UP), UTI, and neoplasia according to their respective diagnoses. UTI was diagnosed when significant bacterial growth was evident in the urine culture. An urethral plug was diagnosed when the urethra was obstructed by plug. An urolith was diagnosed using abdominal radiography and ultrasonography. Neoplasia was diagnosed ultrasonographically by the identification of a mass lesion. FIC was diagnosed by eliminating the other specific possibilities. FLUTD diagnostic methods in this study are based on the parameters used by Dorsch et al. [5] (Table 1). Determination of the obstructive and non obstructive form was based on abdominal palpation of the urinary bladder and the evaluation of urethral patency during catheterisation. The urine samples from the 95 cats displaying urinary tract symptoms were also tested by cytological and microbiological culturing. 10 ml of urine samples were centrifuged for 10 minutes at 1500 rpm and the sediment was examined for the presence of crystal-like formations. Microbiological tests were done by catheteri-

Table 1. Definition of diagnoses in cats with clinical signs of FLUTD [5]

Diagnosis	Definition	Exclusion
Feline idiopathic cystitis	Diagnosis of exclusion	- Urocystoliths identified on abdominal radiographs or ultrasound - Positive urine culture with significant bacterial growth (≥ 10^3 CFU. ml^{-1}) - Evidence of neoplasia on abdominal ultrasound - Struvite crystalluria ("numerous" or "too numerous to count" or +++ on examination of the urine sediment) without obstruction
Bacterial urinary tract infection	Significant bacterial growth (≥ 10^3 CFU.ml^{-1}) in urine samples obtained per cystocentesis or catheterization at the time of presentation	- Urocystoliths identified on abdominal radiographs or ultrasound - Evidence of neoplasia on abdominal ultrasound
Urethral plug	Detection of a urethral plug on catheterization and/or numerous struvite crystals in urine sediment and urethral obstruction	- Urocystoliths or urethroliths identified on abdominal radiographs or ultrasound - Evidence of neoplasia on abdominal ultrasound - Significant bacterial growth (≥ 10^3 CFU.ml^{-1}) in urine samples obtained per cystocentesis or catheterization at the time of presentation
Uroliths	Uroliths identified on radiographs and/or ultrasound	- Evidence of neoplasia on abdominal ultrasound
Neoplasia	Ultrasonographically identified mass lesion (with or without a definitive histologic diagnosis)	- Urocystoliths identified on abdominal radiographs or ultrasound
Miscellaneous	Struvite crystalluria (numerous crystals on examination of the urine sediment) without obstruction Neurologic disorders associated with FLUTD symptoms	- Urocystoliths identified on abdominal radiographs or ultrasound - Evidence of neoplasia on abdominal ultrasound

zation and grown on mannitol salt agar, MacConkey agar, and blood agar (containing 5 % sheep blood). The cultures were incubated at 37 ^{o}C for 24 – 48 h [6].

3. Statistical analysis

Data analysis was performed using Microsoft Excel 2010 and Statistical Data for Social Science (SPSS) 26© IBM Copyright, IBM Corporation and its licensors 1989.2019 IBM, USA. Results were expressed as percentages and 95 % confidence intervals (CIs) were calculated. Univariate analysis was performed to assess each independent variable for its unadjusted association with disease. The chi-square test of homogeneity and independence as well as the Mann-Whitney U test and the Kruskal-Wallis test were performed. The statistical significance level used for this study was $P < 0.05$.

RESULTS

From 1514 cats inspected, 95 (6.27 %), 95 % CI = [5.1 % – 7.5 %] patients were diagnosed with FLUTD. The most frequent diagnosis was FIC in 41 cats (43.15 %), followed by urolithiasis in 25 cats (26.32 %), urinary tract infection (UTI) in 20 cats (21.05 %), urethral plugs in 8 (8.42 %) and neoplasia in one cat (1.05 %). A very high significant difference ($P < 0.00001$) was recorded between the number of cases found in each disease. The clinical signs associated with different groups of FLUTD diagnostic groups were documented in Table 2. Dysuria 73 (76.84 %), hematuria 57 (60 %), pollakiuria 43 (45.26 %), anuria/oliguria 40 (42.10 %), depression 35 (36.84 %), periuria 33 (34.73 %), dark urine 22 (23.15 %), inappetence 18 (18.94 %), fever 5 (5.26 %), polyuria 4 (4.21 %), pyuria 2 (2.10 %), vomiting 1 (1.05 %) were the common signs of FLUTD.

Obstructive FLUTD was diagnosed in 34 cats (35.78 %), all cats with urolithiasis, urethral plugs and neoplasia were classified as obstructed, while non case of obstructive FLUTD was diagnosed in cats with FIC and UTI. The clinical signs of obstructive and non-obstructive FLUTD are shown in Table 3.

Bacterial growth in urine cultures was observed in 20 cats. *Escherichia coli* was isolated from 10 cats, *Staphylococcus* spp. from 4 cats, *Staphylococcus saprophyticus* from 3 cats, mixed infection with *Escherichia coli* and *Pseudomonas* from 2 cats, and polymicrobial culture was found in one cat. The crystal-like formations was found in 25 cats including struvite crystals (9 cats), urate crystals (7 cats), while the other cases showed the presence of calci-

Table 2. Clinical signs of all cats with FLUTD and cats diagnosed with the five different diseases [n (%) of cats]

	All cats	FIC	Urolithiasis	UTI	UP	Neoplasia	P-value
Total	95(100)	41 (43.15)	25(26.04)	20 (21.05)	8 (8.42)	1(1.05)	<0.00001
Dysuria	73(76.84)	28(68.29)	22(88)	16(80)	6(75)	1(100)	<0.00001
Macroscopic Hematuria	57(60)	24(58.53)	13(52)	14(70)	5(62.5)	1(100)	<0.00001
Pollakiuria	43(45.26)	16(39.02)	13(48)	12(60)	2(25)	0	0.0001
Anuria/oliguria	40(42.10)	19(46.34)	11(44)	4(20)	5(62.5)	1(100)	<0.00001
Depression	35(36.84)	15(36.58)	11(44)	5(25)	4(50)	0	0.0004
Periuria	33(34.73)	13(31.70)	9(36)	5(25)	5(62.5)	1(100)	0.0134
Dark urine	22(23.15)	11(26.83)	8(32)	2(10)	1(12.5)	0(0)	--
Inappetence	18(18.94)	9(21.95)	5(20)	2(10)	2(25)	0	--
Fever	5(5.26)	4(9.75)	0	1(5)	0	0	--
Polyuria	4(4.21)	2(4.87)	1(4)	1(5)	0	0	--
Pyuria	2(2.10)	0	1(4)	1(5)	0	0	--
Vomiting	1(1.05)	1	0	0	0	0	--
P-value	<0.0001	<0.0001	<0.0001	<0.0001	-	-	

Table 3. Comparison of the frequency of the reported clinical signs in the cats with the non-obstructive and obstructive form of FLUTD

Group	Non-obstructive form n (%)	Obstructive form n (%)	P-value
Number of cats	61(64.21)	34(35.78)	0.0056
Dysuria	44(72.13)	29(85.29)	0.144
Macroscopic Haematuria	38(62.29)	19(55.88)	0.540
Pollakiuria	28(45.90)	15(44.11)	0.867
Anuria/oliguria	23(37.70)	17(50)	0.244
Depression	20(32.78)	15(44.11)	0.272
Periuria	18(29.5)	15(44.11)	0.151
Dark urine	13(21.31)	9(26.47)	0.393
Inappetence	5(11.9)	7(20.58)	0.155
Fever	5(8.19)	0	0.216
Polyuria	3(4.91)	1(2.94)	----
Pyuria	1(1.63)	1 (2.94)	----
Vomiting	1(1.63)	0	----

um oxalate crystals (3 cats), uric acid crystals (3 cats) and phosphate crystals (3 cats).

Demographic data and risk factors diagnosed with FLUTD are presented in Tables 4 and 5. Using the chi-square test, sex, breed, castration status, living environment, the type of food were found to be significantly associated with FLUTD. Male cats were more common than female cats in all types of FLUTD ($P < 0.0001$), including FIC, UTI, UP, urolithiasis, and neoplasia. The average age of cats with FLUTD was 5.13 ± 3.85 years. Cats aged over 5 years were more affected than those aged between 8 months and 2 years, while the youngest animals from 8 months to 2 years were less affected, no significant difference was found between the 3 classes ($P = 0.158$). However, in UTI and UP, the cats aged from 8 months to 2 years were the most affected, European shorthair breed was more likely to have FLUTD, followed by Persian cats, Siamese cats and Angora cats ($P < 0.0001$). Urolithiasis and neoplasia were more diagnosed in Persian cats. With the exception of neoplasia, spayed/neutered cats were more susceptible to FLUTD than intact cats ($P = 0.00014$). The mean body weight of the cats with FLUTD was $4.40 \text{ kg} \pm 0.89 \text{ kg}$, no significant difference was found between the different diseases (FIC, urolithiasis, UTI, UP and neoplasia). Among all patients diagnosed with FLUTD, 71 (74.73 %) were indoor cats, the prevalence of FLUTD was highly correlated with the indoor living mode of cats ($P < 0.00001$). Almost all cats (98.94 %) with FLUTD were fed only dry food

Table 4. Analyzes of cats affected by FLUTD according to risk factors [n (%) of cats]

Group	All cats	P-value
Number of cats	95	
Sex		
Male	68(71.57)	<0.0001
Female	27(28.42)	
Age		
[8 months- 2 years]	25(26.31)	
[3 -5 years]	30(31.57)	0.158
>5years	40(42.10)	
Breed		
European	46(48.42)	
Persian	25(26.31)	<0.0001
Angora	10(10.52)	
Siamese	14(14.73)	
Sexual state		
Spayed/ Castrated	66(69.47)	0.00014
Intact	29(30.52)	
Weight (kg) Mean ± SD	4.40 ± 0.89	
Living environment		
Indoor	71(74.73)	<0.00001
Outdoor	24(25.26)	
Food		
Dry	94(98.94)	<0.00001
Wet	1(1.05)	
Presence of other cat(s)		
No	38(40)	0.051
Yes	57(60)	

and only one cat diagnosed with UP was fed a wet food, a highly significant difference was recorded between the two types of food ($P < 0.00001$). The prevalence of FLUTD was not correlated with the presence of other cats in the household ($P = 0.051$).

DISCUSSION

The prevalence of FLUTD in this study (6.27 %) was almost similar to 8 % reported for the United States and Canada from 1980 to 1997 [11], but higher than the 2.24 % and 2.29 % reported by Piyarungsri et al. [18] and Kochan and Simsek [8], respectively. These different results may be due to differences in the geographic area, diet, popular breeds of cats in each country and duration of sample collection [18]. In the present study, FIC was the most frequent diagnosis (43.15 %). Similarly, relevant studies from different countries reported FIC as the most prevalent FLUTD with a reported prevalence ranging from 51 % to 60 % [5, 8, 9, 12, 18, 20]. Many theories about the causes of FIC have been proposed, but the specific etiological factor(s) are still unknown [20]. According to Buffington [3], FIC is a result of the

Table 5. Analyzes of cats affected with FIC, urolithiasis, UTI and UP according to risk factors [n (%) of cats]

Group	FIC	Urolithiasis	UTI	UP	Neoplasia	P-value
Number of cats	41	25	20	8	1	<0.00001
Sex						
Male	27(65.85)	20(80)	15(75)	5(62.5)	1(100)	<0.00001
Female	14(34.14)	5(20)	5(25)	3(37.5)	0	<0.001
Age						
[8 months - 2 years]	7(17.07)	6(24)	8(40)	4(50)	0	0.091
[3 - 5 years]	16(39.02)	7(28)	5(25)	1(20)	1(100)	<0.0001
> 5 years	18(43.9)	12(48)	7(35)	3(30)	0	<0.0001
Mean ± SD years	5.12 ± 2.75	5.09 ± 3.1	5.88 ± 5.83	5.58 ± 4.6	5	P = 0.804
Breed						
European	24(58.53)	7(28)	10(50)	5(62.5)	0	<0.00001
Persian	8(19.51)	8(32)	6(30)	2(25)	1(100)	0.066
Angora	6(14.63)	4(16)	0	0	0	----
Siamese	3(7.31)	6(14)	4(20)	1(12.5)	0	----
Sexual state						
Spayed/ Castrated	32(78.04)	15(61.11)	13(65)	6 (75)	0	<0.00001
Intact	9(21.95)	10(38.88)	7(35)	2(25)	1(100)	0.0213
Weight (kg) Mean ± SD	4.43 ± 0.79	4.43 ± 0.74	4.48 ± 1.08	4.10 ± 1.14	3.4	P = 0.851
Living environment						
Indoor	34(82.92)	17(68)	15(75)	4(50)	1(100)	<0.00001
Outdoor	7(17.07)	8(32)	5(25)	4(50)	0	----
Food						
Dry	41(100)	25(100)	20(100)	7(87.5)	1(100)	<0.00001
Wet	0	0	0	1(13.5)	0	----
Presence of other cat(s)						
No	10(24.39)	13(52)	12(60)	2(25)	1(100)	0.0019
Yes	31(75.6)	12(48)	8(40)	6(75)	0	<0.0001

inadequate reaction to variable stressors due to the chronic activation of the central threat response system (CTRS). It is believed, that the CTRS may be sensitized by threatening events that can occur early in life, even before birth, when the CTRS is the most plastic and vulnerable [4]. The second most common cause of FLUTD in our study was urolithiasis (26.32 %). Similar results were reported by Kovarikova et al. [9], Piyarungsri et al. [18], Kochan and Simsek [8]. However, these findings contradict the previous report of Ayoub et al. [1], which indicate urolithiasis is the most frequent cause of FLUTD, followed by FIC. The dietary regimens of the patients included in the study, especially dry food, may account for the high incidence of urolithiasis. The formation of crystal was observed only in urolithiasis cases. The majority of crystal-like formations in our study were struvite crystals of which commercial feed is a main cause. Same results were recorded by Tariq et al. [22], Lew – Kojrys et al. [22] and Nururrozi et al. [16]. Ayoub et al. [1] reported a decrease in the struvite-containing uroliths and an increase in the calcium oxalate-containing uroliths. The urinary tract infection (UTI) reported in the present study (21.05 %) is in line with findings reported by Nururrozi et al. [8], Kraijer et al. [10] and Dorsch et al. [5] who reported rates of 25.3 %, 22.2 % and 18.9 %, respectively. The percentage of UTI cases from this study was higher than 14.2 % reported by Ayoub et al. [21], 11.8 % recorded by Sævik et al. [20], and 8 % reported in Poland by Lew – Kojrys et al. [12]. The different results may be caused by the different methodology with various inclusion criteria. Escherichia coli were the most common isolates, similar finding has been reported by Sævik et al. [20], Lew – Kojrys et al. [12] and Ayoub et al. [1]. However, some reports showed an increasing trend in the prevalence of Staphylococcus species [15, 23]. Most human uropathogenic bacteria originate from the gut [14]. This event was supported in this study as the leading bacteria, Escherichia coli, are also part of the normal feline gut flora [15]. UP was identified as the cause of FLUTD in 8.42 % of the cats included in the present study. The rate of UP was reported by Nururrozi et al. [16] as 4.9 %, Gerber et al. [7] as 10 %, Dorsh et al. [5] as 10.3 %, Kochan and Simsek [8] as 13 %, by Sævik et al. [20] as 21.0 %. In contrast to uroliths, urethral plugs consist of large quantities of organic matter and varying amounts of minerals, mainly struvite [17]. The percentage of neoplasia was 1.05 %, making it the least prevalent cause of FLUTD. Previous findings recorded very low rates of neoplasia ranging between 0.4 % and 3.6 % [1,8,16]. Kochan and Simsek [8] reported that the most common clinical manifestations in cats with FLUTD were pain (100 %), stranguria (81.57 %), pollakiuria (73.68 %), obstruction (60.52 %), hematuria (50.0 %) and periuria (28.9 %). Lew – Kojrys et al. [12] recorded that the clinical signs associated with the disease were stranguria (81.3 %), pollakiuria (71.7 %), obstruction (59.5 %), hematuria (49.9 %) and periuria (25.9 %). Dorsch et al. [5] reported that the the most common clinical signs were stranguria (54.0 %), obstruction and hematuria and their incidence rates were 54.0 %, 52.6 % and 42.4 %, respectively. The clinical signs varied by study and depended on the lifestyle of the cats, including indoor or outdoor husbandry. An owner's ability to observe clinical signs is affected by many factors, including outdoor access, the number of cats and the owner's work schedule. Furthermore, only the prominent clinical signs of FLUTD, such as dysuria or hematuria, can be easily detected in outdoor cats [18].

Most of the cats diagnosed with FLUTD in this study were males, which is similar to the findings by Nururrozi et al. [16], Mazda et al. [15], Ayoub et al. [1]. This might be attributed to the long and narrow urethra in males [20]. In another hand, Kovarikova et al. [9] noted that the urethral plugs were only found in the males while the highest proportion of females was found in the UTI subgroup, this might be explained by the fact that in male cats, where the risk of obstructive uropathy is higher, the treatment is more often required, while in females, clinical symptoms may be subtle and overlooked [9]. The most common breed affected regardless of the cause of FLUTD were European shorthair, which are the most common cats in Algeria. Kovarikova et al. [9] reported that Domestic Shorthair cats, British Shorthair cats, Persian cats and Maine Coon cats had an increased risk of developing FLUTD. In our study, urolithiasis was more often diagnosed in Persian cats, Ayoub et al. [1] noted that Persian cats were the most affected breed with FLUTD and this Persian breed represented the highest percentage of obstructive urolithiasis. According to Nururrozi et al. [16], the oxalate crystals and stones are more often found in some breeds like Himalayan, Persian, Burmese, and Russian blue cats and that genetic factors may contribute to an increased

risk of calcium oxalate urolith formation [16]. However, S æ v i k et al. [20] found no association between cat breed and FLUTD. The variability in the results is probably due to the popularity of particular cat breeds at different times. This variability probably shows that in areas where pedigree cats are more common, they become affected [18]. The current study showed that the most cats with FLUTD were spayed or neutered cats. P i y a r u n g s r i et al. [18] and R e m i c h i et al. [19] revealed that neutering was associated with an increased risk for FLUTD. In contrast, A y o u b et al. [1] reported that most cats with FLUTD were intact. B o r g e s et al. [2] reported that castration affects the density of the elastic and collagen fibers in the periurethral tissues; this decreases the compliance of the periurethral region. Also, most castrated male cats were less active, leading to weight gain, a common risk factor for FLUTD [18]. Almost all cats (98.94 %) with FLUTD were fed only dry food. This finding agreed with previous studies that found that dry food was associated with an increased risk for FLUTD [1,18]. However, B o r g e s et al. [2] reported that there was no significant difference in the diet between cats with FLUTD and clinically normal cats. L u n d and E g g e r t s d ó t t i r [13] considered the dry food as an important risk factor for FLUTD and urolithiasis formation as it influences urinary pH. Also, S k o c h et al. [21] recorded that the dried industrialized food, including cereals, tends to alkalinize the urine and predisposes to the formation of struvite crystals. A higher proportion of the patients were managed completely indoors (74.73 %). Same results were found by D o r s c h et al. [5], L e w – K o j r y s et al. [12], K o v a r i k o v a et al. [9], M a z d a et al. [15]. An inappropriate environment like being more fearful or nervous and having conflicts with other cats in the same household can be risk factors and cause FLUTD among cats. From K o v a r i k o v a et al. [9], the indoor environment with no access to the outside associated with using a litter box, lower activity level, lower hunting behaviour are other risk factors suggesting that an indoor environment frequently does not meet the feline's natural needs and it acts as a stressor.

CONCLUSION

This study highlights the importance of managing social stress in cats to prevent these diseases. The results obtained provide practical recommendations to improve the management of FLUTD, taking into account the specification and practical constraints of each case.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

ETHICAL STATEMENT

There is no ethical approval needed.

CONFLICT OF INTEREST

There is no conflict of interest.

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There is no funding.

AUTHORS CONTRIBUTIONS

Author Dr. Asma Dahmani was responsible for the experimental work and the writing of the article. Author Ms. Safia Zenia was responsible for the statistical analysis.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

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ORIGINAL ARTICLE

MORPHOMETRIC CHANGES IN MALE REPRODUCTIVE ORGANS OF WISTAR RATS FOLLOWING COMBINED MELATONIN AND BISPHENOL-A TREATMENT

Olumide Samuel Ajani[1], Dideolu Osunkoya[1], Olumide Odunayo Akinniyi[2*]

[1]Department of Theriogenology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria; [2]Deartment of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ibadan, Nigeria

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***Correspondence:** olumide.akinniyi@gmail.com

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Bisphenol A (BPA) is a widespread endocrine disruptor known to cause reproductive toxicity. Melatonin has shown promise in ameliorating BPA-induced reproductive damage, but its effects on BPA-induced morphometric changes in reproductive tissues require further investigation. This study investigated the effects of combined melatonin and BPA administration on morphometry of male reproductive organs in Wistar rats. Twenty-four adult male Wistar rats were divided into four groups: control, BPA-treated (10 mg/kg/day), melatonin-treated (10 mg/kg/day), and BPA + melatonin-treated. After 45 days, testes, epididymides, and spermatozoa were analyzed for morphometric parameters. BPA significantly reduced epididymal and testicular tubular density compared to control ($p < 0.05$). Melatonin treatment alone increased epididymal germinal epithelial height and decreased luminal diameter ($p < 0.05$). The combined BPA + melatonin treatment showed the lowest epididymal luminal diameter ($p < 0.05$) and highest testicular tubular density. Spermatozoa head diameter was significantly reduced in the melatonin group compared to the BPA group ($p < 0.05$). The combined treatment group showed a significant decrease in entire sperm length compared to all other groups ($p < 0.05$). Melatonin administration mitigated BPA-induced morphometric anomalies in male rats, primarily by increasing testicular and epididymal tubular density and modulating epididymal luminal diameter. This study demonstrated morphometric effects of BPA and melatonin on male reproductive systems, but the molecular mechanisms remain unexplained. Future research should explore biochemical pathways and long-term reproductive impacts.

Keywords: Bisphenol A; melatonin; morphometry; reproductive toxicity

INTRODUCTION

Bisphenol-A (BPA) has received great attention due to its widespread presence in several consumer products and its adverse effects on reproduction [19]. Reproductive and endocrine disruptions are the major kinds of toxicity induced by BPA [1, 15]. Testicular toxicity induced by BPA has been reported and may account for the increasing frequency of infertility [4]. The reproductive toxicity of BPA is caused through multiple signaling pathways and may lead to the alteration in the process of spermatogenesis in rats [16].

Moreover, BPA reversibly perturbs the integrity of the blood-testis barrier in Sertoli cells *in vitro* [25]. It also induces uterotrophic effects in rats following high oral/and or subcutaneous dosing [6]. In rodents, developmental exposure to BPA leads to increased prostate weight [9], decreased epididymal weight and decreased sperm production [18]. Toxicity of BPA has been reported in mammals through increasing the hydroxyl-radical formation in the rat striatum, it also depletes the endogenous antioxidants in the epididymal sperms and causes defects in the hepatic detoxification mechanism of rats [17]. BPA exhibits endocrine like properties that raise concern about its suitability in consumer products and food containers. It is present in several products, including the interior coatings of food cans, milk containers and baby formula bottles, as well as in dental sealants [24]. It has also been reported to be present in water, feed, dust, and other related media [20, 21] by which animals are also at risk of exposure. When taken orally, it is readily absorbed from the digestive system and can be detected in various body fluids, organs and tissues.

Melatonin (N-acetyl-5-methoxytryptamine, MLT) has been reported to be useful in ameliorating BPA-induced reproductive toxicity in various studies [5]. In addition, Melatonin has been shown to possess antioxidant and prophylactic properties against oxidative stress in several experiments [2]. In addition to its use as an anti-stress, anti-aging, and immune-modulatory agent, MLT has also been used for sexual dysfunctions, gallbladder stones, obesity, and even tumors [13, 22].

Melatonin has demonstrated specific protective effects on testicular tissue and seminiferous epithelium in various studies. It has been shown to maintain the integrity of the seminiferous tubules by protecting Sertoli cells and developing germ cells from oxidative damage [2, 5]. Studies have demonstrated that melatonin helps preserve the height and organization of the seminiferous epithelium, supports spermatogenic cell development, and maintains the blood-testis barrier function [12]. The hormone acts directly on the testes through melatonin receptors present on Leydig cells and seminiferous tubules, helping to regulate local testosterone production and spermatogenesis [10]. Additionally, melatonin's powerful antioxidant properties protect the highly sensitive seminiferous epithelium from reactive oxygen species that can disrupt spermatogenesis [2, 13]. These specific effects on testicular tissue make melatonin a promising protective agent against reproductive toxicants like BPA that are known to disturb seminiferous epithelium structure and function [5, 12].

Although many studies have already shown how melatonin may exert its already well-documented beneficial effects in the treatment of a number of conditions [10, 12], a comprehensive analysis of melatonin's effects on BPA-induced morphometric alterations across the male reproductive system remains to be fully elucidated. Hence, the present work aimed at investigating the effects of combined melatonin and Bisphenol-A (BPA) administration on detailed morphometry of male reproductive organs in Wistar rats, with particular focus on testicular, epididymal, and spermatozoal parameters.

MATERIALS AND METHODS

Experimental animals

Twenty-four (24) adult male Wistar rats (n= 6) weighing between 160 ± 180g were used in this study. The animals were obtained from the Experimental Animal House of the Department of Physiology, University of Ibadan, Nigeria. The rats were housed in plastic cages (60*60*50 cm) where they were acclimatized for 14 days. All the rats were kept under controlled conditions of temperature (25 +/- 2 ˚C), relative humidity (50 +/- 5 %) and normal photoperiod (12 hour light and 12 hour dark). The rats were fed on a standard rat diet (commercial pellet diet) and water was provided *ad libitum*.

Experimental Protocol

The twenty-four (24) male rats were randomly assigned into four (4) groups of six animals each. Group A: rats were orally administered 0.2 mL of olive oil, serving as control. Group B (BPA-intoxicated rats): rats received

a dose of 10 mg.kg^{-1} body weight per day of BPA reconstituted in 0.2 mL olive oil, administered orally. Group C: rats received 10 mg.kg^{-1} body weight of intraperitoneal melatonin (Sigma Aldrich, 98 % pure, dissolved in 0.5 % ethanol in normal saline). Group D (BPA-Melatonin treated rats): rats received orally administered BPA (10 mg.kg^{-1} body weight per day) concomitantly with intraperitoneal melatonin (10 mg.kg^{-1} body weight) [12]. At the end of the 45-day treatment, the rats were fasted overnight and weighed before sacrifice.

Sample collection

The rats were euthanized using an overdose of anesthetic ether [3]. The tunica vaginalis and tunica albuginea of the testes were removed exposing the testicles and epididymis. The testicles were then properly cleaned with tissue paper to remove traces of blood as much as possible in order to prevent interference with the procedure of spermatozoa harvest. The sperm cells were harvested from the cauda epididymis by cutting it open with a clean razor blade and gently placed on a warm glass slide.

Morphometric Analysis (Testes, Epididymis and Spermatozoa)

The testes and epididymis were harvested and weighed. The relative weights of these organs were obtained by standardizing them against the body weight of the rats. These organs were subsequently fixed in buffered neutral formalin solution and processed for histological evaluation. The tissues were embedded in melted paraffin wax, the wax block was then cut on a microtome to yield a thin slice of paraffin containing the tissue. The specimen slice was then applied to a microscope slide, air dried, and heated to cause the specimen to adhere to the glass slide. Residual paraffin was then dissolved which was usually followed by rinsing with acid-alcohol. This was rinsed with water to remove the acid-alcohol. Testes and epididymis (germinal height, luminal diameter and tubular density), the sperm (complete sperm length, head length and head diameter), were measured using analysis world Graph Pad Prism 5.01 software.

Morphometric measurement of the testes (Germinal height, Tubular density and Luminal diameter)

Micrographs of testes was taken at x 40 magnification to specify the parts of the organ, it was retaken at x 100 magnification in which the measurement was made. A straight-line measurement was made by placing the cursor on the basement membrane to the luminal border. About five to six measurements were made on different cell on the same slide with same magnification and recorded. Micrographs of the testes was taken at x 40 magnification after which the number of cells in a tubule was counted and recorded. About five to six counts of the tubules on a slide of the same magnification was made and recorded. Micrographs of the testes was taken at x 40 magnification to specify the parts of the organ to be captured. The capture was then taken at x 100 magnification after which the cells were measured using a straight-line measurement from one end of the basement membrane to the adjacent end.

Morphometric measurement of the epididymis (Germinal height, Tubular density and Luminal diameter)

Micrographs of epididymis were taken at x 40 magnification to specify the parts of the organ, it was retaken at x 100 magnification in which the measurement was made. A straight-line measurement was made by placing the cursor on the basement membrane to the luminal border. About five to six measurements were made on different cells on the same slide with same magnification and recorded. The micrographs of the epididymis were taken at x 40 magnification after which the number of cells in a tubule were counted and recorded. The micrographs of the epididymis were taken at x 40 magnification to specify the parts of the organ to be captured. The capture was then taken at x 100 magnification after which the cells were measured using a straight-line measurement from one end of the basement membrane to the adjacent end.

Morphometric measurement of the sperm cell (Sperm length, Head length, Head diameter and Mid-piece length)

The micrograph of the sperm was taken at x 100 magnification after which an irregular line measurement was used to get the length of the sperm, measured from the acrosome to the extreme end of the end piece). The micrograph of the head was taken at x 100 magnification, after which an irregular line measurement was used to get the length of the head, measured from the acrosomal head to the margin between the head and the mid-piece.

The micrograph of the sperm was taken at x 100 magnification after which a straight-line measurement was used to get the diameter of the sperm head, measured from one end to the adjacent head of the head.

The micrograph of the sperm was taken at x 100 magnification after which an irregular line measurement was used to get the length of the mid-piece, which is the junction between the head and the tail.

Data analysis

The Statistical Package for Social Sciences (SPSS®, version 26) was used for the analysis. The Shapiro-Wilk test was done and it showed that the parameters were Gaussian. Given the normal distribution of the data, parametric tests were employed for further analysis. The 4 groups were subjected to a One-Way Analysis of Variance (ANOVA) to compare their means. Specific group differences were identified by conducting post-hoc pairwise comparisons using Tukey's Honest Significant Difference (HSD) test. Each group's data is represented by mean ± standard deviation (SD), offering information on both the central tendency and dispersion. Mean values of $p \leq 0.05$ were considered significant.

RESULTS

Effect of melatonin against Bisphenol-A induced morphometric anomalies of epididymis of albino rats

The results indicate that the epididymal germinal epithelial height did not differ significantly between the control group (A: 103.3 ± 13.0 µm) and the Bisphenol-A-treated group (B: 118.9 ± 11.8 µm) ($p > 0.05$). However, the melatonin-treated group (C: 189.3 ± 95.1 µm) exhibited a significantly greater germinal height than groups A and B ($p < 0.05$). The combination treatment group (D: 135.3 ± 17.4 µm) had an intermediate value, which was not statistically different from any other group ($p > 0.05$). In terms of epididymal luminal diameter, no significant differences were observed between the control (A: 1088.0 ± 45.3 µm) and Bisphenol-A (B: 1002.3 ± 73.5 µm) groups ($p > 0.05$). However, the melatonin group (C: 855.2 ± 207.3 µm) exhibited a significant reduction in diameter compared to groups A and B ($p < 0.05$), while the combination group (D: 585.7 ± 32.0 µm) had the lowest luminal diameter, which was significantly different from all other groups ($p \leq 0.05$). Regarding epididymal tubular density, the Bisphenol-A group (B: 10.3 ± 0.6) had a significantly lower density compared to all other groups ($p < 0.05$). The control (A: 16.0 ± 1.73), melatonin (C: 16.0 ± 1.0), and combination treatment (D: 19.0 ± 2.0) groups did not differ significantly from each other ($p > 0.05$) (Table 1).

Effect of melatonin against Bisphenol-A induced morphometric anomalies of testes of albino rats

The results for the height of the germinal epithelium showed no significant differences across the groups ($p > 0.05$). The combined treatment group (D: 311.2 ± 39.4 µm) had the lowest value, while the melatonin-treated group (C: 326.6 ± 31.5 µm) had the highest. For testicular luminal diameter, the control (A: 1131.8 ± 17.4 µm), Bisphenol-A (B: 1012.5 ± 66.6 µm), and combined treatment (D: 1077.4 ± 40.5 µm) groups did not differ significantly from one another ($p > 0.05$). However, the melatonin group (C: 890.0 ± 265.7 µm) showed a significant decrease compared to the control ($p \leq 0.05$), though it did not differ significantly from the Bisphenol-A or combined treatment groups ($p > 0.05$). Regarding testicular tubular density, the control group (A: 16.3 ± 1.5) was not significantly different from the melatonin (C: 14.0 ± 1.0) or combined treatment (D: 18.3 ± 2.1) groups ($p > 0.05$). However, the Bisphenol-A group (B: 11.0 ± 1.7) showed a significant decrease compared to the control and combined treatment groups ($p < 0.05$) but was not significantly different from the melatonin group ($p > 0.05$). The combined treatment group (D) had the highest value, which was significantly different from the Bisphenol-A group ($p < 0.05$) (Table 2).

Effect of melatonin against Bisphenol-A induced morphometric anomalies of spermatozoa of albino rats

The spermatozoa head diameter in the melatonin group (C: 34.9 ± 0.8 µm) was significantly reduced compared to

Table 1. Effect of melatonin against Bisphenol-A induced morphometric anomalies of epididymis of albino rats

Parameters	Group A (mean ± SD)	Group B (mean ± SD)	Group C (mean ± SD)	Group D (mean ± SD)
Epididymal germinal epithelial height (µm)	103.3 ± 13.0^{a}	118.9 ± 11.8^{a}	189.3 ± 95.1^{b}	135.3 ± 17.4^{ab}
Epididymal luminal diameter (µm)	1088.0 ± 45.3^{a}	1002.3 ± 73.5^{a}	855.2 ± 207.3^{b}	585.7 ± 32.0^{c}
Epididymal tubular density	16.0 ± 1.73^{a}	10.3 ± 0.6^{b}	16.0 ± 1.0^{a}	19.0 ± 2.0^{a}

Values with different superscript (^{a,b,c}) letters are significantly different ($p \leq 0.05$).

Table 2. Effect of melatonin against Bisphenol-A induced morphometric anomalies of testes of albino rats

Parameters	Group A (mean ± SD)	Group B (mean ± SD)	Group C (mean ± SD)	Group D (mean ± SD)
The height of the germinal epithelium (μm)	317.8 ± 54.4	315.2 ± 42.5	326.6 ± 31.5	311.2 ± 39.4
Testicular luminal diameter (μm)	1131.8 ± 17.4^{a}	1012.5 ± 66.6^{ab}	890.0 ± 265.7^{b}	1077.4 ± 40.5^{a}
Testicular tubular density	16.3 ± 1.5^{ac}	11.0 ± 1.7^{b}	14.0 ± 1.0^{ab}	18.3 ± 2.1^{c}

Values with different superscript (^{a,b,c}) letters are significantly different ($p \leq 0.05$).

the Bisphenol-A group (B: 45.3 ± 6.9 μm) ($p < 0.05$). The control (A: 37.5 ± 1.6 μm) and combined treatment (D: 37.9 ± 1.0 μm) groups did not differ significantly from any other group ($p > 0.05$). For spermatozoa head length, no significant differences were observed across the groups ($p > 0.05$), with values ranging from 225.5 ± 5.7 μm in the melatonin group (C) to 249.7 ± 5.8 μm in the Bisphenol-A group (B). Similarly, spermatozoa mid-piece length did not show significant differences among groups ($p > 0.05$), with values ranging from 293.8 ± 32.1 μm in the control group (A) to 320.0 ± 24.5 μm in the Bisphenol-A group (B). In terms of entire sperm length, the combined treatment group (D: 839.2 ± 77.2 μm) displayed a significant decrease compared to all other groups ($p < 0.05$). The control (A: 920.3 ± 47.3 μm), Bisphenol-A (B: 874.2 ± 52.4 μm), and melatonin (C: 898.6 ± 38.0 μm) groups were not significantly different from each other ($p > 0.05$) (Table 3).

DISCUSSION

Results from the present study indicated significant levels of variations, especially in the epididymal parameters for the different treatment groups. The melatonin-treated group had an epididymal germinal epithelial height that is significantly increased as compared with what was seen in both control and BPA-treated groups. Such could potentially indicate that melatonin has a stimulatory effect on the epididymal epithelium. These agree with other studies that were reported by Q i et al. [14] where melatonin promoted the proliferation and migration of the epithelial cells. The most striking result was that the epididymal luminal diameter was significantly reduced only in the BPA + melatonin group when compared to all other groups; this may be considered as a suspected synergistic action of BPA and melatonin on the epididymis, possibly affecting sperm maturation and storage. Similar structural changes in the epididymis following BPA exposure have been reported by G u r m e e t et al. [7], who observed decreased epithelial height and increased lumen diameter in rats exposed to BPA. BPA treatment significantly reduced epididymal tubular density, consistent with previous studies. For instance, O l u k o l e et al. [11] demonstrated that BPA exposure caused degenerative changes in the epididymis, including a reduced tubular density. Thus, maintenance of tubular density by melatonin treatment alone or combined with BPA at the level comparable to the control group suggests a protective role of melatonin against BPA-induced damage.

Regarding testicular parameters, no differences were statistically significant in the height of the germinal epithelium among all groups. Thus, it may indicate that neither BPA nor melatonin has succeeded in showing significant changes in the thickness of the seminiferous epithelium during this study. However, the result obtained disagreed with some earlier studies. For instance, J i n et al. [8] indicated that mice had a decrease in the height of the seminiferous epithelium following BPA exposure. There was, however, a significant decrease in the testicular luminal diameter in the melatonin-treated group when compared with the control, which was not evident in the BPA and BPA + melatonin groups. What this actually means bio-

Table 3. Effect of melatonin against Bisphenol-A induced morphometric anomalies of spermatozoa of albino rats

Parameters	Group A (mean ± SD)	Group B (mean ± SD)	Group C (mean ± SD)	Group D (mean ± SD)
Spermatozoa head diameter (μm)	37.5 ± 1.6^{abc}	45.3 ± 6.9^{ab}	34.9 ± 0.8^{c}	37.9 ± 1.0^{abc}
Spermatozoa head length (μm)	225.9 ± 10.5	249.7 ± 5.8	225.5 ± 5.7	237.0 ± 30.3
Spermatozoa mid piece length (μm)	293.8 ± 32.1	320.0 ± 24.5	305.5 ± 3.2	296.7 ± 12.6
Entire sperm length (μm)	920.3 ± 47.3^{a}	874.2 ± 52.4^{a}	898.6 ± 38.0^{a}	839.2 ± 77.2^{b}

Values with different superscript (^{a,b,c}) letters are significantly different ($p < 0.05$)

logically remains speculative and as such requires further study. Melatonin may affect the fluid dynamics in the seminiferous tubules; however, this is purely hypothetical and needs to be verified in future. BPA treatment significantly reduced testicular tubular density compared to the control and BPA + melatonin groups, aligning with previous studies showing that BPA exposure can lead to testicular damage. For example, T a i n a k a et al. [23] observed that prenatal exposure to BPA resulted in reduced seminiferous tubule volume in mice. The BPA + melatonin group had the highest tubular density, suggesting a potential protective effect of melatonin against BPA-induced testicular damage.

Regarding spermatozoa parameters, the melatonin-treated group showed a significantly reduced spermatozoa head diameter compared to the BPA-treated group, indicating that melatonin might influence sperm head morphology. No significant differences were observed in spermatozoa head length or mid-piece length across the groups. The BPA + melatonin group showed a significant decrease in entire sperm length compared to all other groups, suggesting a potential interaction between BPA and melatonin that affects sperm morphology, which could impact sperm motility and fertility.

CONCLUSION

In conclusion, melatonin mitigates some of the morphometric changes induced by BPA in male rats with respect to the maintenance of the density of both the epididymal and testicular tubules. However, the study again revealed unexpected effects when BPA and melatonin were administered together – for example, the significant reduction in the diameter of epididymal lumina and the whole length of sperm with regard to the BPA + melatonin group. This study has indeed shown the morphometric effects of BPA and melatonin on the male reproductive system, but it has not explained 'how' it is done at a molecular level. Thus, future studies may investigate the biochemical and molecular pathways through which these interactions occur and further determine the long-term implications of such morphological changes with regard to reproductive function and fertility.

ETHICAL APPROVAL

All the animals (rats) used in this study were handled in accordance with good animal practice requirements of the Animal Ethics Procedures and Guidelines with approval obtained from the Animal Care and Use Research Ethics Committee (UI-ACUREC /17/0069) of the University of Ibadan.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

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AUTHORS CONTRIBUTIONS

All authors contributed to the study revision. Material preparation, data collection and analysis were performed by Olumide Samuel Ajani, Dideolu Osunkoya, and Olumide Odunayo Akinniyi. All authors read and approved the final manuscript.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

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ORIGINAL ARTICLE

DETERMINATION OF GESTATION TIME IN DOGS BY MEASUREMENT OF FOETAL SIZE

Nela Vargová[1], Alexandra Dohnal[2], Slavomír Horňák[1], Gabriela Kacková[1], Alexandra Valenčáková[1], Ľubica Horňáková[1*]

[1]Small animal clinic, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81, Košice, Slovakia; [2]Veterinären Nära Dig, Östergatan 39, 231 44, Trelleborg, Sweden

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***Correspondence:** lubica.hornakova@uvlf.sk

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ABSTRACT

Establishing the gestation period in pregnant dogs is important to minimize neonatal loss and complications. This study aimed to predict the day of parturition using ultrasound to measure the inner chorionic cavity (ICC), biparietal parameter (BP) and crown to rump length (CRL) in 10 pregnant dogs. The measurements were taken once between days 21–34 of gestation. The results were inserted into various formulas to calculate days before parturition (DBP) and compared with actual parturition dates. The ICC measurement, using Luvoni and Grioni's and Groppetti et al.'s formulas, showed the highest accuracy (94 %). The study enhances methods for predicting parturition in dogs, improving prenatal care and reducing neonatal losses.

Key words: dog; foetal size; formula; gestation

INTRODUCTION

Determination of gestation time in pregnant bitches is crucial just as in humans, because the pregnancy is occuring during a short period of time. It is critical for the foetuses to be fully developed before parturition, to prevent and reduce the loss of neonates. An approximate due date is not only necessary for prevention of neonatal mortality, but also for planning an eventual caesarean section in case of high-risk pregnancies, or in case of oversized foetuses. The determination of gestation not only allows delivery of viable puppies, it also encourages health of the pregnant bitch [9]. This approach enables the scheduling of a planned caesarean section and also provides veterinarians with the ability to predict the day of parturition, even when the mating date is unknown. As a result, this technique improves the management of reproductive health for animals [19].

Different methods can be used to predict the time of parturition, however a combination of these methods is the key for a precise determination. Ultrasonographic examination can be performed during the first appearance of embryonic or foetal structures, radiographical examination can be used during the later stages of pregnancy, once bones are formed. During these examinations, different structures are observed in relation to the day of gestation. Ultrasonography is the most frequent and most accurate method used for the measurement of the foetuses and extra foetal structures during pregnancy. Determination of the due date after measurement of the embryos or foetuses with ultrasound is done by using special formulas. The formulas are dependent on the parameters measured by the ultrasound, but also other important factors must be taken into consideration, such as the size of the bitch. Apart from radiographic and USG examinations, hormonal assays can be performed, not only to determine the parturition date but also to determine the state of ovulation [1].

The aim of this study was to evaluate the precision of formulas suggested by Beccaglia et al. [1], Luvoni and Grioni [12], Milani et al. [13], Groppetti et al. [5], and Luvoni and Beccaglia [11], utilizing measurements of three different ultrasonographic parameters: inner chorionic cavity (ICC) measurement, crown-to-rump length (CRL) measurement and biparietal (BP) parameter. A comparison was conducted between the accuracy determined by these formulas and the actual length of gestation, with the objective of evaluating their efficacy in predicting parturition.

This suggests that employing the parameter closer to the latter stages of gestation enhances the precision of predicting the parturition.

Factors influencing gestational length in dogs

The accuracy of predicting the parturition ultimately based on the date of mating may be compromised, as a result of the variability of the gestational length among individual dogs [11]. Causes behind the variability of gestational length in bitches can be different.

Mating can take place as early as three days before ovulation [23]. However, once ovulation has taken place, the oocyst must undergo maturation to reach the suitable stage for fertilization; this process typically takes 48–72 hours [17]. It is also important to consider the fact that the spermatozoa can survive in the uterus for up to 9 days in comparison to other domestic animals. This prolonged viability of the spermatozoa can facilitate a successful fertilization of the oocyte, even in cases where natural mating has occurred 9 days before the actual ovulation, ultimately resulting in pregnancy and production of a litter [15].

It has also been demonstrated that there is a clear correlation between the litter size and gestational length [18]. In litters consisting of four puppies or fewer, there is typically an observation of prolonged gestation time, however in litters with four or more puppies, the gestational length decreases [2].

Additionally, it is important to take into consideration that gestational length and even pregnancy termination can be influenced by various factors beyond normal physiological conditions, including infections as Canine herpes virus and Brucellosis [24]. Canine herpesvirus-1 (CHV-1) is common in Europe [25] and is a virus transmitted through oronasal secretions or by venereal transmission in adult dogs, however transplacental transmission affects the foetuses and may cause embryonic resorption, abortion and stillbirth [16]. Canine Brucellosis is caused by *Brucella canis*, and is a zoonotic bacterial disease which is associated with reproductive failure in dogs characterized by abortions and infertility [6].

During the breeding period, the luteinizing hormone (LH) surge and increase in progesterone levels can offer information on the gestational length in the bitch. However, once the pregnancy is confirmed, ultrasonography stands out as the most effective tool for predicting the date of parturition [11].

Ultrasonographic examination and the appearances

Pregnancy in the bitch can be diagnosed as early as on day 18–20 through a ultrasonographic examination, which is considered being the most accurate method today [7]. This technique is particularly helpful in the assessment of gestational stage when the date of ovulation is unknown [1].

The first appearing structure that can be seen during an ultrasonographic examination after mating is the gestational sac. The gestational sac is seen on the ultrasound as an anechogenic structure as early as on day 18 after ovulation has taken place [1]. As the pregnancy progresses, the diameter of the gestational sacs increases; initially these structures are spherical and maintain the spherical shape until approximately day 25 prior to parturition. However,

if the gestational sac with the conceptus appears flattened upon the ultrasonographic examination, it indicates that the gestational age is greater than 25 days before parturition [3].

On day 23 after the ovulation, an embryo can be seen with a heartbeat inside the chorionic cavity [1]. The size of the embryo at this stage is approximately 1 cm in diameter [9]. Later, between days 27–31, the embryo changes in shape and becomes a bipolar structure with limb buds which can be detected upon ultrasonography [1].

Between days 23–26, the zonary placenta can be observed as a cylindrical structure [8].

When it comes to the detection and presence of organs, the first abdominal viscera to appear is the stomach and the urinary bladder between days 29–33. Approximately around the same time, the skeleton can be visualized in the form of a hyperechoic structure between days 29–33 after ovulation [1]. The first ossification is seen on the mandible, maxilla, frontal lobe and the clavicle, followed by ossification of the palatine, incisive, zygomatic and nasal bones around day 33 of gestation, as well as soft ossification of several long bones including humerus, radius and femur among others, can be observed [14]. Only a few days later, between day 32–34, the foetus starts to move and this can be seen on during the ultrasonographic examination [1].

Abdomen and the thorax become properly visible between days 34–36, the liver and lungs can be seen around this time as well. However, the lungs appear more hyperechoic than the liver which is a hypoechoic structure in comparison to the rest of the abdomen. Around day 41–43, the kidneys start to be visible [1].

Between days 48–57 of pregnancy lungs become more mature and the foetus should theoretically be ready for the extra-uterine life; further development of the lungs take place during the neonatal period [19].

The last organ to be seen on the ultrasound is said to be the bowel, which does not appear until the end of the pregnancy around days 57–63 of gestation [1]. Detection of the bowel around day 57 marks the end of the foetal organogenesis [4]. Between day 58 and 62 of gestation, all layers of the intestinal wall become visible and gastrointestinal peristalsis can also be seen [19]. However, the intestine surpasses the available room within the abdominal cavity around day 30, and protrude into the umbilical stalk. This phenomenon is referred to as physiological herniation [14].

Predictive tools for parturition date estimate

Precisely predicting the day or parturition based on the time of mating is not possible. Parturition prediction can be achieved through various methods, including hormonal and/or cytological approaches. However, one of the drawbacks of these methods is their expenses [20]. By measuring the extra foetal and foetal parameters with the help of ultrasound, prediction of whelping date can be determined, even if the day of ovulation is unknown. During initial stages of pregnancy, between day 19 and 37 after the LH peak, measurement of the Inner chorionic cavity (ICC) and foetal Crown-to-rump length (CRL) can be obtained [1]. Meanwhile, it is suggested that the measurement of the biparietal parameter (BP), should be obtained during the second half of pregnancy, around 25 days before whelping. This is because ossification is more clearer, making measurements more precise [21].

Measurements of the Inner chorionic cavity (ICC)

The measurement of the ICC involves taking the average of two diameters of measurement of the ICC, each measured at a 90 degree angle [11]. It has been suggested that the measurement of the ICC should be performed during the first half of the gestation, 42 to 30 days before whelping to obtain the highest accuracy [21].

Specific formulas for measurement of the ICC have been developed for dogs up to 10 kg, and for dogs with a weight of 11–25 kg. For giant breeds with a weight of above 40 kg, formula for medium sized dogs should be used, however with a deviation factor of -2 days. The precise prediction of parturition can be determined when the size-specific formulas for small and medium sized breeds are applied:

ICC in small sized bitches: DBP = (mm – 68.68)/1.53

ICC in medium sized bitches: DBP = (mm – 82.13)/1.8 [1]

In addition to formulas provided by Beccaglia et al. [1], other formulas for the measurement of the ICC in dogs have been suggested by Luvoni and Grioni [12], Milani et al. [13], and Groppetti et al. [5].

Luvoni and Grioni: DBP = 45.628–0.556 x ICC (mm)

Milani et al.: DBP = 48.121–0.5237 x ICC (mm)

Groppetti et al.: DBP = 44.76–0.434 x ICC (mm) [22]

Measurements of the foetal Crown-to-rump length (CRL)

The CRL is measured as the distance from the most rostral point of the crown to the caudal edge of the perineum [10]. This parameter can be obtained during the first half of pregnancy [1]. Equation for the prediction of gestational age is:

Days after LH peak = 24.64 + 4.54 x cm – 0.24 x cm^2 [11]

Measurements of the foetal Biparietal parameter (BP)

The BP is determined by measuring the distance between the two parietal bones [10].

Formulas for the measurement of the BP are also based on the weight of the bitch, for small breeds (up to 10 kg) and for medium-sized breeds (11–25 kg). For dogs above 40 kg of weight, formula for medium sized breeds is used with a deviation factor of -2 days [1]. Measurement of this parameter is the most accurate to predict the day of parturition during the later stages of pregnancy [11]:

BP in small sized bitches: DBP = (mm – 25.11)/0.6

BP in medium sized bitches: DBP = (mm – 29.18)/0.7 [1]

MATERIALS AND METHODS

The research sample consisted of 10 bitches of different breeds, and different age categories ranging from 2–6 years of age. All females that participated in this study had a weight between 10 kg–40 kg. Certain criteria had to be met for the females to participate in this study, including the exact date of mating (which was determined by a progesterone test) and exact day of parturition to know the exact duration of the pregnancy. All patients were admitted to the Small Animal Clinic of the University of Veterinary Medicine and Pharmacy in Košice, for the purpose of pregnancy diagnosis. Females comprising the research sample underwent a complex clinical and gynaecological examination, before the actual ultrasonographic examination was conducted. The ultrasonographic examination was performed by the ultrasound ALOKA ProSound Alpha 6 using a linear probe up to 16 MHz. All examinations were performed with the consent and authorization of the owners. The ultrasonographic examination was performed in a latero-lateral position between days 21–34 of gestation. In the research, three key parameters were used to obtain the results including the measurement of the Inner chorionic cavity (ICC), Biparietal parameter (BP) and Crown-to-rump length (CRL). The measurements were either obtained during the actual examination of the patients, through the utilization of the electronic callipers integrated in the ultrasound, or after the examination by using the same method and performing measurements on saved prints. All examinations were performed under controlled conditions to ensure accuracy and consistency of the results. Furthermore, the results of the measurements were inserted to formulas suggested by Beccaglia et al. [ICC in medium sized bitches: DBP = (mm – 82,13)/1,8 and BP in medium size bitches: DBP = (mm – 29,18)/0,7)], Luvoni and Grioni, Milani et al. and Groppetti et al., to determine the days before parturition (DBP) based on the measurement of the Intra chorionic cavity (ICC) [1], [11], [13]. Measurements of the ICC were performed across two axes to obtain an average value, which was then inserted into the formula to obtain DBP. Accuracy of the formulas was assessed by comparing the results of the formulas with reality. Beccaglia et al.'s formula for medium sized breeds was used to obtain DBP based on BP and Luvoni and Beccaglia's formula was used to obtain DBP based on the measurement of CRL [1], [11]. Furthermore, the determination of gestation time was assessed by using another formula suggested by Beccaglia et al. This mathematical formula was based on the measurement of the Biparietal parameter (BP) of the head of the foetus to determine DBP [1]. Formula for the measurement of medium sized bitches was used to obtain results. In this study, the CRL parameter was determined by using the formula suggested by Luvoni and Beccaglia to obtain days after LH peak [11].

After calculating the days after LH peak, the results were converted into DBP to obtain the results of all measurements in the same values. The objective of using these parameters was to gain an insight into the developmental stages of canine gestation and their relationship to foetal size and days before parturition.

Ultrasonographic parameters

In the research, three key parameters were used to obtain the results including the measurement of the Inner chorionic cavity (ICC), Biparietal parameter (BP) and Crown-to-rump length (CRL). The mentioned parameters were selected due to their significance in the foetal development, and they were measured by the using ultrasound imagining techniques. The objective of using these parameters was to gain an insight into the developmental stages

of canine gestation and their relationship to foetal size and days before parturition.

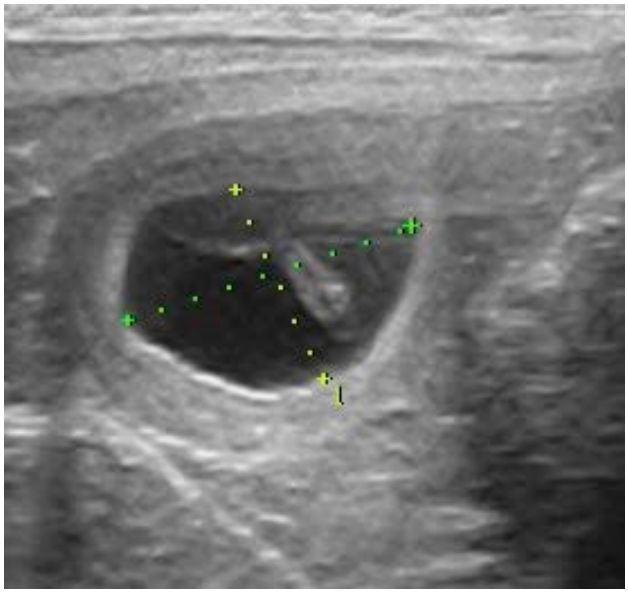

Fig. 1. Measurement of the Intra chorionic cavity (ICC).
The figure represents the ICC measurement, which refers to the space within the chorionic sac, where the embryo develops. Measurement of the longitudinal and transversal axis of the ICC was performed to obtain an average size in mm.

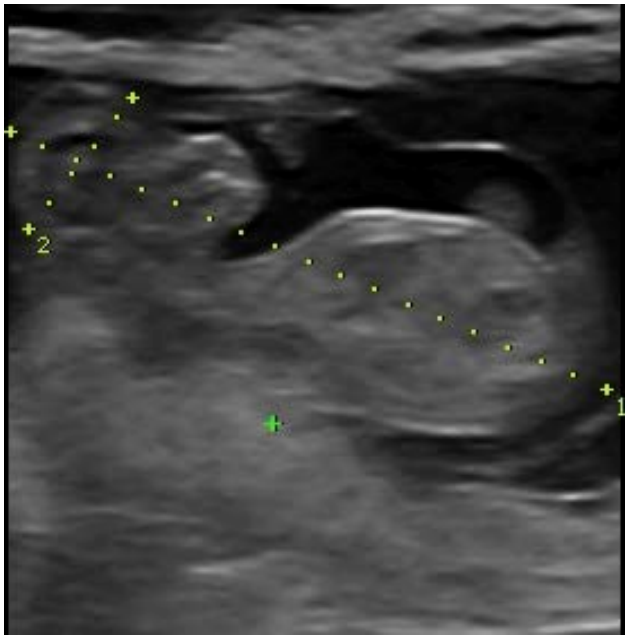

Fig. 2. Measurement of the Crown-to-rump length (1) and measurement of the Biparietal parameter (2).
The figure represents the measurements of CRL of the foetus by measuring the highest (crown of the head) and the lowest point of the body (crown) and the BP measuring the distance between the parietal bones.

Data analysis

Data analysis was performed by the utilization of four pre-existing mathematical formulas suggested by B e c c a g l i a et al. [1] (ICC in medium sized bitches), L u v o n i and G r i o n i [12], M i l a n i et al. [13] and G r o p p e t t i et al. [5] to determine the days before parturition (DBP) based on the measurement of the Intra chorionic cavity (ICC).

Measurements of the ICC were performed across two axes to obtain an average value, which was then inserted into the formula to obtain DBP. Accuracy of the formulas was assessed by comparing the results of the formulas with reality.

Table 1. Formulas used in the research to obtain DBP by the use of ICC.

Beccaglia et al.	DBP = (mm – 82,13) / 1,8
Luvoni and Grioni	DBP = 45,628 – 0,556 x ICC (mm)
Milan et al.	DBP = 48,121 – 0,5237 x ICC (mm)
Groppetti et al.	DBP = 44,76 – 0,434 x ICC (mm)

Source: Alexandra Dohnal, 2024

Furthermore, the determination of gestation time was assessed by using another formula suggested by B e c c a g l i a et al. [1]. This mathematical formula was based on the measurement of the Biparietal parameter (BP) of the head of the foetus to determine DBP. Formula for the measurement of medium sized bitches was used to obtain results.

Table 2. Formula used in the research to obtain DBP by the use of BP.

Beccaglia	DBP = (mm – 29,18) / 0,7

Source: Alexandra Dohnal, 2024

Finally, in this study, the CRL parameter was determined by using the formula suggested by L u v o n i and B e c c a g l i a (2006) to obtain Days after LH peak.

After calculating the days after LH peak, the results were converted into DBP to obtain the results of all measurements in the same values.

Table 3. Formula to obtain Days after LH peak by the use of CRL.

Luvoni and Beccaglia	Days after LH peak = 24,64 + 4,54 x cm – 0,24 x cm^2

Source: Alexandra Dohnal, 2024

RESULTS

By obtaining three different measurements of the Inner chorionic cavity (ICC), Biparietal parameter (BP) and the

Crown-to-rump length (CRL) through USG in pregnant bitches, we obtained an average size of the parameters. This allowed us to gather valuable information about the foetal development and the stage of pregnancy. Once the measurements were obtained from the USG examination, the values could be inserted into the various mathematical formulas which were designed specifically for assessment of the stage of pregnancy in the bitch based on the mentioned parameters.

Formula suggested by Groppetti et al. [5] based on the measurement of ICC, predicted the exact date of parturition in two of the ten females that participated in the study, which aligned with the actual date of delivery provided by the owner. Luvoni and Grioni's formula, which was based on the measurement of ICC as well, predicted the exact date of parturition in one of the ten females [12]. Overall average accuracy rate of these two formulas was 94 %.

Furthermore, Luvoni and Beccaglia's [11] formula for prediction of parturition based on the measurement of CRL accurately forecasted the precise delivery date in one out of the ten patients, with an average accuracy rate of 88 % [11]. However, Beccaglia's [1] formula for prediction of parturition based on the measurement of the BP did not align with the actual delivery dates in any of the cases measured. It's worth noting that this measurement was only taken in six out of the ten females, and the average accuracy rate was 85 %.

After calculation of the results obtained from the examination, with the help of the mathematical formulas suggested by Beccaglia et al. [1], Luvoni and Grioni [12], Milani et al. [13], Groppetti et al.[5] and Luvoni and Beccaglia [11], we were able to assess the days before parturition (DBP) and we could further compare the accuracy of the formulas with the actual DBP, based on the known reproductive history and day of parturition of the females.

An average of the days before parturition (DBP) was calculated based on the formulas to compare them with the average DBP based on the actual date of parturition; this to obtain the average accuracy in %.

Patient information

The following tables provide a summary of data on pregnant bitches, measured parameters, and calculations of days before parturition (DBP) using mathematical formulas by Beccaglia et al. [1], Luvoni and Grioni [12], Milani et al. [13], Groppetti et al.[5] and Luvoni and Beccaglia [11]. The tables include identification numbers and breed information of the patients, as well as recorded values of the Inner chorionic cavity (ICC), Biparietal parameter (BP), and Crown-rump length (CRL) at different stages of pregnancy. Based on these measurements, formulas from various authors were applied to predict the length of gestation, and the accuracy of these predictions was compared with the actual date of parturition. The results offer an overview of the reliability of different methods in determining the expected delivery date in bitches.

DISCUSSION

The data that were presented in the work results, give us an insight into the determination of DBP in pregnant bitches and thereby making it possible to predict the day of parturition. By the use of various mathematical formulas based on the ICC measurement, BP parameter and CRL measurement, we could determine the DBP.

Every female DBP based on ICC were calculated by using four different formulas suggested by different authors including Beccaglia et al. [1], Groppetti et al. [5] Luvoni and Grioni [12], and Milani et al. [13]. After obtaining results from each formula, we could compare them to the actual DBP using the exact known day of mating and the exact day of whelping.

In females where the BP was measurable, we used Beccaglia's formula for medium sized breeds to obtain results, as all of the dogs weighted between 10 kg and 40 kg. However, it is important to note that the BP was not measurable in all patients. This was primarily due to the fact that the examination was performed at an early stage of gestation, where obtaining precise measurements of this parameter was not possible. As a result of this, our analysis was limited to the 6 females in whom we could measure this parameter.

The CRL parameter could be measured in all 10 females, and was calculated by the formula suggested by Luvoni and Beccaglia [11], to obtain Days after LH peak, which were further converted into DBP.

However, obtaining precise measurements of this parameter was in some of the cases challenging, particular-

Table 4. Identification number, breed of patients and length of pregnancy.

Patient ID	Breed	Actual length of pregnancy (given in days)
1	Tatra hound	62
2	German shepherd	60
3	Golden retriever	66
4	Czechoslovak wolfdog	62
5	Sheltie	61
6	Rhodesian ridgeback	63
7	Bavarian mountain dog	62
8	Kooikerhondje	63
9	Hovawart	63
10	Boxer	61

Source: Alexandra Dohnal, 2024

Table 5. Day of gestation, measurement of the ICC, CRL and BP in mm.

Patient ID	Day of gestation	ICC (mm) average	CRL (mm) average	BP (mm) average
1	23	6,57 mm	7,0 mm	-
2	21	14,9 mm	9,5 mm	-
3	26	22,9 mm	25,8 mm	7,5 mm
4	21	8,4 mm	4,5 mm	-
5	26	20,1 mm	12,6 mm	5,5 mm
6	25	14,15 mm	8,6 mm	4,5 mm
7	21	9,3 mm	5,2 mm	14,0 mm
8	34	29,6 mm	24,7 mm	8,2 mm
9	21	22,7 mm	8,2 mm	-
10	21	24,35 mm	22,8 mm	6,5 mm

Source: Alexandra Dohnal, 2024

Days before parturition based on measurement of the ICC

Table 6. Calculation of DBP based on the size of the ICC according to Beccaglia, Groppetti et al., Luvoni and Grioni and Milani et al.

Patient ID	DBP based on ICC (Beccaglia)	DBP based on ICC (Groppetti et al.)	DBP based on ICC (Luvoni and Grioni)	DBP based on ICC (Milani et al.)	Actual DBP based on actual date of birth
1	42d	42d	42d	44d	39d
2	37d	38d	37d	40d	39d
3	32d	34d	33d	36d	40d
4	40d	41d	40d	43d	41d
5	34d	36d	34d	37d	35d
6	37d	38d	37d	40d	38d
7	40d	40d	40d	43d	41d
8	30d	31d	29d	32d	29d
9	33d	34d	33d	36d	28d
10	32d	34d	32d	35d	33d

d - days of pregnancy
Source: Alexandra Dohnal, 2024

Table 7. Accuracy rate of Beccaglia formula, accuracy rate of Groppetti formula, accuracy rate of Luvoni formula, accuracy rate of Milani formula and average accuracy of each formula expressed in % based on the measurement of ICC

Patient ID	Accuracy Beccaglia	Accuracy Groppetti et al.	Accuracy Luvoni & Grioni	Accuracy Milani et al.
1	92 %	92 %	92 %	87 %
2	95 %	97 %	95 %	97 %
3	80 %	85 %	83 %	90 %
4	98 %	100 %	98 %	95 %
5	97 %	97 %	97 %	94 %
6	97 %	100 %	97 %	95 %
7	98 %	98 %	98 %	95 %
8	97 %	93 %	100 %	90 %
9	82 %	79 %	82 %	71 %
10	97 %	97 %	97 %	94 %
Average accuracy	93 %	94 %	94 %	91 %

Source: Alexandra Dohnal, 2024

Days before parturition based on measurement of the BP

Table 8. Calculation of DBP based on the size of the BP parameter according to Beccaglia et al.

Patient ID	DBP based on BP (Beccaglia et al.)	Actual DBP based on actual date of birth	Accuracy Beccaglia et al.
3	31d	40d	78 %
5	34d	35d	97 %
6	35d	38d	92 %
7	21d	41d	51 %
8	30d	29d	97 %
10	32d	33d	97 %
Average accuracy			85 %

d - days of pregnancy
Source: Alexandra Dohnal, 2024

Days before parturition based on measurement of the CRL

Table 9. Calculation of "Days after LH peak" by Luvoni and Beccaglia based on the size of CRL, converted values to DBP and actual DBP based on the known date of birth. (Luvoni and Beccaglia, 2006)

Patient ID	Days after LH peak (Luvoni and Beccaglia	Converted values to DBP	Actual DBP based on actual date of birth	Accuracy
1	27d	35d	39d	90 %
2	28d	32d	39d	82 %
3	34d	32d	40d	90 %
4	26d	36d	41d	88 %
5	31d	30d	35d	86 %
6	28d	35d	38d	92 %
7	26d	36d	41d	88 %
8	34d	29d	29d	100 %
9	28d	35d	28d	75 %
10	33d	28d	33d	85 %
Average accuracy				88 %

d - days of pregnancy
Source: Alexandra Dohnal, 2024

ly during the later stages of gestation, as the entire foetus could not be completely fitted within a single frame.

Accuracy of DBP prediction based on measurement of the ICC

Following the measurements of the ICC, we proceeded to calculate the average accuracy rate of the formulas proposed by the different authors, aiming to compare them with the actual DBP based on the exact date of mating.

The analysis demonstrated that the formulas suggested by Luvoni and Grioni [12] and Groppetti et al. [5] were the most accurate. Both formulas exhibited an average accuracy rate of 94 %, which suggests that they are particularly reliable in estimating the DBP and prediction of parturition. It is however essential to take into consideration the fact that all four formulas yielded an average accuracy rate exceeding 90 %, where formula suggested by Beccaglia [1] yielded an average accuracy of 93 % and formula by Milani et al. [13] yielded an average accuracy of 91 %.

The high average accuracy rates of all the formulas, surpassing the 90 % threshold, may be attributed to the fact that the parameters were measured during the first half of gestation (between day 21 and 34 of pregnancy), which lasted till day 37 as suggested by Beccaglia et al. [1]. Beccaglia et al. [1] also states that the most accurate prediction based on the ICC parameter can be obtained during this time of gestation, which has been confirmed by our results.

It is further suggested by Luvoni [10] that the most accurate prediction can be done, when the bitch is examined between day 20–30 or day 31–43 of gestation.

Measurement of particularly patient number 8 was performed during a later stage of gestation (on day 34), in comparison to the other patients. A 100 % accuracy rate was calculated using the formula proposed by Luvoni and Grioni [12], which suggest that this statement is correct.

However, patient number 9 exhibited the lowest accuracy rates across all of the formulas, varying from 71–82 %, despite the fact that the measurement was obtained during the first half of pregnancy, on day 21, as suggested by the previously mentioned authors. These lowered accuracy rates may be attributed to several factors including the litter size, as suggested by Eilts et al. [2], but also other variations in the canine physiology. Other factors that may have lowered the accuracy include subjective interpretation and difficulty in obtaining precise measurements of this parameter. However, it seems less likely that the accuracy rates stem directly from the formulas themselves, given the fact that all formulas exhibited lower accuracy rate. Therefore, it is more probable that the discrepancies arise either from the measurement technique or inherited physiological variations of the dog itself, in this particular patient.

Accuracy of DBP prediction based on measurement of the foetal CRL

Another method employed in this study to determine the DBP and compare them with the reality, was the measurement of the foetal CRL, followed by the use of formula proposed by Luvoni and Beccaglia [11]. The accuracy rate of this parameter ranged between 75–100 % with an average accuracy rate of 88 %. Once again, it was suggested by different authors including Beccaglia et al. [1] that this measurement should be obtained during the first half of pregnancy. However, Siena and Milani [19] states that the measurement of the CRL should not be performed earlier than day 26, and later than day 45 of pregnancy, as the body of the foetus undergoes a change and the measurement is less accurate. Siena and Milani [19] further states that the optimal time for the CRL measurement is on day 30 of gestation. This statement finds support in our study as evidenced by the lowest accuracy rates observed in Table 9.

Patient number 9, where the measurement of CRL was conducted on day 21 of gestation, exhibits an accuracy rate of 75 %. Additionally patient number 2, also measured on day 21 of gestation, exhibits the second lowest accuracy at 82 %. This statement can also be viewed from the other perspective, where the patient number 8, who was measured on day 34 of gestation, shows a 100 % accuracy rate, followed by patient number 6, measured on day 25 of gestation, exhibiting the second highest accuracy rate of 92 %. Based on our observation, it appears that the accuracy of predictions increases as we approach day 30 of pregnancy. Despite this, there are patients in our study who do not support this statement. A comparison of patient number 3 and patient number 5 can be done, where both were measured on day 26 of pregnancy, however a difference was observed in these patients, where number 3 yielded a higher accuracy by 4 % (90 %). This lower accuracy rate can once again be attributed to different factors including the

litter size and physiological differences among dogs, maybe even breed and size, as patient 3 is a Golden Retriever and patient 5 is a Sheltie.

In general, the lower average accuracy rate of 88 % in our study may not only be attributed to the timing of the measurement before day 30 of pregnancy, several other factors may have contributed to this trend. For instance, the positioning and movement of the foetus during the ultrasonographic examination can significantly affect the clarity and precision of the measurement of the CRL. Additionally, during the second half of gestation, after day 37, it may become more challenging to capture the entire foetus in a single frame due to its larger size and increased mobility. Hence, the timing timing of measurement of this parameter is crucial.

Accuracy of DBP prediction based on measurement of the foetal BP

The third and final method utilized in this study for determining the DBP and subsequently comparing it with the actual DBP involved the assessment of the BP.

Measurement of the BP parameter was conducted in only 6 out of the 10 females participating in this study and a 85 % average accuracy rate was calculated. This limitation was a result of the fact that the ultrasonographic examinations of the bitches were performed during an early stage of pregnancy, which made it challenging to obtain this parameter. Furthermore, even among patients in whom the measurement was performed, its precision was compromised, as indicated by the results in Table 8. In comparison to the two other parameters used in this study, which each yielded at least one 100 % match with the actual DBP, the Biparietal parameter did not achieve a single instance of perfect alignment with reality. We believe that this discrepancy primarily stems from the fact that the measurements were obtained early in gestation, spanning from day 21 to day 34 as indicated in Table 5.

According to Siena and Milani [19], the optimal time for obtaining the Biparietal parameter is from day 30 of gestation, with even better results anticipated if the measurement is acquired from day 35 and later, when the parietal bones are more visible.

In our study, a relatively clear trend can be seen which indicates that the accuracy of the parameter is higher as the gestational length progresses. This suggests that measurements obtained closer to day 30 of pregnancy yield more accurate results. This phenomenon can particularly be seen in patient number 5, 6 and 8, where the accuracy rate is above 90 %. However, in two of the patients (7, 10), BP was measured on day 21 of gestation. In patient 7, a 51 % accuracy was calculated, and in patient 10, a 97 % accuracy rate was calculated despite the fact that the measurement was performed early during the pregnancy. This suggests that a larger sample size would enhance the objectivity and, more importantly, the accuracy of the study. However, our study was limited in this regard, particularly concerning the Biparietal parameter, as it was only measured in 6 out of the 10 dogs.

Our study further indicates the importance of utilizing the BP later in gestation, aligning with the recommendations of previously mentioned authors. This suggests that employing the parameter closer to the latter stages of gestation enhances the precision of predicting the parturition.

CONCLUSION

In conclusion, this study investigated the accuracy of formulas proposed by Beccaglia et al., Luvoni and Grioni, Milani et al., Groppetti et al., and Luvoni and Beccaglia for estimating DBP in dogs, utilizing three different parameters including the Inner chorionic cavity (ICC), foetal Crown-to-rump length (CRL) and the Biparietal parameter (BP) obtained through ultrasonographic examination.

Our analysis revealed that the measurement of the Inner chorionic cavity (ICC) achieved the highest accuracy rate when utilizing the formulas of Luvoni and Grioni, and Groppetti et al., exhibiting a 94 % accuracy rate. Following were the formulas proposed by Beccaglia et al. with a 93 % accuracy rate and Milani et al. with a 91 % accuracy rate. These findings suggest that measuring the Inner chorionic cavity during the early stages of pregnancy can serve as a reliable method for predicting parturition.

Furthermore, our study revealed that the CRL measurement exhibited an accuracy rate of 88 %, slightly lower than that of the ICC. Importantly, our findings highlighted the significance of timing in obtaining accurate measurements. Specifically, measurements taken closer to day 30 of pregnancy demonstrated a higher accuracy rate, aligning with recommendations from Siena and Milani.

Lastly, the BP demonstrated an average accuracy rate of 85 %, which was the lowest among the three parameters. This may be attributed to the fact that the majority of measurements were obtained before day 30 of gestation, when the parietal bones were less visible. Additionally, the lower accuracy rate could be influenced by the limited amount of patients, as we performed this measurement in only 6 out of the 10 females. Notably, accuracy tended to increase in patients closer to the day 30 of gestation.

Ultimately, our study highlights that the most accurate prediction of parturition can be determined by the measurement of the Inner chorionic cavity (ICC) during early gestation. Furthermore, our findings emphasize the importance of timing in obtaining accurate measurements, especially with regards to the BP and CRL, aligning with recommendations from prior research. Further investigation into refining measurement techniques could benefit from considering variables such as weight categories, breed of the dogs and size of the litter. By exploring these factors, we may gain a deeper insight into how variations in canine physiology influence the accuracy of prenatal measurements and their predictive value for parturition. Such a comprehensive approach could contribute significantly to enhancing the precision and reliability of early gestational assessments in dogs.

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CONFLICT OF INTEREST

We declare that all listed authors are without a Conflict of Interest.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

ETHICAL STATEMENT

This study did not require any Ethical approval.

AUTHORS CONTRIBUTIONS

Ľ. H.: Conceived and designed the study, developed the research methodology, supervised the project execution, contributed to defining the research objectives and study parameters; N. V.: performed ultrasonographic examinations, collected measurements (ICC, BP, CRL), ensured data accuracy and consistency, assisted in refining the methodology based on initial findings; A. D.: conducted statistical analyses, compared predictive formulas with actual parturition dates, interpreted the accuracy of different methods, played a key role in data processing and validation; G. K.: conducted literature review, provided background context, contributed to the interpretation of findings, assisted in drafting the introduction and discussion sections; A. V.: drafted the manuscript, ensured scientific rigor, clarity, and coherence, provided insights into improving data presentation and refining conclusions; S. H.: critical revision of the manuscript, final editing, supervision, project administration, coordinated team efforts, provided final approval for manuscript submission and managed funding acquisition. All authors contributed significantly to the study and approved the final version of the manuscript.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

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CASE REPORT

NOTIFICATIONS OF FEATHER MITES (ASTIGMATA: PTEROLICHIDAE) FROM THE EURASIAN COOT *FULICA ATRA* (GRUIFORMES: RALLIDAE) IN TÜRKIYE: *GRALLOBIA FULICAE* (TROUESSART, 1885) AND *GRALLOLICHUS PROCTOGAMUS* (TROUESSART, 1885)

Gökhan Eren[1]*, Furkan Eren[2]

[1]Department of Aquatic Animal Health, Mediterranean Fisheries Research, Production and Training Institute, Antalya, Türkiye; [2]Sakarya University, Institute of Natural Sciences, Sakarya, Türkiye

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***Correspondence:** gokhaneren54@gmail.com

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ABSTRACT

Feather mites (Astigmata: Analgoidea and Pterolichoidea) are external symbionts that inhabit the feathers and skin of avian hosts. Studies on detecting feather mite fauna of avian hosts in Türkiye have increased in recent years, but they are still not at the desired level. The material of this study consists of feather mite specimens collected from the wing and tail feathers of an Eurasian coot (*Fulica atra*) cadaver that was found dead in a natural area and subjected to ectoparasitic examination. As a result of detailed microscopic examination carried out in the light of the keys and illustrations in the relevant literature, the mite specimens were identified as *Grallobia fulicae* (Trouessart, 1885) and *Grallolichus proctogamus* (Trouessart, 1885). Both species are new records for the feather mite fauna of Türkiye.

Key words: bird parasites; faunistic records; new records

INTRODUCTION

Feather mites (Astigmata: Analgoidea and Pterolichoidea) are a group of ectosymbiotic organisms with high species diversity and density that live on the feathers and skin of their avian hosts. There are also species that settle in the respiratory system (Cytoditidae and Turbinoptidae). These mites, which have high host specificity, cannot survive outside the host because they are dependent on their hosts (permanent-obligate) [3, 7, 12]. The symbiotic relationship of feather mites with birds is somewhere between commensalism and parasitism. Transmission is mostly

vertical, occurring through contact during parental care and mating [10, 13].

Fulica atra (Gruiformes: Rallidae), also known as the Eurasian coot or common coot (''sakarmeke'' in Turkish), is a water bird with a wide distribution range across the world (Eurasia, Western Sahara, Australia, and New Zealand), consisting of four taxonomic subspecies: *Fulica a. subsp. atra*, *Fulica a. subsp. lugubris*, *Fulica a. subsp. novaeguineae*, and *Fulica a. subsp. australis* [11, 14].

In the present work, we report detected *Grallobia fulicae* (Trouessart, 1885) and *Grallolichus proctogamus* (Trouessart, 1885) species from the Eurasian coot (*Fulica atra*) (Gruiformes: Rallidae) in Sakarya province, Türkiye.

MATERIALS AND METHODS

In December 2024, a dead Eurasian coot (*Fulica atra*) carcass found near Lake Sapanca was examined for feather mites. As a result of the examination, feather mite infestation was detected, and then mite specimens were collected with blunt-ended forceps and stored in Eppendorf tubes containing 70% Ethanol. For identification purposes, representative numbers of mite specimens were first cleared with Lactophenol for 24–48 hours and then mounted on glass slides with Hoyer's solution. Finally, species identification of mite specimens was performed under a light microscope (CX23 Binocular Microscope, Olympus Corporation, Tokyo, Japan) using keys in the relevant literature [4, 9]. Additionally, permanent glass slides of the identified species are deposited in G. Eren's personal collection.

RESULTS

As a result of microscopic examination, the mite specimens were identified as *Grallobia fulicae* (Trouessart, 1885) (Fig. 1. A-D) and *Grallolichus proctogamus* (Trouessart, 1885) (Fig. 1. E-I) from the Pterolichidae. Since no data could be determined for these two mite species in Türkiye in the literature research, both mite species are new records for the feather mite fauna of Türkiye.

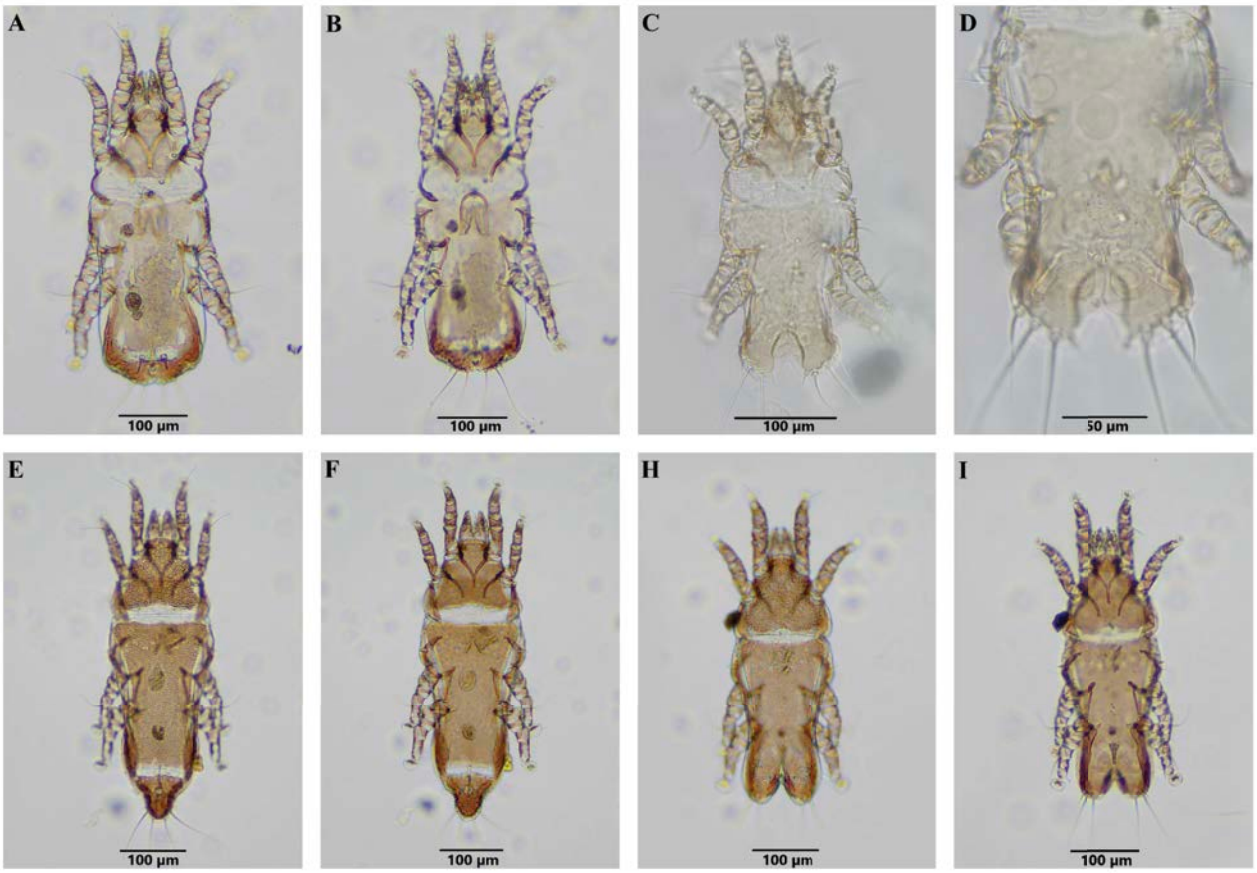

Fig. 1. Feather mites identified from the Eurasian coot (*Fulica atra*): *Grallobia fulicae*, female, dorsal (A) and ventral (B); *G. fulicae*, male, dorsal (C) and ventral (D); *Grallolichus proctogamus*, female, dorsal (E) and ventral (F); *G. proctogamus*, male, dorsal (H) and ventral (I).

DISCUSSION

Grallobia Hull, 1934 and *Grallolichus* Gaud, 1960 genera identified in the present study are currently classified in Pterolichidae Trouessart & Mégnin, 1884 [7]. The *Grallobia* genus consists of 10 species associated with rails (Rallidae) and flufftails (Sarothruridae) [5], while the *Grallolichus* genus consists of 16 species associated with rails (Rallidae), flufftails (Sarothruridae), finfoots (Heliornithidae) and jacanas (Charadriiformes: Jacanidae) [2].

Grallobia fulicae was first described by Trouessart (1885) [15] based on specimens collected from the Eurasian coot (*Fulica atra*) in Europe. It was subsequently reported from the same host in Russia (formerly USSR) [4], France [6], Botswana (formerly Bechuanaland), Congo and Rwanda [5] and Korea [9].

Similarly, *Grallolichus proctogamus* was first described by Trouessart (1885) [15] based on specimens collected from the Eurasian coot (*F. atra*) in Europe. Thereafter, it has been reported from the same host in Russia (formerly USSR) [4], Bulgaria [16], Czechia [1], France [6], Botswana (formerly Bechuanaland) and Rwanda [5] and Korea [9]. In previous studies conducted in Türkiye, only *G. minutus* Gaud et Mouchet, 1963 from the genus *Grallolichus*, has been reported in association with the Western swamphen (*Porphyrio porphyrio*) [8].

CONCLUSIONS

This study aims to report feather mite specimens collected from a dead host, as stated in the material method section. In conclusion, with this paper presentation, *Grallobia fulicae* and *Grallolichus proctogamus* were reported for the first time in Türkiye. Studies based on feather mite specimens collected from spontaneously developing, i.e. dead avian hosts, contribute to the diversity of the feather mite fauna in Türkiye, although not as significantly as comprehensive studies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this short communication/case report.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

ETHICAL STATEMENT

Ethical approval is not required as the study material consists of parasite specimens collected on the dead bird carcass.

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AUTHORS CONTRIBUTIONS

Gökhan Eren: Methodology, conception/design of the study, data acquisition, identification, drafting the manuscript, final approval and accountability. Furkan Eren: Methodology, collection of specimens, preservation, transportation, field work.

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ORIGINAL ARTICLE

HABITUATION MITIGATES ADVERSE EFFECTS OF HEAT STRESS IN BROILER CHICKEN DURING THE HOT-DRY SEASON

Tunde Ogundeji[1]*, Joseph Olusegun Ayo[2]

[1]Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, University of Benin, Benin City, PMB 1154, Benin City, Nigeria; [2]Department of Veterinary Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, P. O. Box 2555, Zaria, Nigeria

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***Correspondence:** tunde.ogundeji@uniben.edu

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ABSTRACT

Heat stress is a significant challenge in broiler chicken production, particularly in tropical and subtropical regions. This study evaluates the effectiveness of habituation in mitigating adverse effects of heat stress in broiler chickens during the hot-dry season. Sixty-day-old male broiler chicks (Arbor Acres) were randomly divided into control (n = 30) and habituated groups (n = 30). The habituated group was subjected to 4 days of habituation training from days 17 to 20. The two groups were subjected to tonic immobility (TI) test on day 21. Heterophil-to-lymphocyte (H:L) ratio, serum heat-shock protein 70 (Hsp70) expression, and erythrocyte osmotic fragility were assessed after the TI test. The results indicated that the thermal environment during the study period was characterised by high dry-bulb temperatures and high temperature-humidity index (THI), indicating significant thermal challenge on the broiler chickens. Habituated chickens showed significantly reduced TI durations compared to control. The H:L ratio and serum Hsp70 concentrations were markedly decreased ($P < 0.05$) in habituated chickens relative to controls. There was decreased ($P < 0.05$) erythrocyte membrane fragility in the habituated group, with reduced haemolysis compared to the control group. In conclusion, these findings show that the adverse effects of heat stress during the hot-dry season was mitigated in the habituated broiler chickens, as evidenced by reduced TI durations, a lower H:L ratio, decreased serum Hsp70 concentrations, and enhanced erythrocyte membrane stability. The study highlights the importance of habituation as a behavioural strategy in reducing sensitivity to heat stress in broiler chickens.

Keywords: erythrocyte osmotic fragility; habituation; heat-shock proteins 70; heterophil-lymphocyte ratio; tonic immobility

INTRODUCTION

Habituation in broiler chickens involves adaptation to repeated exposure to stimuli [22]. While initial exposure to a stressor typically elicits strong behavioural and physiological responses, subsequent exposures often result in a diminished response, indicating habituation [28]. Unlike associative learning, which involves linking two or more stimuli, habituation is a non-associative learning process that enables organisms to filter out irrelevant or repetitive information, thereby optimising energy for productive purposes [10, 24]. It plays a crucial adaptive role by mitigating the negative effects of chronic stress [17, 18]. Previous studies have explored the use of habituation to reduce adverse stress responses in poultry. For example, J o n e s [19] highlighted the importance of habituation to reduce fear and stress responses in poultry when exposed to humans, suggesting that regular, positive human interaction can lower stress-induced behaviours. Additionally, habituation through regular handling has been shown to improve the behavioural responses of laying hens, resulting in a decrease in stress-related behaviours [5]. However, a significant gap in the literature exists, particularly regarding the influence of habituation on poultry during thermal environmental stress conditions. In the Northern Guinea Savannah zone of Nigeria, the seasonal environmental temperature fluctuations during the hot-dry season pose significant challenges for broiler chickens [3]. High ambient temperature and relative humidity (RH) during the season are important thermal environment factors that cause heat stress, which is a major issue in broiler production in tropical and subtropical regions.

Neurologically, habituation reduces the activation of the hypothalamic-pituitary-adrenal (HPA) axis, a key component of the body's stress response system. The dampening effect of habituation protects the body against the adverse effects of prolonged stress [23]. Studies have shown that habituation mechanisms in animals help modulate stress responses, preventing the unnecessary depletion of resources and enabling adaptive responses to genuine threats [17]. Habituation to repetitive handling in chickens can lead to reduced physiological stress, enhancing overall welfare and resilience [20, 28]. Stress response in broiler chickens can be effectively measured via fear-related behavioural tests such as tonic immobility (TI) tests [30], and the TI test has been modified to study habituation in broiler chickens [24]. Studies have shown that broiler chicken exhibiting reduced TI durations indicates enhance welfare [13]. Thus, this study aimed to investigate the mitigating effects of habituation in broiler chickens during environmental heat stress conditions such as the hot-dry season.

MATERIALS AND METHODS

Experimental site and location

The experiment was performed in the poultry house of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria (110 10' N, 070 38' E), located in the Northern Guinea Savannah Zone of Nigeria. The study was carried out from March to April, during the hot-dry season [3].

Experimental flock and management

A total of sixty-day-old male chicks (Arbor Acres) were used for the experiment. They were raised on a deep litter system and properly identified via tags. They were fed a standard diet from day 1 to day 21 (Table 1). The broiler chickens were given access to water and feed *ad libitum*. Biosecurity measures were followed throughout the experiment.

Table 1. Nutrient contents of the broiler chickens feed

Nutrient contents	Amount
Metabolisable energy (kcal.kg^{-1})	2800
Proximate analysis* (%):	
i) Dry matter	97.50
ii) Crude protein	24.74
iii) Crude fibre	4.61
iv) Oil	3.40
v) Ash	4.50
vi) Nitrogen-free extract	62.75

* Analysed in the biochemical laboratory, Department of Animal Science, Ahmadu Bello University, Zaria, Nigeria

Thermal microenvironmental parameters

The dry- and wet-bulb temperatures of the micro-environment of the poultry pen were measured three times daily using a dry- and wet-bulb thermometer (Aura Labtech, India) at 07:00 h, 13:00 h, and 19:00 h (GMT+1) from days 8 to 28. From the data, the RH was calculated each day using an online relative humidity calculator (www.1728.org/relhum.htm). The temperature-humidity index (THI) on each day was determined using the following formula as described by T a o and X i n [29]:

$$THI = 0.85 \times DBT + 0.15 \times WBT$$

(where DBT = dry-bulb temperature and WBT = wet-bulb temperature).

Experimental design and groupings

Sixty-day-old broiler chicks were divided into two groups of 30 chickens. Using simple random sampling, the birds were assigned to control and habituated groups. Each broiler chicken in the habituated group was subjected to 4-days of habituation training from day 17 to 20 as described by Nash and Gullap [24]. Briefly, the habituation training consists of manually restraining a bird on dorsal recumbency for 15 seconds in a U-shaped cradle covered with cloth by trained personnel. The duration of each resulting immobility episode was terminated after about 15 seconds by gentle prodding. Each bird was given five inductions per day, with a 15-second intertrial interval. On day 21, all broiler chickens in both groups were subjected to TI test. The habituation training and tonic immobility test were conducted once on each of the designated days and commenced at 07:00 h (GMT+1). On day 21, a total of 2 mL of blood per bird was collected from 7 broiler chickens per group via the wing vein, after the TI test, into anticoagulant (K_2EDTA) and anticoagulant-free sample bottles. The blood samples containing anticoagulant were taken immediately to the laboratory for the determination of heterophil and lymphocyte count, and erythrocyte osmotic fragility (EOF). The anticoagulant-free sample bottles containing blood were allowed to clot for 10–20 minutes at room temperature and were subsequently centrifuged at 2000 ×g for 20 minutes to obtain the serum. The separated serum was frozen until the determination of Hsp70 expression.

Tonic immobility test

The TI test was carried out as described by Ogundeji and Ayo [25].

Determination of heterophil-lymphocyte ratio

Heterophil and lymphocyte counts were determined using conventional staining techniques [8]. From the data, H:L ratios were obtained.

Determination of serum heat shock protein 70 expression

The serum level of Hsp70 was determined using a commercially available ELISA kit (Wuhan Fine Biotech Co., Ltd., Wuhan, China) as described by the manufacturer's protocol.

Determination of erythrocyte osmotic fragility

The EOF was determined according to the method described by Faulkner and King [12] on day 21. Briefly, 0.02 ml of blood collected from each bird in each group were added to tubes, containing increasing concentrations (0, 0.1, 0.3, 0.5, 0.7, and 0.9%) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4. The tubes were gently mixed and incubated at room temperature (25–26 °C) for 30 minutes. The contents in each tube were then mixed and centrifuged at 400 ×g for 10 minutes, after which the supernatant was decanted. The optical density of the supernatant was determined spectrophotometrically at 540 nm. The degree of haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0 % NaCl) as 100 %. The percentage haemolysis was calculated using the following formula:

$$\text{Percentage haemolysis (\%)} = \frac{\text{Optical density of test solution x 100}}{\text{Optical density of standard solution}}$$

Data analyses

The data obtained were expressed as mean ± standard error of the mean (mean ± SEM). The TI duration was analysed using the Mann-Whitney test. The H:L ratio, Hsp70, and EOF were analysed using the Students' t-test. Values of $P < 0.05$ were considered significant. The analyses were performed using GraphPad 8.02 for Windows (San Diego, CA, USA).

RESULTS

Thermal micro-environment parameters during the study period

The thermal environment parameters inside the broiler chickens' house during the study period are presented in Table 2. The DBT and THI range from 24.14 ± 0.66 °C to 38.29 ± 0.97 °C and 23.83 ± 0.66 °C to 37.02 ± 0.98 °C, respectively. RH fluctuated between 55.71 ± 5.57 % and 23.83 ± 0.66 %. The overall average daily DBT, RH, and THI during the study period were 31.38 ± 0.61 °C, 64.29 ± 2.84 %, and 30.50 ± 0.61 °C, respectively.

Table 2. Thermal environment parameters inside the broiler chickens house during the study period

Parameter	Hour of the Day			
	7:00 h	13:00 h	19:00 h	Average Daily
DBT (°C)	24.14 ± 0.66 (19 – 28)	38.29 ± 0.97 (32 – 43)	31.71 ± 0.90 (27 – 38)	31.38 ± 0.61 (27.33 – 33.67)
RH (%)	82.97 ± 1.84 (68.50 – 92.00)	54.50 ± 3.51 (35.30 – 93.80)	55.71 ± 5.57 (16.70 – 79.90)	64.29 ± 2.84 (43.20 – 78.90)
THI	23.83 ± 0.66 (18.85 – 27.70)	37.02 ± 0.98 (31.10 – 41.65)	30.64 ± 0.80 (25.95 – 35.75)	30.50 ± 0.61 (26.68 – 32.97)

Values in parentheses are the minimum and maximum. DBT: Dry-bulb temperature; RH: Relative humidity; THI: Temperature-humidity index; *n* = 14 days.

Tonic immobility duration of the broiler chickens

The control group exhibited a significantly ($P < 0.05$) longer TI duration of 81.42 ± 12.80 seconds compared to the habituated group, which had a TI duration of 40.61 ± 4.56 seconds (Fig. 1).

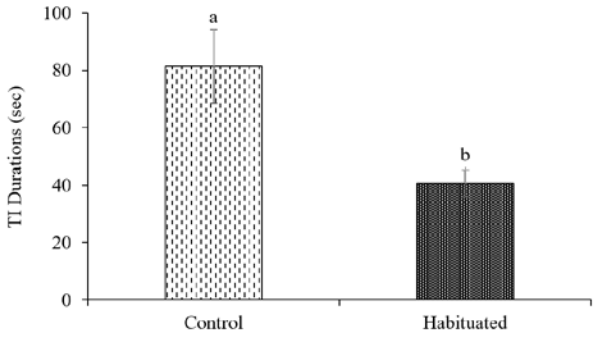

Fig. 1. Effects of habituation on tonic immobility (TI) duration in broiler chickens during the study period

Heterophil-to-lymphocyte (H:L) ratio in the broiler chickens

A significant decrease ($P < 0.05$) in the heterophil-to-lymphocyte (H:L) ratio was recorded in the control group compared with the habituated group (Table 3).

Table 3. Effects of habituation on haematological responses in broiler chickens during the study period

Parameters	Control	Habituated
Heterophil (× 10^3 µL)	1.36 ± 0.11	0.97 ± 0.20
Lymphocyte (× 10^3 µL)	6.36 ± 0.41	6.23 ± 1.03
H:L Ratio	0.22 ± 0.03^a	0.14 ± 0.01^b

^{ab} Means for the same parameter having different superscript letters across the row are significantly ($P < 0.05$) different; H/L: heterophil lymphocytes ratio.

Serum heat shock protein 70 concentration in broiler chickens

The results showed a significant ($P < 0.05$) decrease in the serum heat-shock protein 70 (Hsp70) concentration in the habituated group of broiler chickens compared with the control group (Fig. 2). The habituated group presented an Hsp70 concentration of 5.90 ± 0.27 ng.mg^{-1}, whereas the control group presented a higher concentration of 6.66 ± 0.15 ng.mg^{-1}.

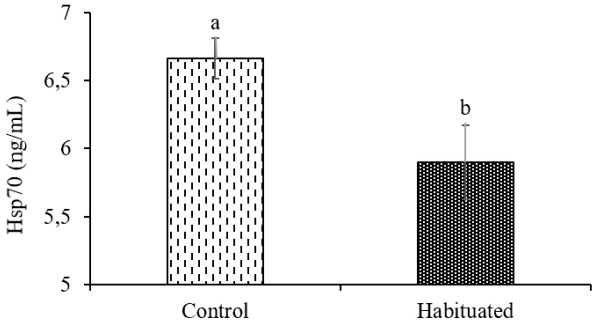

Fig. 2. Effects of habituation on serum heat-shock protein 70 concentration in broiler chickens during the study period

Erythrocyte osmotic fragility in broiler chickens

Erythrocyte osmotic fragility in broiler chickens was assessed during the study period (Fig. 3). At a NaCl concentration of 0.7 %, the percentage haemolysis recorded in the habituated group was significantly lower ($P < 0.05$), measuring 4.15 ± 0.42 %, compared to the control group, which presented a higher haemolysis value of 7.65 ± 0.64 %. No significant differences in haemolysis were detected across the experimental groups at NaCl concentrations of 0.9 %, 0.5 %, 0.3 %, and 0.1 %.

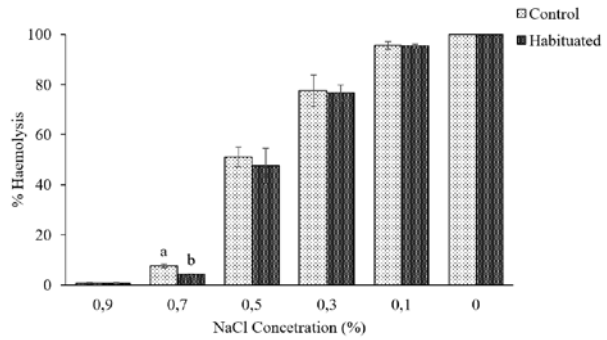

Fig. 3. Effects of habituation on erythrocytes osmotic fragility in broiler chickens during the study period

DISCUSSION

The results of this study highlight the impact of the Northern Guinea Savannah's thermal microenvironment on broiler chickens during the hot-dry season, revealing that the birds were exposed to significant heat stress. The fluctuations in DBT ranging from 24.14 ± 0.66 °C to 38.29 ± 0.97 °C, along with RH levels between 54.50 ± 5.57 % and 82.97 ± 1.84 %, indicate the presence of both mild and extreme heat conditions that impede the chicken's ability to thermoregulate. This aligns with previous studies [31], which reported that broilers in similar climates experience considerable physiological stress, diverting energy from growth to thermoregulation. The study's findings on the THI, with values ranging from 23.83 ± 0.66 °C to 37.02 ± 0.98 °C, suggest that broilers were subjected to moderate to severe heat stress, particularly as THI values above 30 °C are considered critical for poultry [21, 29]. These results emphasise the necessity for effective heat mitigation strategies in poultry management.

The result of the present study indicates that habituated broiler chickens exhibit reduced TI duration when subjected to TI test. This is in agreement with the report by N a s h and G u l l a p [24], who demonstrated that habituation reduces the TI response in broiler chickens. The TI test is a fear-realated behavioural test capable of activating the stress response mechanism in chickens [13]. Several studies have shown a link between stress and fear responses in poultry. For example, H e m s w o r t h et al. [17] reported that handling stress impacts fear responses and physiological stress indicators in broiler chickens. S u l i m o v a et al. [27] further highlighted that chronic stress influences physiological responses and emphasised the physiological impact of fear. D e H a a s et al. [9] illustrated these associations in commercial laying hens, revealing that heightened fear responses correspond with physiological stress indicators. Thus, this finding of the present study suggests that the habituation may be reducing the physiological burden on the animal stress mechanism. Since the neurobiology of fear and stress response is superimposed, the damping effects of habituation in the broiler chickens may cause a decrease in stress response during the hot-dry season.

The H:L ratio is a reliable and non-invasive marker for chronic stress [16]. In this study, a significant difference was observed in the H:L ratio between the habituated group and the control group. The H:L ratio in the habituated group was significantly lower compared to the control group, suggesting that habituation helped reduce the physiological stress response of broilers to heat stress. These findings align with previous research where stress mitigation strategies have been shown to reduce the H:L ratio in broilers. For instance, A l t a n et al. [2] reported that broilers subjected to heat stress exhibited a lower H:L ratio when provided with preconditioning or acclimatisation techniques. Similarly, habituation to stressors, such as human handling or sound exposure, could decrease the H:L ratio in chickens, implying that repeated exposure to mild stressors can desensitize the birds to more severe stressors [7]. The reduction in the H:L ratio observed in the habituated group in the present study indicates that by gradually exposing the birds to controlled fear stimuli, the stress response may have been damped, resulting in lower heterophil counts relative to lymphocytes.

The observed reduction in Hsp70 concentrations in the habituated group provides evidence for a reduced physiological stress response resulting from habituation. Typically, elevated ambient temperatures or stressful conditions trigger an increase in Hsp70 expression as part of the chicken's defence mechanism to maintain cellular homeostasis [6]. However, the reduced Hsp70 levels in the habituated group suggest that habituation might alleviate the need for an intense stress-induced response, thereby diminishing the physiological burden on the birds. This finding aligns with previous research that has explored the stress-mitigating strategies in poultry. For example, G o u d a et al. [14] emphasise that heat shock proteins, including Hsp70, are critical components of the chicken's defence system against thermal stress. However, this study's results indicate that habituation – a process by which birds become accustomed to stressors through repeated

exposure – can also play a significant role in reducing the expression of these proteins. This could be because the habituated group, through habituation, experienced less fear and anxiety, which in turn mitigated their overall stress response, as evidenced by the lower Hsp70 levels. Additionally, Balakrishnan et al. [4] highlights that Hsp70 serves as a key indicator of how broilers respond to stress at a molecular level. The lower Hsp70 levels in the habituated group suggest that psychological conditioning can influence these molecular pathways, reducing the activation of stress responses in a manner similar to other stress alleviation strategies, such as nutritional supplementation.

The findings of this study highlight a substantial difference in erythrocyte osmotic fragility between the habituated and the control groups, reinforcing the protective effects of habituation during heat stress. The lower percentage of haemolysis in the habituated group, particularly at 0.7 % NaCl concentration, suggests that habituation could play a critical role in enhancing erythrocyte membrane stability. This reduced fragility may be attributed to decreased oxidative stress, which has been identified as a major contributor to erythrocyte membrane damage in heat-stressed broilers [1]. Lower haemolysis values, such as those observed in the habituated group, align with previous studies suggesting that oxidative stress is reduced through interventions that improve erythrocyte resilience [11]. Thus, the observed improvement in erythrocyte membrane integrity in the habituated group may result from reduced heat-induced oxidative damage due to habituation.

CONCLUSION

In conclusion, the findings from this study show that habituation mitigated the adverse effects of heat stress in broiler chickens during the hot-dry season. This is evidenced by reduced TI durations in the habituated broiler chickens, indicating reduced fear and stress levels. The habituated birds exhibited significantly decreased H:L ratio and serum Hsp70 concentrations, suggesting reduced physiological stress; and enhanced erythrocyte osmotic fragility, demonstrating improved resilience to oxidative stress and better membrane stability. The study shows the importance of habituation in reducing sensitivity to heat stress in broiler chickens.

DATA AVAILABILITY STATEMENT

The corresponding author will provide the raw data of this article on reasonable request.

ETHICAL APPROVAL

This study with reference number ABUCAUC/2021/025 was approved by the Ethical Committee on Animal Use and Care of Ahmadu Bello University, Zaria.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

No funding was received for this study.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

AUTHORS CONTRIBUTIONS

The authors contributed equally to this study.

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ORIGINAL ARTICLE

OCCURRENCE AND COMPARISON OF HEMATOLOGICAL AND SERUM BIOCHEMICAL PARAMETERS OF PESTE DES PETITS RUMINANTS' VIRUS (PPRV) INFECTED AND NON-INFECTED BREEDS OF GOATS IN NIGERIA

Sunday Charles Olaogun[1]*, Precious Ifeoma Obiora[1], Amos Ayodele Oladipupo[2], Osereime Adah[1], Adeniyi Charles Adeola[3], Emmanuel Adeoye Adenaike[4], Hafiz Ayinde Badmus[5]

[1]Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria; [2]Department of Theriogenology, Faculty of Veterinary Medicine, University of Ibadan, Oyo State, Nigeria; [3]State Key Laboratory of Genetic Resources and Evolution & Yunnan Laboratory of Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China; [4]Department of Veterinary Medicine, Micheal Opara University of Agriculture, Umudike, Nigeria; [5]Department of Crop Protection and Environmental Biology, University of Ibadan, Ibadan, Nigeria

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***Correspondence:** sc.olaogun@ui.edu.ng

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Peste des Petits ruminant's virus infection (PPRV) is of great economic importance in the small ruminant production industry and is recognized among the top ten diseases threatening small ruminant production and productivity globally and, most especially, in the tropics. This study investigates the occurrence of PPRV and establishes and compares hematobiochemical parameters of PPRV-infected and non-infected Nigerian goats to establish the extent of deviations of these parameters. A total of 58 goats, involving 32 healthy and 26 PPRV-infected goats manifesting clinical symptoms and confirmed with PPRV Antibody Rapid test kit, were analysed. Blood and serum samples were collected aseptically during June to August of 2024 at the Akinyele Livestock Market and the University of Ibadan Teaching and Research Farm, Ibadan, Oyo State, Nigeria. Breeds and sex were morphologically identified, and age was determined using the rostral dentition technique. Hematobiochemical analyses were done by the adoption of standard procedures. Findings revealed that crossbred goats were the breed with the highest incidence (57.1 %), while the lowest incidence was observed in West African Dwarfs (30.0 %). Also, higher incidence was observed in bucks (58.3 %) compared to does (35.3 %). There were no significant differences ($p < 0.05$) observed in all hematological and biochemical parameters, but lymphocytes and neutrophils had p-values of 0.08, respectively, when the values were compared. There was lymphocytosis, neutrophilia, hypoproteinemia, hyperglobulinemia, hypoglycemia, generalized increased liver and kidney enzymes, and an increase in the concentration of sodium and potassium ions detected in PPRV-infected goats compared to non-infected goats.

Keywords: biochemistry; goats; hematology; Nigeria; PPRV

INTRODUCTION

The rearing and production of small ruminant animals are of great global significance, as sheep and goats constitute approximately 56 % of the world's ruminant population, while producing roughly 1.5 million tons of meat and 25.6 million tons of milk [19]. The small ruminant production sector contributes significantly to the preservation of ecological systems and landscapes, aids in biodiversity conservation, and supplies products to niche markets [22]. Several issues hampered optimal production of small ruminants in Nigeria, with diseases being major challenges. One of these major, economically important clinical viral diseases is Peste des Petits Ruminants (PPR). PPR is an acute, infectious, febrile, highly contagious viral disease of small ruminants, which include sheep, goats, and wild ungulates, with a high mortality rate. The disease commonly presented with fever, mucopurulent ocular and nasal discharges, necrotizing and erosive stomatitis, severe enteritis, and pneumonia, ultimately leading to death [5].

There have been reports implicating PPR as the cause of high morbidity and mortality in small ruminants; especially with concurrent bacterial, viral, or parasitic infections, when the morbidity and mortality can be as high as 100 %. The severity and mortality tend to be higher in young animals compared to adults, with high neonatal mortality and abortion rates associated with PPR infection [1, 18].

Studies showed that of the total global population of 2.1 billion sheep and goats, about 80% of their population is at risk of PPR with a global economic loss of about US$ 1.2–1.7 billion annually [14, 21]. A whopping financial loss of about 1.5 million United States dollars (US) has been estimated as annual losses due to PPRV infection in Nigeria [9, 32]. Despite the significant losses associated with this important viral condition, especially at the smallholder, rural/communal level, where access to vaccination is very low in Nigeria. Little is known about the blood picture of PPR-infected goats vis-à-vis its comparison with the apparently healthy goats. The significance of assessment of hematological and biochemical parameters as a diagnostic, prognostic, and analytical tool for treatment options and monitoring the subsequent response to a specific treatment regimen has been previously reported [24, 25].

The examination of the blood components is essential in the investigation of several metabolites and other constituents in the body of animals, which are important in the determination of the physiological, nutritional, and pathological status of an animal [26].

Since PPR has become an endemic disease in Nigeria, with its yearly economic losses and inability to effectively control the disease through proper vaccination, treatment of the condition has become a necessity. The extent of variation in susceptibility to PPRV infection among Nigeria's indigenous goat breeds, sex, and ages has not been previously elucidated. There have been no previous data comparing the hematological and biochemical parameters of PPRV-infected and non-infected Nigerian goats. Considering the evident nearly global economic loss this disease has proven to be responsible for, there is an increasing demand for a thorough understanding of its clinical manifestations, vis-à-vis hematological and biochemical profiling of infected goats and its comparison with non-infected goats. This will guide clinicians in making appropriate diagnostic, prognostic, and therapeutic decisions when confronting PPRV cases in Nigerian indigenous goats.

This study, therefore, highlights the incidence of PPRV infection among different breeds, sexes, and ages of Nigerian goats and compares the levels of hematological and biochemical parameters in Peste des Petits ruminant's virus (PPRV)-infected and non-infected goat breeds in Nigeria.

MATERIALS AND METHODS

Study locations

The study was carried out at two locations, the Akinyele cattle market and the University of Ibadan Teaching and Research Farm, Ibadan, Oyo State, Nigeria. The Akinyele cattle market is located along the Oyo-Ibadan expressway and within longitude 30 45 (E) and 40 0 (E) and latitude 30 15 (N) and 70 30 (N) of the equators. The climatic conditions in the two locations were similar, with a high rainfall pattern (1,200–1,350 mm per year), temperatures varying between 27 °C and 32°C, and relative humidity ranging from 70 % to 90 % [23]. The vegetation pattern is typically that of a rainforest (Fig. 1).

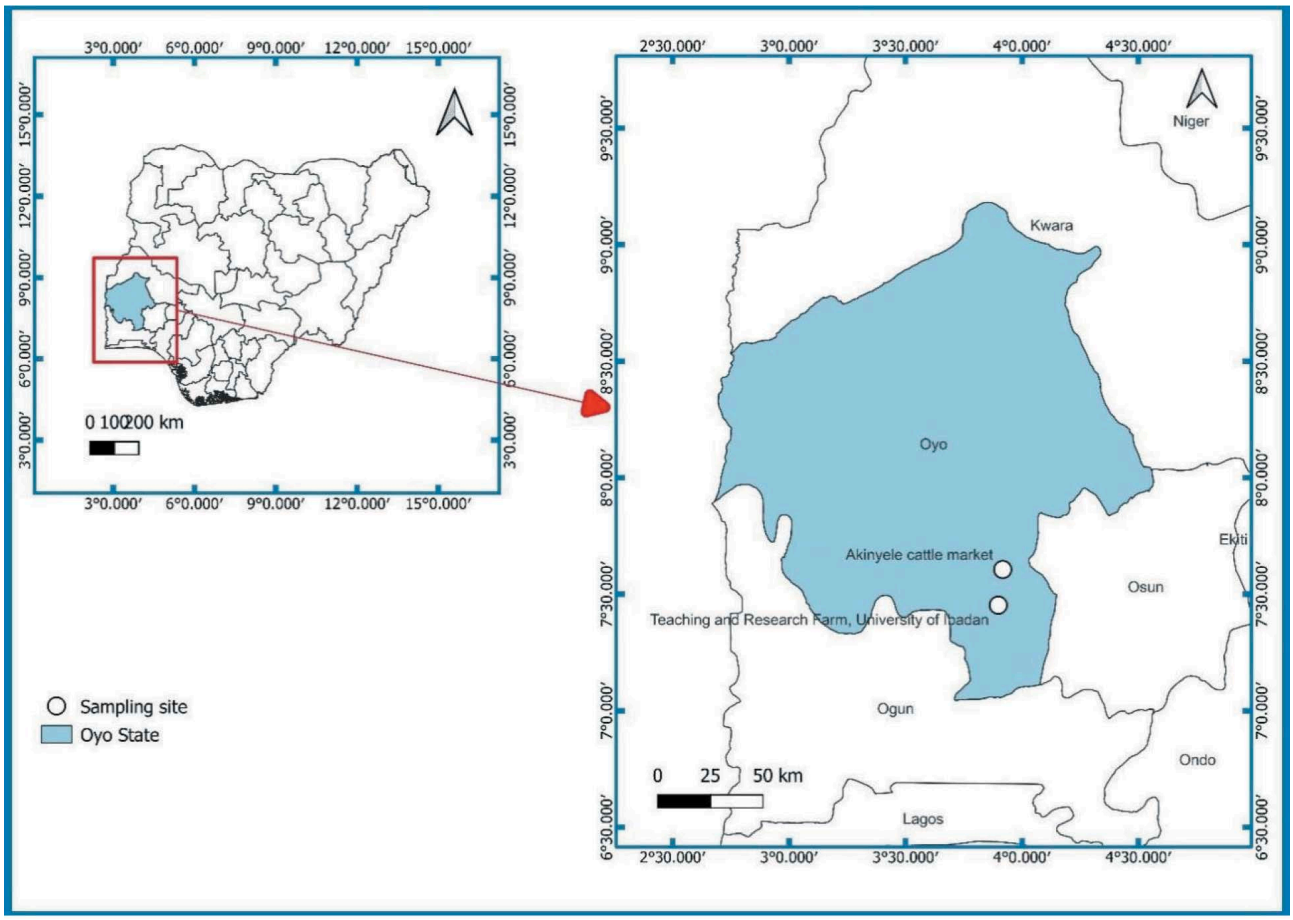

Fig. 1. Map showing the location of the study sites.

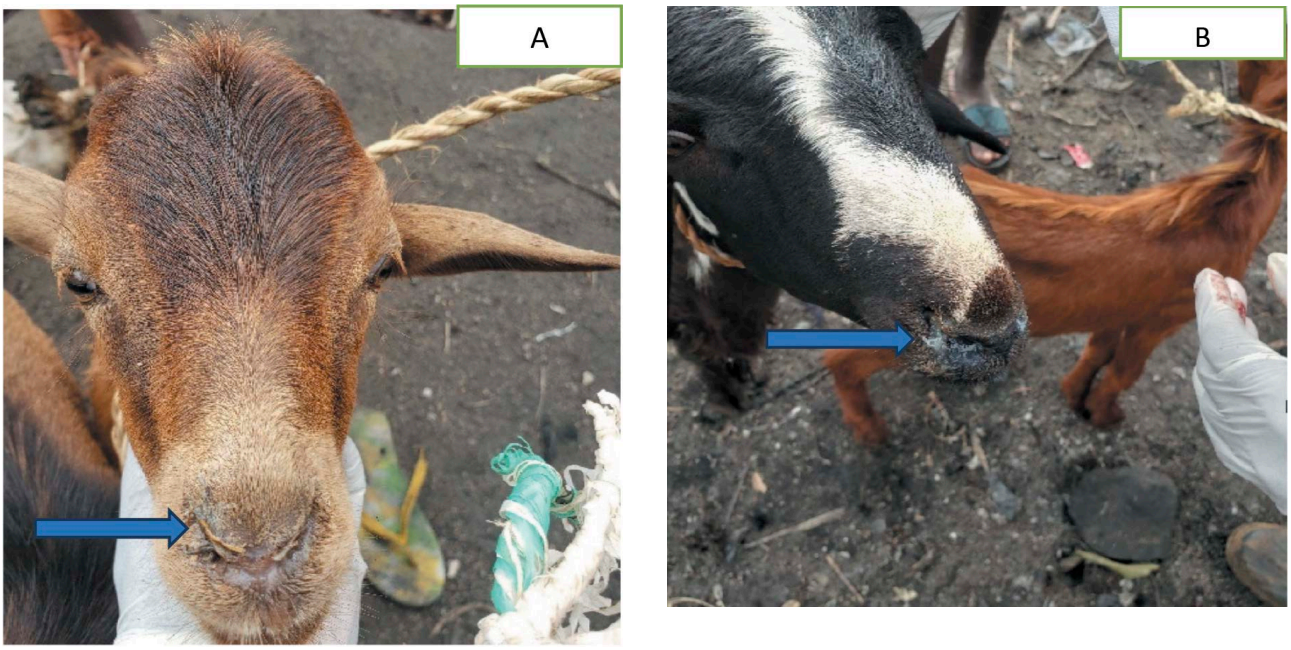

Fig. 2 A, B. PPRV infected goats with arrows showing occulo-nasal discharge.

Studied animals

Both PPRV-infected and non-infected goats were sampled during the slaughtering of goats at the abattoir beside the market, and some were sampled from the University of Ibadan Teaching and Research Farm. The breeds of goats sampled were Red Sokoto, West African Dwarf, Sahel, and crossbreed goats. Breed and sex were morphologically categorized based on their specific features and sexual organs, respectively [23]. Age was broadly classified into young (0 to 2½ years) and adult (> 2½ years) goats using dentition according to Dubie et al. [13].

A total of 58 goats were sampled, and these include 32 apparently healthy that were negative for PPRV and 26 PPRV positive goats using rapid diagnostic kit. The positive goats were manifesting clinical signs of rough hair coat, occulo-nasal discharge, respiratory distress, and diarrhea, mainly characterized by pasted vent (Fig. 2).

Sample collection

10 mls of blood was collected through jugular venipuncture from each animal using a vacutainer set and subsequently divided into 5 mls each in BD vacutainer® (clot activator tube) and BD vacutainer® (EDTA) for whole blood and serum, respectively. They were then covered and rolled gently before being placed in slanting positions inside the thermos flask containing ice packs to prevent lysing of the cells and transported to the general laboratory of the Department of Veterinary Medicine, University of Ibadan, Nigeria.

Procedure for the rapid test kit

Shenzhen Finder Biotech Co., Ltd. PPRV Antibody Rapid Test Kit was used for the confirmatory diagnosis of PPRV infection in the sampled animals. The test kits work on the principle of immunohistochemistry and capture the antibodies developed during the infection. 3 drops of whole blood from the goats collected via venipuncture were used for the kit following manufacturer's protocol.

Hematological and biochemical analyses

The hematological parameters were analyzed using standard methods such as microhematocrit, cyanomethemoglobin, hemocytometer, and others as previously adopted by Adedokun et al., Olaogun et al. and Olaogun et al. [4, 26, 27]. Biochemical parameters such as total protein, albumin, globulin, glucose, cholesterol, triglycerides, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, sodium, and potassium were analyzed using commercial test kits supplied by Fortress Diagnostics Limited (UK) as previously described by Charles and Adeday o, Olaogun et al. and Olawuwo et al. [10, 28, 29].

Statistical analyses

We used descriptive statistics to summarize the collected data, establish frequencies and percentages, and present them in the tables. The Student's T-test was employed to compare the mean ± SD of PPRV-infected and non-infected cattle. All statistical tests were conducted using the statistical package for social sciences (SPSS) version 26 (SPSS Inc., Chicago).

RESULTS

Out of the 58 animals sampled, 26 (44.8%) were confirmed PPRV positive and 32 (55.2%) were PPRV negative.

Table 1 gives a summary of the numbers and percentages of PPRV-infected and non-infected goats that were studied based on breed classification. Out of a total of 16 Red Sokoto goats sampled, 8 (50.0 %) were positive for PPRV, while 8 (50.0 %) were negative. Out of 20 West African Dwarf goats sampled, 6 (30.0 %) were PPRV positives, while 14 (70.0 %) were negatives for PPRV infection. Out of 8 Sahel goats sampled, 4 (50.0 %) were positive for PPRV, while 4 (50.0 %) were negative. The 14 crossbreed goats sampled revealed 8 (57.1 %) positives and 6 (42.9 %) negatives. Out of 24 bucks sampled, 14 (58.3 %) were positives and 10 (41.7 %) were negatives for PPRV. Out of a total of 34 does sampled, 12 (35.3 %) were positives, while 22 (64.7 %) were negatives for PPRV infection. Out of 26 young goats, 14 (53.8 %) were positives and 12 (46.2 %) were negatives for PPRV infection, while the 32 adult goats sampled indicated 12 (37.5 %) positives and 20 (62.5 %) negatives for PPRV infection.

Table 2 reveals the mean ± SD of the erythrogram values of the PPRV-infected and non-infected goats sampled with their P-values when statistically compared. The mean ± SD values of the erythrogram of the PPRV-infected and non-infected goats were shown as follows: packed cell volume (PCV) 28.54 ± 7.59 and 26.44 ± 6.21, respective-

Table 1. Breed, sex, and age distribution of goats sampled and their percentage of occurrence.

Parameters	Number positive/Percentage n=26	Number negative/Percentage n=32	Total/Percentage n=58
Breeds			
Red Sokoto	8/30.8	8/25.0	16/27.6
West African Dwarf	6/23.1	14/43.8	20/34.5
Sahel	4/15.4	4/12.5	8/13.8
Cross	8/30.8	6/18.8	14/24.1
Sex			
Male	14/53.8	10/31.3	24/41.4
Female	12/46.2	22/68.7	34/58.6
Age			
0 to 2½ (young)	14/53.8	12/37.5	26/44.8
Above 2½ (Adult)	12/46.2	20/62.5	32/55.2

Table 2: The mean values of the erythrogram of the PPRV-infected and non-infected goats (Mean ± SD).

Erythrocytic Parameter	Infected group n=26	Range	Non-infected group n=32	Range	P-values	Normal values Feldman et al. [15]
PCV (%)	28.54 ± 7.59	16.00–39.00	26.44 ± 6.21	15.00–35.00	0.419	22–38
Hb count(g/dl)	9.45 ± 2.50	5.20–12.90	8.69 ± 2.07	4.90–11.50	0.378	8–12
RBC count($\times 10^6/\mu L$)	17.77 ± 8.01	9.82–36.42	15.95 ± 5.80	9.04–26.02	0.484	8–18
MCV (fL)	19.19 ± 9.83	6.34–34.77	18.64 ± 7.86	10.40–31.17	0.866	16–25
MCH (pg)	6.64 ± 3.30	2.06–11.47	6.16 ± 2.58	3.38–10.24	0.595	2.20–8
MCHC (g/dL)	33.02 ± 0.86	31.67–35.60	32.77 ± 0.29	32.00–33.18	0.274	30–36

*$p < 0.05$ = statistical significance between the infected group and non-infected group. PCV = packed cell volume, Hb = Hemoglobin, RBC = Red blood cells, MCV = Mean corpuscular volume, MCH = Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration, NA = Not available, SD = Standard deviation

ly; hemoglobin concentration (Hb) 9.45 ± 2.50 and 8.69 ± 2.07, respectively; red blood cell count (RBC) 17.77 ± 8.01 and 15.95 ± 5.80, respectively; mean corpuscular volume (MCV) 19.19 ± 9.83 and 18.64 ± 7.86, respectively; mean concentration hemoglobin (MCH) 6.64 ± 3.30 and 6.16 ± 2.58, respectively. While the mean corpuscular hemoglobin concentration (MCHC) is 33.02 ± 0.86 and 32.77 ± 0.29, respectively. None of these parameters showed any statistical significance at $P < 0.05$ when the values were compared between PPRV-infected and non-infected goats.

Table 3 provides the summary of the mean ± SD of the leucogram of the PPR-infected and non-infected goats sampled with their respective p-values that indicated levels of significance. The mean ± SD of the leucogram values of the PPRV-infected and non-infected goats were stated as follows: White blood cell count (WBC), 7.62 ± 2.13 and 8.08 ± 2.05, respectively; lymphocyte, 68.85 ± 4.24 and 65.63 ± 5.10, respectively; neutrophil, 29.77 ± 4.19 and 32.94 ± 5.13, respectively; monocyte, 1.31 ± 0.48 and 1.44 ± 0.51, respectively; absolute lymphocyte, 5.25 ± 1.54 and 5.28 ± 1.36, respectively; absolute monocyte, 0.25 ± 0.52 and 0.22 ± 0.29, respectively; absolute neutrophil, 2.10 ± 0.88 and 2.67 ± 0.84; and platelet, 9.08 ± 1.04 and 8.75 ± 1.00, respectively.

Table 4 reveals summary of the mean ± SD of serum biochemical values of the PPRV-infected and non-infected goats sampled with their respective p-values that implied significances. The mean ± SD of the serum biochemical values of the PPRV-infected and non-infected goats were stated as follows: Total serum protein, 31.8 ± 0.59 and 32.3 ± 0.61, respectively; albumin, 11.8 ± 0.18 and 11.1 ± 0.08, respectively; globulin, 19.6 ± 0.48 and 21.2 ± 0.58, respectively; glucose, 57.33 ± 10.87 and 57.50 ± 6.56, respectively; cholesterol, 29.00 ± 9.24 and 31.38 ± 15.21, respectively; triglycerides, 39.69 ± 9.18 and 40.00±13.66, respectively; creatinine, 15.8 ± 0.45 and 14.5 ± 0.51, respectively; AST, 39.44 ± 6.86 and 38.46 ± 9.83, respectively; ALT, 29.50 ± 6.97 and 29.00 ± 8.51, respectively;

Table 3: The mean values of the total and differential leucocytic counts of the PPRV- infected and non-infected goats (Mean ± SD).

Leucocytic Parameter	Infected group n=26	Range	Non-infected group n=32	Range	P-values	Reference values Feldman et al. [15]
WBC (×10^3/µL)	7.62 ± 2.13	5.20–11.60	8.08 ± 2.05	4.80–12.60	0.560	3-13
Lymphocytes (%)	68.85 ± 4.24	60.00–75.00	65.63 ± 5.10	57.00–73.00	0.080	50–70
Neutrophils (%)	29.77 ± 4.19	24.00–39.00	32.94 ± 5.13	26.00–42.00	0.084	30–48
Monocytes (%)	1.31 ± 0.48	1.00–2.00	1.44 ± 0.51	1.00–2.00	0.491	0–4
Absolute lymphocyte (%)	5.25 ± 1.54	3.12–8.47	5.28 ± 1.36	3.36–8.69	0.954	NA
Absolute monocyte (%)	0.25 ± 0.52	0.05–1.98	0.22 ± 0.29	0.06–1.18	0.846	NA
Absolute neutrophil (%)	2.10 ± 0.88	0.06–3.24	2.67 ± 0.84	1.34–4.70	0.093	NA
Platelet (x10^9/L)	9.08 ± 1.04	8.00–10.00	8.75 ± 1.00	8.00–10.00	0.397	NA

*$p < 0.05$ = statistical significance between the infected group and non-infected group. WBC = White blood cells, NA = Not available, SD = Standard deviation

Table 4: Biochemical parameters of the PPRV- infected and non-infected goats (Mean ± SD).

Biochemical Parameter	Infected group n=26	Range	Non-infected group n=32	Range	P-values	Reference values. Feldman et al. [15]
Total serum protein (g/L)	31.80 ± 0.59	21.30–43.20	32.30 ± 0.61	22.00–4.21	0.843	34.9–83.5
Albumin(g/L)	11.80 ± 0.18	10.10–10.80	11.10 ± 0.08	10.00–1.25	0.174	22.3–55.1
Globulin(g/L)	19.60 ± 0.48	12.20–30.11	21.20 ± 0.58	11.90–3.10	0.436	9.9–50
Glucose (mmol/L)	57.33 ± 10.87	40.00–72.00	57.50 ± 6.56	42.00–66.00	0.960	1.3–6.8
Cholesterol (mg/dl)	29.00 ± 9.24	10.00–46.00	31.38 ± 15.21	10.00–56.00	0.607	NA
Triglycerides (mg/dl)	39.69 ± 9.18	22.00–55.00	40.00 ± 13.66	20.00–62.00	0.942	NA
Creatinine (µmol/L)	15.80 ± 0.45	10.00–20.15	14.50 ± 0.51	10.00–2.32	0.489	11.4–221
AST(U/l)	39.44 ± 6.86	26.00–52.00	38.46 ± 9.83	24.00–54.00	0.756	7.9–299
ALT(U/l)	29.50 ± 6.97	16.00–44.00	29.00 ± 8.51	18.00–44.00	0.863	2.3–49
ALP (U/l)	33.31 ± 7.09	22.00–48.00	32.69 ± 6.70	22.00–42.00	0.812	7.7–950
Sodium (mmol/L)	49.13 ± 6.37	34.00–57.00	48.31 ± 10.88	30.00–64.00	0.803	120–180
Potassium (mmol/L)	39.69 ± 7.12	25.00–48.00	38.31 ± 11.11	22.00–55.00	0.688	3.7–6.3

*$p < 0.05$ = statistical significance between the infected group and non-infected group. NA = Not available, AST = Aspartate aminotransferase, ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, SD = Standard deviation. The reference interval is as referenced in Feldman et al. [15]

ALP, 33.31 ± 7.09 and 32.69 ± 6.70, respectively; sodium ions, 49.13 ± 6.37 and 48.31 ± 10.88; and potassium ions, 39.69 ± 7.12 and 38.31 ± 11.11, respectively. There were no significant differences in all the biochemical parameters when comparisons were made between PPRV-infected and non-infected goats.

DISCUSSION

The distinct variation observed in hematological and biochemical parameters in PPRV-infected and non-infected Nigerian goats confirmed the importance of blood indices in assessing the general health status of animals. PPR is an important endemic disease of goats in Nigeria. The outcome of this present study becomes very germane in making therapeutic, prognostic, and diagnostic decisions about PPRV infection. This agrees with prior findings of Tambuwal et al. [31], who also identified that because the life of all flesh is in the blood, therefore, its role in the assessment of the health status, chemical evaluation for the survey, physiological and pathological conditions, and diagnostic and prognostic disease evaluation in animals is indisputable.

The West African Dwarf (WAD) breed of goats was observed to constitute the highest population of goats in the sampled locations. This supports the earlier observation of Yakubu et al. [35], who reported that WAD goats were the most widely distributed goats in Nigeria. The greater percentage of PPRV infection among crossbred goats seen in this study may be because crossbred goats were generally more prevalent in the sampled locations, and it may also be associated with the genetic characteristics or inherited

traits that may make them more susceptible compared to other breeds.

The smaller percentage of occurrence of PPRV infection among WAD goats compared to other breeds, as seen in this study, does not agree with the findings of Victor et al. [34], who reported the highest PPRV infection among WAD goats compared to other breeds. These differences may be associated with the differences in locations and period/season of the two studies. The demographic data revealed that there was a higher population of female animals than male animals, and this may be a result of the preference for the rearing of female animals by farmers for breeding and milk production. The occurrence of PPRV in Nigerian breeds of goats appeared to be higher in male goats than in female goats, as revealed by the findings in this study. This, however, contradicts the observation of Saeed et al. [30], who reported more seroprevalence among females than males. This may be due to generally higher stress in the male animals compared to female animals that can lead to immunosuppression and subsequent exposure. It may also be related to differences in breeds and locations of the two studies.

According to the demographic data, the population of adult animals sampled was higher than that of the young animals. This could be because adult animals were generally being brought to the market for sale. The observation of more PPRV cases in young animals compared to adult animals may be attributed to the fact that older animals have acquired post-exposure immunity to the infection following repeated exposure to the virus compared to naïve young goats with inadequate immune defenses, especially after the colostral antibodies have waned. This also corroborates the previous findings of more PPRV infection in young goats compared to adult goats as reported by Abubakar et al. [2].

Also, according to the results obtained, animals obtained from the open market showed a higher prevalence rate than those sampled at the farm. This agrees with the report of Victor et al. [34], who reported open markets as a major risk factor for PPRV infection in small ruminants in Benue State, Nigeria. This may be because a good number of goats obtained at the open livestock market are generally compromised in health, which owners put up for sale to minimize their loss. Another reason are animals that become infected with the disease via contact with infected animals in the market before being bought since the PPRV is transmitted via close contact with secretions and excretions of infected animals as previously described by Abu – Elzein et al. [3].

Though the PCV and Hb concentrations of the infected goats were within the normal range, they were, however, observed to be higher than that of the non-infected group. This is in tandem with the findings of Maina et al. and Ugochukwu et al. [20, 33], who also reported increased PCV and HbC in PPRV infection in goats. Hemoconcentration associated with diarrhea in the later stage of PPR infection in goats may be the cause of these increases. This also agrees with the report of another study that recorded an increase in total RBC count and Hb concentration, which may be because of diarrhea causing dehydration and polycythemia or hemoconcentration [16].

The WBC of the PPRV-infected goats is slightly lower than that of the non-infected goats as observed in this present study. This agrees with the findings of Begum et al. [7], who also reported a significant decrease in WBC in PPRV-infected goats compared to non-infected Bengal goats in Bangladesh.

The lymphocyte count of the infected goats was higher than that of the non-infected goats. This agrees with the result of Begum et al. [8], who attributed this increase to the immune reaction triggered by the PPR virus. But this disagrees with the observation of Islam et al. [16], whose report shows lymphopenia, probably attributed to necrosis of the lymphocytes in lymph nodes, spleen, and Peyer's patches, as the virus is lymphotropic, like that of the rinderpest virus.

However, the neutrophilia observed in PPRV-infected goats compared to non-infected goats as observed in this study agrees with the findings of Das et al. and Kataria and Kataria [12, 17]. This may be due to stress and viral effect and secondary bacterial superimposition.

Decreased levels of total protein and globulin in infected compared to non-infected goats, as seen in this present study, agree with the hypoproteinemia observed by Begum et al. [7] in PPRV-infected compared to non-infected goats in black Bengal goats in Bangladesh. This also corroborates with the earlier findings of Islam et al. [16], who also reported hypoproteinemia in infected goats and stated that nephritic damage to the glomeruli that results in increased permeability of capillary walls of the glomerulus leading to excretion of high levels of pro-

tein from blood to urine may be the probable reason. The relative hypoglycemia observed in PPRV-infected goats compared to non-infected goats is similar to the observation of B a l o g u n et al. [6], who also reported reduced glucose levels in PPRV-infected goats. This could be attributed to the occurrence of diarrhea and glycogenolysis impairment, associated with the pathogenesis of PPR in general. A relatively higher levels of creatinine observed in PPRV-infected goats compared to non-infected goats may be due to the involvement of kidney dysfunction by the virus. This agrees with the observations of B e g u m et al. and U g o c h u k w u et al. [7, 33], who reported an increase in the level of creatinine in PPRV-infected goats.

Relatively higher levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) observed in PPR-infected goats compared to non-infected goats may be due to the deleterious effects of PPRV on the liver. This agrees with the observation of B e g u m et al. [7], who reported a gradual increase in the levels of all four liver and kidney enzymes assayed for in PPRV-infected compared to non-infected goats in their experimental study.

Also, higher levels of sodium (Na) and potassium (K) ion concentrations were detected in the serum of PPRV-infected goats compared to non-infected goats. This corroborates with the observation of a significant increase in the concentration of sodium and chloride ions in PPRV-infected goats compared to non-infected goats. The probable reason had been attributed to severe diarrhea and dehydration [8, 11]. Higher potassium ion concentration in PPRV-infected goats recorded in this study compared to non-infected goats is also in agreement with the findings of I s l a m et al. [16], who reported higher serum potassium ion in PPRV-infected goats compared to non-infected. This may probably be linked to renal disease, which causes excessive potassium retention, or may be because of electrolyte imbalance due to diarrhoea-induced dehydration, associated with PPR in goats.

CONCLUSION

This study further confirmed the endemicity of PPRV infection in Nigerian goats using clinical symptoms and a rapid diagnostic kit. The highest incidence of PPRV was observed in crossbred goats, males, and young goats compared to other breeds, females, and adult goats, respectively. There was generalized lymphocytosis, neutrophilia, hypoproteinemia, hyperglobulinemia, hypoglycemia, generalized increased liver and kidney enzymes, and an increase in the concentration of sodium and potassium ions in PPRV-infected goats compared to non-infected goats.

Recommendation

Generalized lymphocytosis, neutrophilia, hypoproteinemia, hyperglobulinemia, hypoglycemia, generalized increased liver and kidney enzymes, and an increase in the concentration of Na^+ and K^+ ions should be considered when making treatment plans and management protocols for PPRV in goats. The endemicity of PPR in southwest Nigeria is real, and more strategic approaches should be formulated by the stakeholders to mitigate against the deleterious effect of the condition.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

ETHICAL STATEMENT

All applicable national and institutional guidelines for the care and use of animals were duly adhered to. The study was carried out in accordance with the recommendation of the ethical standard of the University of Ibadan's Research Ethics Committee (UI-ACUREC). The protocol and procedures employed were reviewed and approved by the Ethical Standard of Research Committee (Ref. No. NHREC/UIACUREC/05/12/2022A Date: 18/11/2024).

CONFLICT OF INTEREST

None declared.

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GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

AUTHORS CONTRIBUTIONS

Conceptualization: S.C.O., O.P.I., O.A.A., A.O., A.C.A., B.H.A.; Sampling and methodology: S.C.O., O.P.I., O.A.A., A.O., A.C.A., B.H.A., A.E.A.; Formal analysis: S.C.O., O.P.I., O.A.A.; Writing – original draft preparation: S.C.O.; Writing – review and editing: S.C.O., O.P.I., O.A.A., A.O., A.C.A., A.E.A., B.H.A.

All authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

OCCURRENCE OF MULTIDRUG RESISTANCE IN COAGULASE POSITIVE METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREAUS* FROM OPEN WOUNDS IN DOGS IN JOS, NORTH CENTRAL NIGERIA

Adamu Ahmad Kaikabo[1]*, Diana Adamu Tanko[2], Lubabatu Ibrahim[1], Maryam Muhammad[1]

[1]Bacterial Research Division, National Veterinary Research Institute, PMB 01, Vom, Nigeria; 2Department of Veterinary Science Laboratory Technology, Federal College of Animal Health and Production Technology, Vom, Nigeria

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***Correspondence:** karkafa@yahoo.co.uk

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

***Staphylococcus aureus* is an important cause of wound infections in companion animals, and infections with methicillin-resistant *Staphylococcus aureus* (MRSA) are of particular concern due to limited treatment options and their zoonotic potentials. The aim of this study was to investigate the occurrence of methicillin-resistant *Staphylococcus aureus* in open wounds in dogs brought for routine veterinary care in Jos and environs. Only coagulase positive *Staphylococcus aureus* isolates were used to determine the antimicrobial susceptibility using 8 antibiotics. These were found to be resistant to 4 antimicrobials; penicillin, methicillin, cefoxitin and oxacillin, at the concentrations tested. Suggesting that, the *Staphylococcus aureus* were methicillin resistant phenotypes. However, the isolates also showed sensitive and intermediate susceptibility to gentamicin, with only one coagulase positive *Staphylococcus aureus* isolate showing resistance to gentamicin. Similarly, the results on kanamycin susceptibility indicated majority of the isolates were susceptible, with only one isolate showing intermediate susceptibility, however few of the isolates were resistant. All the isolates showed resistance to cephalothin. Similarly, resistance to neomycin was observed in almost all the isolates, except two isolates, that were found to be sensitive. Sensitivity to ceftiofur was observed in 10 out of the 13 isolates; remaining 3 isolates were resistant to ceftiofur. The results showed that methicillin-resistant *Staphylococcus aureus* occurred in open wounds in dogs and could be a recurring issue in clinical management of wound infections in dogs, and a risk to public health.**

Key words: coagulase positive; dogs; methicillin resistance; open wound; *Staphylococcus aureus*

INTRODUCTION

Staphylococci are Gram-positive bacteria, and they are classified into two groups, coagulase-positive (CoPS) and coagulase-negative (CoNS), based on their ability to produce the enzyme coagulase [1]. *Staphylococcus aureus* and *Staphylococcus pseudintermedius* are the most important species in the CoPS group as they are major pathogens for both humans and animals, especially S. aureus. Although CoNS are saprophytic and rarely pathogenic [5], multidrug-resistant (MDR) strains have been associated with severe cases of difficult to treat infections, especially in immunocompromised individuals [19].

Methicillin-resistant staphylococci (MRS) are among the most important bacteria in both human and veterinary medicine and of major clinical, public health and economic concern [7]. The problem is aggravated by the fact that MRS, in addition to β-lactam antibiotics, are commonly resistant to other classes of antimicrobial agents, including aminoglycosides, macrolides, phenicols, tetracyclines and fluoroquinolones [8,6]. Methicillin resistance is conferred by the mecA gene which encodes an altered penicillin-binding protein (PBP2a or PBP2') with a low affinity for β-lactam antimicrobials [18].

Even though C h a h et al., 2014 [3] have characterized methicillin-resistant CoNS from dogs in Nsukka, Nigeria, there are, however, no documented reports on the characterization of CoPS methicillin-resistant staphylococci from dogs in Nigeria. Thus, we conducted a small survey to investigate the occurrence of CoPS methicillin-resistant *Staphylococcus aureus* from clinical wound infections in dogs in Jos, North Central Nigeria. The MRSA was identified from phenotypic *Staphylococcus aureus*-positive samples subjected to antimicrobial susceptibility testing to determine their resistance to methicillin. Due to the fact, that we could not find a literature reporting CoP MRSA in open wounds in dogs in Nigeria, this represents the first report on CoP MRSA in dogs in Jos, North Central Nigeria. The data provides important baseline measurements for future surveillance of CoP MRSA in dogs in Nigeria. The multidrug resistance pattern observed in this study is important in guiding clinical judgement on which antibiotic to use for the treatments of wound infections in dogs in the study area.

MATERIALS AND METHODS

Study area

Dogs from Jos and Bukuru were included in the study. A total of 100 dogs with open wounds presented to the ECWA Veterinary clinic, Bukuru Jos Metropolis for routine veterinary care were sampled.

Sample collection, preservation and transportation

The cotton tip of each sterile swab stick was moistened in sterile normal saline and gently rolled over the wound for about 10 seconds and transferred into bijou bottle containing transport media; the samples were adequately labelled. All samples were transported on ice packs to the Bacteriology Division, National Veterinary Research Institute, Vom for processing.

Bacterial culture, isolation, and identification

Each swab sample was inoculated and streaked on mannitol salt agar (Oxoid, Basingstoke, UK). All inoculated plates were incubated at 37 °C from 24 to 48 h. On each plate that produced growth, discrete colonies were randomly selected and purified on nutrient agar.

Purified colonies were subjected to Gram's staining and catalase test and presumptive staphylococcal colonies were further tested for coagulase production (slide coagulase test using rabbit plasma), DNase activity, and hemolysis on 5 % sheep blood agar using standard procedures [12]. Representative colony which appeared yellow on mannitol salt agar and were gram positive cocci was subjected to catalase test for preliminary confirmation of Staphylococcus species. Those colonies positive for catalase test were further tested for coagulase activity to confirm *Staphylococcus aureus* from other nonpathogenic Staphylococci. Isolates were preserved on nutrient agar slants for further analysis, including antimicrobial susceptibility test.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing of *Staphylococcus aureus* was performed using disc diffusion method as described [2]. Antibiotic discs used included: penicillin, methicillin, oxacillin, gentamicin, kanamycin, neomycin, cephalothin and ceftiofur in various disc concentrations. The minimum inhibitory concentration results were interpreted based on the approved standards of the Clinical

and Laboratory Standards Institute [16,17]. All MRSA positive isolates were stored in glycerol stocks at −80 °C at Bacteriology Division National Veterinary Research Institute, Vom.

Statistical analysis

Data obtained were imputed into Microsoft excel 2010 and percentage of resistance showed by the isolates to various antibiotics used were calculated.

RESULTS

The outcome of the study is highlighted below.

Demography of dogs

Out of the 100 dogs sampled, 62 and 33 were male and female dogs, respectively. Sampled dogs were 62 and 23 adult and young dogs, respectively. The breed demography indicated that 54 were foreign breeds and 31 were local breeds. This shows that high numbers of dogs brought for veterinary care in the study area during the period of the study were foreign breeds compared to local breeds that were in low numbers (Fig. 1).

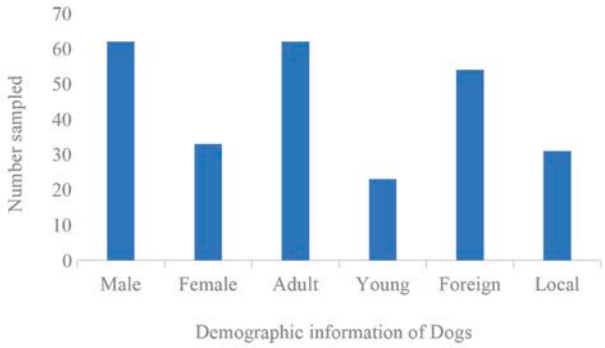

Fig. 1. Demography of the dog samples based on breed, sex and age.

Cultural characteristics of *Staphylococcus aureus* isolated from open wounds in dogs

All samples (N = 100) processed grew on mannitol salt agar producing yellow colonies suggestive of Staphylococcus species; of these, 13 were coagulase positive, and 17 showed beta hemolytic activity. However, none of the isolates were positive for DNase activity. Only 13 out of the 17 beta hemolytic staphylococci isolated were coagulase positive. Thus, only 13 beta hemolytic coagulase positive staphylococci were used for the antibacterial susceptibility. These two characteristics indicated that the isolates were pathogenic. Therefore, determining their sensitivity to antibiotics is crucial.

Antimicrobial susceptibility of *Staphylococcus aureus* isolated from open wounds in dogs

We have performed disc diffusion test on all coagulase positive *Staphylococcus aureus* isolates to determine their susceptibility to 8 antibiotics (Table 1). Resistance to 3 antibiotics (penicillin, methicillin and oxacillin) was observed in all the *Staphylococcus aureus* isolates at the concentrations tested (Table 1). The result showed *Staphylococcus aureus* isolates were resistant to methicillin at the tested concentrations. However, 6 and 4 of the *Staphylococcus aureus* isolates tested for susceptibility to gentamicin were found to be sensitive and showing intermediate sensitivity to gentamicin, respectively. Perhaps only one *Staphylococcus aureus* isolate was resistant to gentamicin at the concentration tested (Table 1). Similarly, the results on kanamycin susceptibility showed that majority of the isolates were susceptible, with only one isolate showing intermediate susceptibility, however few of the isolates were resistant. All the isolates showed resistance to cephalothin and neomycin. However, two isolates showed sensitivity to neomycin. 10 out of the 13 isolates showed sensitivity to ceftiofur of which the remaining 3 isolates were resistant. In all, the susceptibility pattern showed that at least 63.3 % and 36.36 % of the isolates were susceptible to at least two (2) and one (1) antimicrobials, respectively. Similarly, 62 %, 23 %, 8 % of the isolates tested showed resistance to 3, 4, 5, and 2 antimicrobials, respectively.

The resistance profile showed in Table 2 indicates that all isolates showed complete resistance to penicillin, methicillin and oxacillin. However, nearly all the isolates were susceptible to kanamycin, with very few of the isolates showing resistance.

The isolates showed greater phenotypic intermediate resistance to kanamycin, and lower to-gentamicin. However, greater number of the isolates were sensitive to gentamicin.

The results in Table 4 indicated that 69.2 %, 23.07 % and 7.67 % of the coagulase positive MRSA showed phenotypic resistance to 3, 4 and 5 antibiotics, respectively. Overall, patterns of phenotypic resistance were summarized in Table 3.

Table 1. Antibiotic Susceptibility profile of coagulase positive MRSA isolated from open wounds in dogs in Jos, North Central Nigeria

	Antibiotic susceptibility pattern based on RIS							
Sample ID	Methicillin (30)	Oxacillin (1)	Penicillin (30)	Kanamycin (30)	Gentamicin (30)	Neomycin (30)	Cephalothin (30)	Ceftiofur (30)
EJS/002/2021	R	R	R	S	S	R	R	S
EJS/003/2021	R	R	R	R	S	I	R	S
EJS/007/2021	R	R	R	I	I	R	R	S
EJS/008 /2021	R	R	R	S	S	R	R	S
EJS/009 /2021	R	R	R	R	R	R	R	S
EJS/010/2021	R	R	R	S	S	I	R	S
EJS/011/2021	R	R	R	S	R	R	R	R
EJS/015/2021	R	R	R	S	S	R	R	S
EJS/019/2021	R	R	R	S	S	R	R	R
EJS/020/2021	R	R	R	S	I	R	R	S
EJS/021/2021	R	R	R	S	R	R	R	R
EJS/022/2021	R	R	R	S	I	R	8	S
EJS/027/2021	R	R	R	S	S	R	R	R

R= resistance, I = intermediate, S = sensitive

Table 2. Antibiotic susceptibility pattern based on resistance, intermediate and sensitive susceptibility (RIS)

	Zone of inhibition (mm) of antibiotics (µg)							
Sample ID	Methicillin (30)	Oxacillin (30)	Penicillin (30)	Kanamycin (30)	Gentamicin (30)	Neomycin (30)	Cephalothin (30)	Ceftiofur (30)
EJS/002/2021	6	6	9	24	18	13	11	34
EJS/003/2021	6	6	6	12	20	15	6	27
EJS/007/2021	6	6	8	16	14	9	9	30
EJS/008 /2021	6	6	10	23	19	12	12	40
EJS/009 /2021	6	6	10	10	12	12	6	28
EJS/010/2021	6	6	6	22	19	14	6	28
EJS/011/2021	6	6	6	18	12	10	6	6
EJS/015/2021	6	6	6	20	18	13	6	26
EJS/019/2021	6	6	6	18	16	12	6	8
EJS/020/2021	6	6	7	18	14	9	10	30
EJS/021/2021	6	6	6	19	0	10	6	16
EJS/022/2021	6	6	6	19	14	10	8	32
EJS/027/2021	6	6	6	18	15	13	6	17

DISCUSSION

Companion animals, particularly dogs and cats, are frequently implicated as potential reservoirs of methicillin-resistant staphylococci [15]. Studies on Staphylococcal species in companion animals is important because of their potential for zoonotic infections and possibility of resistance genes transfer [13].

Our study aimed at investigating the occurrence of MRSA in an open wound in dogs brought to the clinics by families for routine veterinary care. One hundred samples were collected and processed.

In this study, the demographic characteristics of the dogs studied showed, out of the one hundred dogs sampled, sixty-two were male dogs and thirty-three were female dogs. Although our study was not a dog's population study, our results were not in concordance with the findings of O g b u et al., [11] who reported female dogs were more prevalent than the male dogs (1:1.2) in the study area. This small survey which was mainly carried out to

Table 3. Intermediate and sensitive resistant phenotypes in coagulase positive MRSA isolated from open wounds in dogs in Jos, North Central Nigeria

Sample ID	Intermediate	Sensitive
EJS/002/2021	K	CN
EJS/003/2021		CN
EJS/007/2021	K	CN
EJS/008 /2021	K	CN
EJS/009 /2021		
EJS/010/2021	K	CN
EJS/011/2021	CN K	
EJS/015/2021	K	CN
EJS/019/2021	K	CN
EJS/020/2021	CN K	
EJS/021/2021	K	
EJS/022/2021	CN K	
EJS/027/2021	K	

CN = Gentamicin; K = Kanamycin

Table 4. Percentage of phenotypic antibiotic resistance of coagulase positive MRSA isolated from open wounds in dogs in Jos, North Central Nigeria

Sample ID	Resistant phenotypes	No. resistant	(%)
2	PEN MET OX	3	(69.23)
3	PEN MET OX K	4	
7	PEN MET OX	3	
8	PEN MET OX	3	
9	PEN MET OX K CN	5	(7.67)
10	PEN MET OX	3	
11	PEN MET OX	3	
15	PEN MET OX	3	
19	PEN MET OX	3	
20	PEN MET OX	3	
21	PEN MET OX CN	4	
22	PEN MET OX	3	
27	PEN MET OX CN	4	(23.07)

PEN = Penicillin; MET = Methicillin; OX = Oxacillin; K = Kanamyin; CN = Gentamicin

determine *Staphylococcus aureus* colonization of open wounds in dogs and the susceptibility or resistance of the isolates to methicillin and only used a small number of dogs (N = 100) brought for routine veterinary care in some selected veterinary clinics in the study area. Therefore, it may not present the exact population of dogs reported by O g b u et al. [11]. Similarly, O b o e g b u l e m and N w a k o n o b i [10] and R a n a et al. [14] have reported higher number of female dogs compared to male dogs in their studies of dogs' population in Lagos urban and rural areas. Their results are also not in accordance with our study. This showed preference for female over male dogs. This may be attributed to use of female dogs for breeding and cultural purposes [11].

The age characteristics of the dogs sampled in this study were sixty-two (62) for adult dogs and thirty-three (33) for young dogs. The report of the study conducted on dog population in the study area based on age showed majority of the dogs were adult (48.6 %), above 12 months of age. This was due to the fact that younger dogs were sold out after weaning, due to breeding value or security reasons. The same reason was observed by R a n a et al. [14] who reported that most dog owners were mostly interested in security or companionship purposes.

Visits at the clinics for routine veterinary care were higher in exotic than local breeds. Out of the hundred dogs sampled, fifty-four (54) were foreign or exotic breeds and thirty-one (31) were local breeds. In their dog population study, O g b u et al. [11] had reported high population of indigenous breed (71.3 %) compared to local and mixed breeds (28.9 %) in the study area. They observed that indigenous breeds are kept mainly for cultural beliefs and delicacy. Exotic breeds are mainly kept by the elites who can afford their feeding, housing and veterinary care compared to local or mixed breeds whose owners are majorly poor and allow their dogs to freely roam and scavenge for their foods. They also observed that dogs are mainly kept for security reasons due to increased security challenges. Guard duties have been identified as the primary reason for keeping dogs in a number of states in Nigeria [10].

In our microbiological study for methicillin resistance, the samples collected were processed by culture, and identification of the isolates were performed using phenotypic characteristics on selective media, morphology, and biochemical tests such as catalase, coagulase and DNase tests. All the samples (N = 100) cultured grew luxuriantly on mannitol salt agar producing phenotypic yellow colonies, suggestive of *Staphylococcus* species. The result showed that 63 (63%) isolates were catalase positive and 13 (21 %) were coagulase positive, respectively, and none of the isolates were positive for DNase activity. Beta hemolytic activity on 5 % sheep blood agar was observed in seventeen (4) of the isolates recovered. The coagulase positive isolates were further identified as *Staphylococcus aureus,* based on their morphology and biochemical characteristics [11]. Coagulase positive *Staphylococcus aureus* also

frequently occurs in dogs [19]. Study has reported a prevalence of 16 % coagulase-positive *Staphylococcus aureus* in dogs in Bangladesh.

In our study, only the coagulase positive *Staphylococcus aureus* (COPS) was used to determine the antimicrobial susceptibility. The antibiotics susceptibility of the 13 coagulase-positive (CoPS) *Staphylococcus aureus* were determined using methicillin (30 µg), oxacillin (30 µg), cefoxitin (30 µg), penicillin (30 µg), gentamicin (30 µg), kanamycin (30 µg), neomycin (30 µg), cephalothin (30 µg), and ceftiofur (30 µg) discs. All the thirteen (13) coagulase-positive *Staphylococcus aureus* isolates tested showed resistance to penicillin, methicillin, cefoxitin and oxacillin indicating they were methicillin resistant *Staphylococcus aureus* strains (Table 1). This study has demonstrated that the dogs sampled carried CoP MRSA in the exposed wounds. The characterization of staphylococcal species that colonize pets, especially in exposed wounds, is important for maintaining animal health and to minimize the risk of transmission to owners.

Furthermore, the antimicrobial susceptibility patterns of the 13 CoP MRSA showed that only 1 isolate showed resistance to 2 (8 %) antimicrobials, 8 (62 %) to 3 antimicrobials, 3 (23 %) were resistant to 4 antimicrobials, and 1 (8 %) to 5 antimicrobials tested in various disc concentrations. Magiorakos et al., [9] have proposed that isolates showing resistance to three or more antimicrobial classes interpreted by clinical breakpoints were classified as multidrug-resistant (MDR). This signifies that 12 CoP MRSA isolated in this study were multidrug resistant. In the same vein, only 7 (63.63 %) isolates were susceptible to 2 antimicrobials, 4 (36.36 %) were susceptible to 2 antimicrobial and 1 (0.16 %) had intermediate susceptibility to 1 antimicrobial.

This study shows that antimicrobial resistance occurs in coagulase-positive staphylococci cultured from open wounds in dogs in the study area. We could not find any literature on any collection of isolates which represents CoP MRSA in dogs in Nigeria. A study by Chah et al [3] had only reported coagulase negative (CoN) methicillin-resistant strains in dogs in Nsukka, Nigeria.

CONCLUSION

The study is of clinical significance since without the knowledge of antibiotics resistance, especially methicillin resistance in *Staphylococcus aureus*, treatment of wounds in pet dogs would be a recurring issue due to resistance of the organisms involved. It is of a public health importance, as the spread of MRSA to humans is palpable and it would be an issue in the medical settings as well as it could lead to livestock acquired MRSA (LA-MRSA). Acquired MRSA in hospitals, humans and livestock has been a global challenge. We encourage continuous surveillance for MRSA to give a broader picture of the problem and a future way forward to address this recurring veterinary and medical risk.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

ETHICAL APPROVAL

Even though the sample collection procedure was not invasive, approval was sought and granted by the animal care and use committee (ACUC) of the National Veterinary Research Institute, Vom, Nigeria. Similarly, informed consent of the dog owners was obtained prior to sample collection. The reason for the research was fully explained to the clients.

CONFLICT OF INTEREST

We declare that authors have no competing interests.

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GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

AUTHORS CONTRIBUTIONS

KAA: Conceived the idea, wrote the proposal and manuscript.

TAD: Samples collection.

IL: Samples processing.

MM: Approved the manuscript and permission to publish.

All authors have read and proofread the manuscript and agreed for publication.

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ORIGINAL ARTICLE

REASONS AND RISK FACTORS FOR THE INCIDENCES OF PERINATAL CALF MORTALITY AND TWIN BIRTHS IN LOCALLY-BORN HOLSTEIN DAIRY COWS UNDER HOT ENVIRONMENTAL CONDITIONS

Lazoumi Ouarfli[1], Abdelmadjid Chehma[2]

[1]Laboratoire Valorisation et Conservation des Ecosystèmes Arides (LVCEA). Faculté des Sciences de la Nature et de la Vie et Sciences de la terre, Université de Ghardaïa, Ghardaïa, Algérie; [2]Laboratoire de Bio Ressources Sahariennes. Préservation et Valorisation 30000 Ouargla, Algérie

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***Correspondence:** lazoumi.ouarfli@univ-ghardaia.dz

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ABSTRACT

Perinatal calf mortality (PCM) and multiple pregnancies (twins) present significant challenges in dairy cattle breeding, impacting both economic viability and reproductive performance. This study examined risk factors for stillbirths and twin births in a dairy farm located in Algeria's Northern Sahara, analyzing 1047 full-term calving records of locally-born purebred Holstein cows. Binary logistic regression models were employed to identify factors influencing PCM and twin births. During the study period (1995 – 2017), 7.07 % of calvings resulted in stillbirths, and 3.15 % resulted in twins. PCM was strongly associated (p = 0.0001) with severe dystocia, calf gender, and retained placenta, as well as gestation length (p = 0.023), dam parity (p = 0.019), and temperature-humidity index (THI) at breeding (p = 0.025). The likelihood of twin births was higher in multiparous cows and significantly associated (p = 0.034) with heat stress conditions at parturition (THI-P). These findings highlight the need for targeted management strategies during the peripartum period to mitigate reproductive disorders and reduce financial losses in dairy herds.

Key words: dystocia; heat stress; Holstein; stillbirth; twinning births

INTRODUCTION

Perinatal calf mortality or stillbirth (SB), has been defined by many researchers as the mortality of full-term calves (normal pregnancy >260 days), within 24 to 48 h after parturition [31, 37, 39]. During the last decade, several studies have been reporting an incessant increase in the frequency of PCM in many countries [38]. Therefore, the high stillbirth rates are a serious problems in cattle breeding, characterised by a high economic and genetic loss and increased costs for replacements [9]. Recently, in necropsy studies internationally defined dystocia (35 %) and anoxia (30 %) as major causes of PCM, to a lesser degree, other causes (15 %), infections (5 %) and congenital defects (5 %) [43]. Besides, the greater risk of perinatal mortality is in primiparous compared to multiparous cattle [40].

Hence, the primary causes of dystocia in primiparae cows are relative fetal oversize and in pluriparae cows there are maldispositions [44].

Otherwise, some findings indicated that maternal diet during different stages of gestation could induce physiological and epigenetic changes in fetal tissues of different species, which could have serious postnatal consequences [30]. Evidence suggests that dairy cows under heat stress, during late gestation, in hot environmental conditions, have been related to lower birth weight, decreased total plasma protein concentration and haematocrit, and compromised immune function of the new-born calf [57].

Multiple pregnancies (twins) are undesirable in dairy cattle herds as they compromise the reproductive performance such as increased abortion, dystocia, retained placenta, calf mortality, occurrence of freemartins, postpartum therapy, and longer rebreeding intervals, and an economic burden of up to $225 per twin pregnancy [14, 33]. Despite this, cows are predominantly a monovular species. The incidence of multiple ovulations and thus twinning in dairy cows has increased considerably alongside milk production in the last 30 years [32]. In effect, global warming is likely already having a negative impact on reproductive functions in mammals [4]. Thus, López–Gatius et al. [33] indicated that the warm climate is the main factor compromising the fate of multiple pregnancies. In addition, the breeding synchronization protocols for fixed-time artificial insemination (FTAI), have become routine components of the dairy herd reproductive management. Therefore, the hormone combinations used for FTAI can increase the risk of double ovulation [14]. Nevertheless, some short protocols appear to reduce the rate of double ovulation compared to spontaneous estrus [11]. Nevertheless, the risk of twin pregnancy is much more common in older cows [15]. In effect, it has been observed by Garcia–Ispierto and López–Gatius [15] that the twin pregnancies may account for 25 % of all pregnancies on day 90 of gestation in cows in their third lactation or more.

This study sets out to explore the influence of risk factors of the incidences of stillborn calves and twinning births in locally-born purebred Holstein dairy cows raised under Saharan climate in Ghardaia region.

MATERIALS AND METHODS

Herd management

The study population was a commercial dairy herd of native purebred Holstein-Friesian lactating dairy cows in Algerian Northern Sahara (32°41'06.7 "N latitude and 4°44'10.8 "E longitude). During the study period (January 1995 to December 2017), the mean number of lactating cows in the herd was 312 and mean annual milk yield was 7030.3 kg per cow. Cows were milked three times daily and fed total mixed ration (roughage: 40 %, concentrate feed: 60 %). All cows were artificially inseminated (n = 2512) 12 h after the expression of signs of estrus (EDAI). Alternatively, by programmed-timed artificial insemination (TAI) using the (GnRH - PGF2α – GnRH) protocol, (OVSYNCH®, CEVA Santé Animale, France). Pregnancy was diagnosed by rectal palpation and transrectal ultrasound using "My's-A001-N", with 1164 pregnancy cases, i.e. a mean pregnancy rate in herd (PR % = 46.33 %). A 1047 full term calving were recorded by farm manager and personnel for each calving event in Excel sheet. Information recorded included dam parity, date of calving, occurrence of dystocia (unassisted or assisted calving), calf gender, calve mortality at 24 h to 48 h post-calving and placental expulsion status (spontaneous expulsion or placenta retention). Calf mortality during calving and after parturition (within the first 24-48 h after birth) is termed as perinatal mortality and it mainly occurs due to dystocia [19].

Climatic factors

Climatic factors were obtained from the regional meteorological station which reflects the weather conditions on the farm. It includes the maximum daily temperatures in °C (T) and the maximum relative humidity (% RH). These were used to calculate the temperature-humidity index (THI) [36]: $THI = (0.8 \times T) + [(\% RH / 100) \times (T - 14.4)] + 46.4$.

Date of breeding and calving were used to allocate the THI values at breeding and late gestation (7th, 8th and at parturition), hence, it were divided into 4 ranges, according to Hahn et al.' [20] classification. They evaluated the intensity of heat stress, as follows: normal, alert, danger and emergency, which corresponds with the following values, THI < 74, [75–78], [79–83] and THI > 84, respectively.

Statistical analysis

To investigate the association between PCM, twinning births and the potential risk factors, the statistical analyses were performed for Holstein cows. According to the binary nature of PCM and twin birth as the response variables, a logistic regression model was constructed in Minitab® 18.1 (Minitab, Inc., in the United States and other countries) as the following models:

Model 1: Logit (π) = $\alpha + AFC_i + Bi\text{-}Y_j + Br\text{-}Y_k + Ca\text{-}Y_l + CS_m + GLR_n + DP_o + BT_p + CE_q + PR_r + THI\text{-}B_s + THI\text{-}7_t + THI\text{-}8_u + THI\text{-}P_v$

Model 2: Logit (π) = $\alpha + AFC_i + Bi\text{-}Y_j + Br\text{-}Y_k + Ca\text{-}Y_l + DP_m + BT_n + THI\text{-}B_o + THI\text{-}P_p$

Where: π = the probability of PCM and twin birth; t α = the intercept parameter; AFC = fixed effect of age at first calving (day) (1 = <720, 2 = [720–900], 3 = [900–1080],

Table 1. Odds ratios (OR) and 95 % confidence intervals (CI) of the animal factors associated with the incidence of stillbirth.

Variable	N	PCM (%)	OR	95 % CI	p-value
Calf sex					
Female	24	32.43	Referent		
Male	35	47.30	1.43	(0.83; 2.44)	0.0001
Twin	15	20.27	16.42	(7.39; 36.49)	
Parity of dam					
Multiparous	34	45.95	Referent		
Primiparous	9	12.16	0.54	(0.25; 1.16)	0.019
Nulliparous	31	41.89	1.51	(0.91; 2.52)	
Gestation length range					
[270-282]	41	55.41	Referent		
[251-269]	12	16.22	2.85	(1.42; 5.70)	0.023
[283-300]	21	28.38	1.18	(0.68; 2.04)	
Age at first calving					
<720	6	8.11	Referent		
[720-900]	43	58.11	0.8	(0.32; 1.95)	
[900-1080]	16	21.62	0.76	(0.28; 2.01)	0.666
[1080-1260]	7	9.46	1.34	(0.42; 4.23)	
>1260	2	2.70	1.71	(0.31; 9.46)	
Breeding type					
EDAI	71	95.95	Referent		0.279
TAI	3	4.05	0.27	(0.02; 3.25)	
Calving ease					
Unassisted	13	17.57	Referent		
Little intervention	1	1.35	0.07	(0.007; 0.67)	0.0001
Difficult	15	20.27	2.63	(0.85; 8.08)	
Very difficult	45	60.81	76.19	(18.76; 309.46)	
Placental expulsion status					
Spontaneous expulsion	9	12.16	Referent		0.0001
Placenta retention	65	87.84	65.97	(31.85; 136.66)	
Twinning birth					
Singleton	59	79.73	Referent		0.0001
Twin	15	20.27	13.49	(6.47; 28.10)	

N = number of cases; OR = Odds ratios; CI = 95 % confidence intervals; PCM = perinatal calf mortality; EDAI = artificial insemination by estrus detection; TAI = timed artificial insemination.

4 = [1080–1260], 5 = >1260); Bi-Y = fixed effect of dam's birth year (1992-2014); Br-Y = fixed effect of dam's breeding year (1994–2017); Ca-Y = fixed effect of dam's calving year (1995-2017); CS = fixed effect of calf sex: (1 = male, 2 = female, 3 = twin); GLR = fixed effect of gestation length range (day) (1 = [251–269], 2 = [270–282], 3 = [283–300]); DP = fixed effect of dam's parity (1 = Nulliparous, 2 = Primiparous, 3 = Multiparous); BT = fixed effect of breeding type (1 = EDAI (artificial insemination by estrus detection), 2 = TAI (timed artificial insemination); CE = fixed effect of calving ease (1 = unassisted, 2 = little intervention, 3 = difficult, 4 = very difficult); PR = fixed effect of placental expulsion status (1 = spontaneous expulsion, 2 = placenta retention); THI-B; THI-7; THI-8; THI-P = fixed effect of temperature-humidity-index (THI) at breeding, at 7th month of gestation, 8th month of gestation and at parturition (1 = <74, 2 = [75–78], 3 = [79–83], 4 = >84).

RESULTS

Perinatal calf mortality occurrence

The analysis of binary regression indicate that out of 1047 full term calving studied, 74 stillborn calves (7.07 %) were recorded. Table 1 provides the odds ratios (OR) and the prevalence rates for SB. It is apparent that the PCM incidence was significantly (p = 0.0001) higher, 13.49 times higher in twins than singleton births. Similarly, in the pregnant dams that had dystocia during the calving process and placenta retention, cases were 76.19 higher and 65.97 times more susceptible to having stillborn calves than unassisted calving and spontaneous expulsion of placenta at the parturition, respectively.

Moreover, females with a short [251–269] and prolonged [283–300] gestation lengths had a significant increased risk (p = 0.023) of stillborn calves, 2.85 and 1.18 times higher than females with average pregnancies [270–282]. Whereas, the calf gender was significantly (p = 0.0001) associated with the occurrence of PCM incidence, as bull calves were 1.43 times more likely to be stillborn than heifer calves. In the same way, there was a significant association between dam's parity and stillbirths (p = 0.019), hence, the incidence of PCM in nulliparous dams was 1.51 times higher compared to the multiparous dams. Otherwise, there was no linking between the AFC (p = 0.666) and the BT (p = 0.279) and SB occurrence.

As shown in Figure 1, the possibility to having a stillborn calf with assisted calving (dystocia) was 92.43%

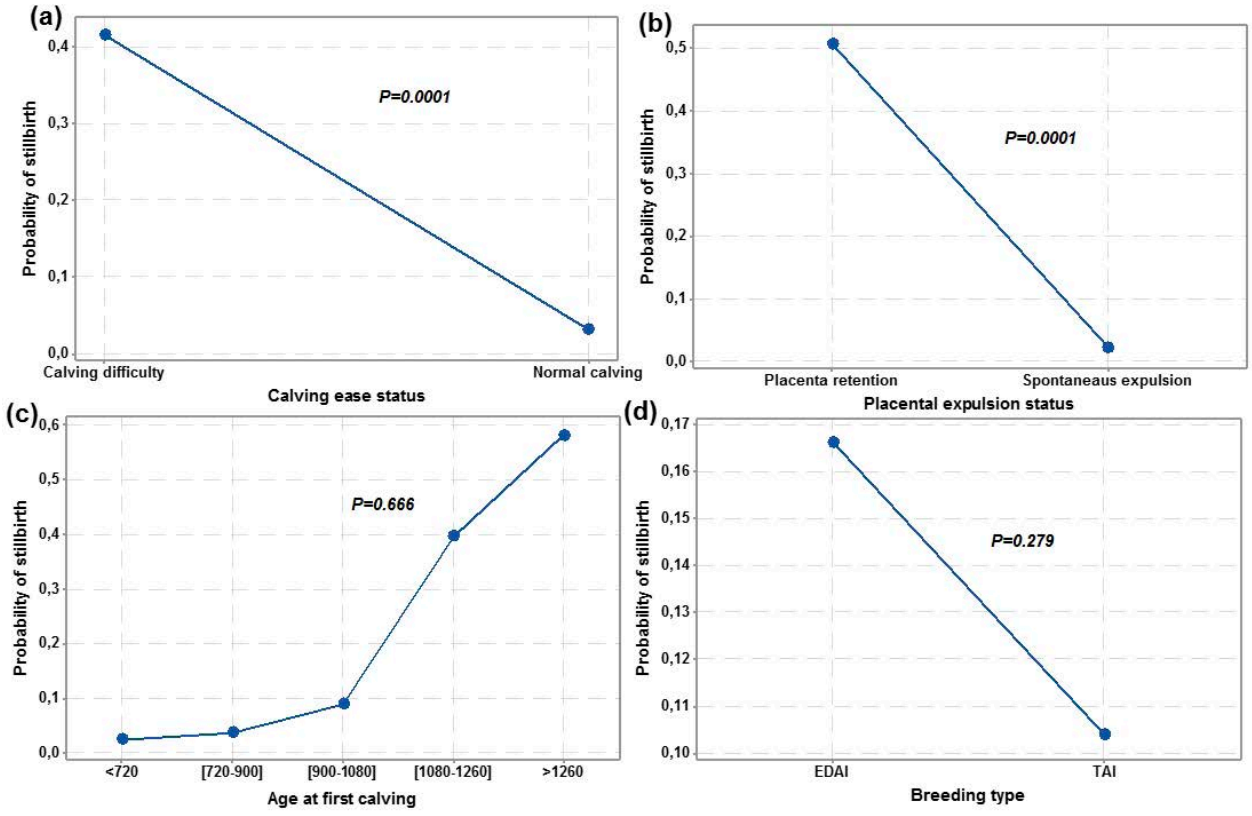

Fig 1. Main effects plot of calving ease (a), placental expulsion (b), AFC (c), and breeding type (d) for the incidence of PCM.

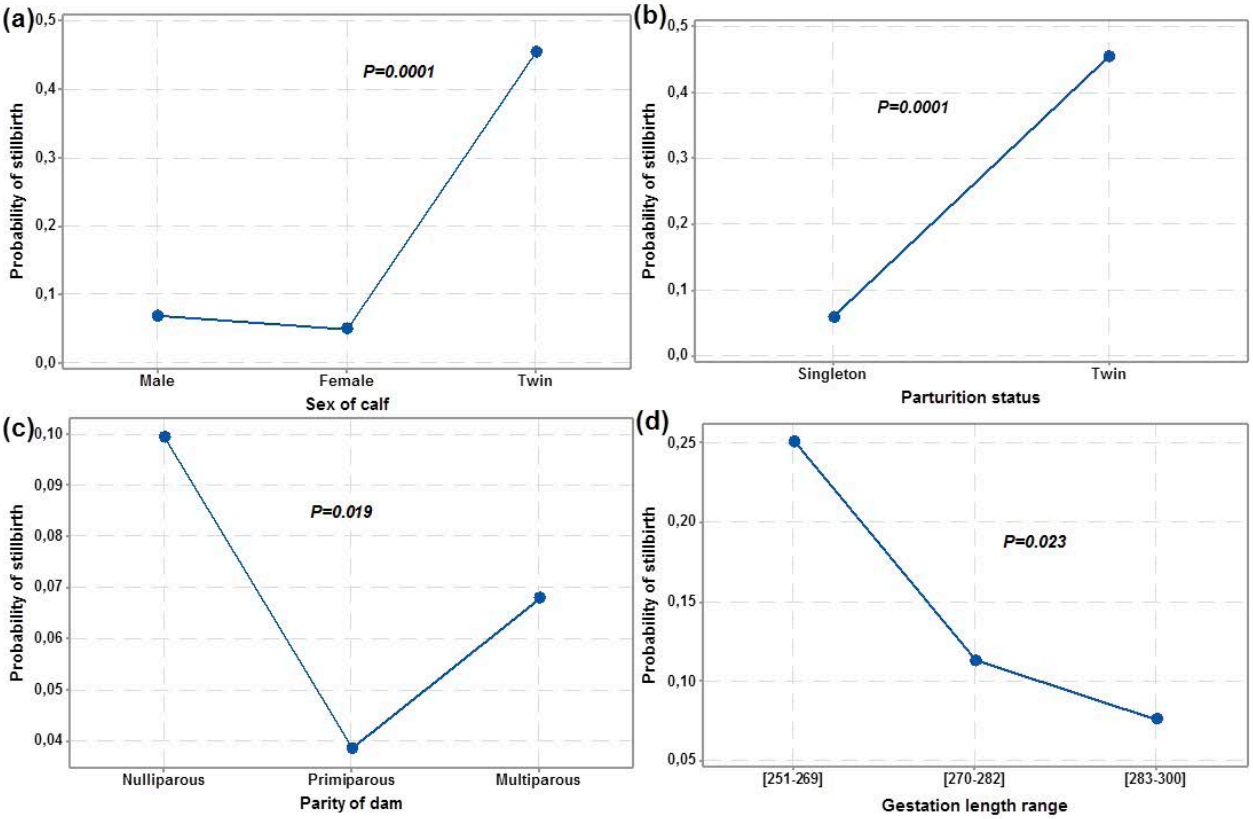

Fig 2. Main effects plot of sex of calf (a), parturition status (b), parity of dam (c), and gestation length range (d) for the incidence of PCM.

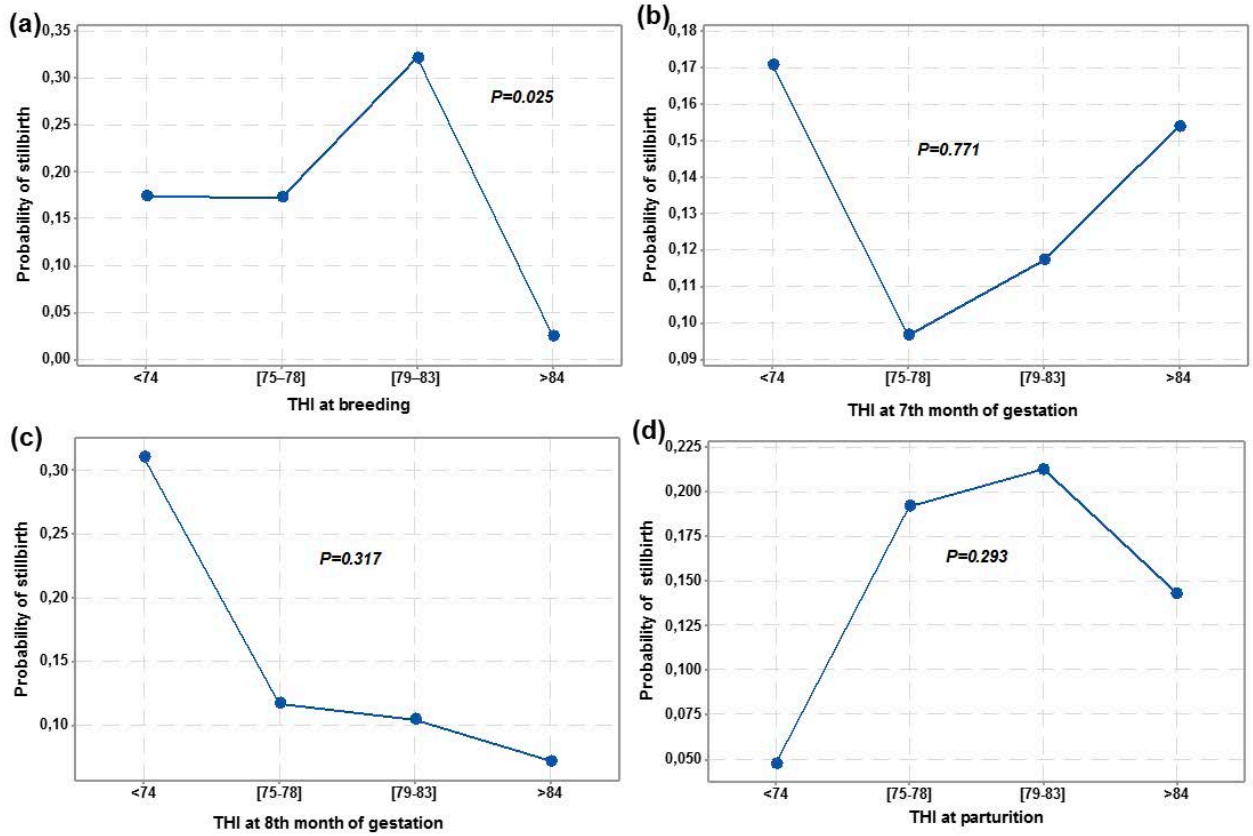

Fig 3. Main effects plot of THI at breeding (a), THI at 7th month of gestation (b), THI at 8th month of gestation (c) and THI at parturition (d) for the incidence of PCM.

higher than normal calving. In addition to the retained placenta cases, which had a high significant linking (p = 0.0001) with the incidence of SB (probability: 50.06 % vs. 2.1 %). On the contrary, no significant (p = 0.279) differences were found between PCM and BT. On the other hand, when AFC >1260d, the risk of SB is 58.1 %, although the association between the two variables is not significant (p = 0.666).

Looking at Figure 2, it is apparent that gestation length (GL) ranges had a significant relationship (p = 0.023) with incidence of SB, especially in shorts GL (251 to 269 days), with a probability of 25.1 %. Another significant aspect of mortality risk in twin births (45.45 %). In addition, the gender calf showed a high significant difference (p = 0.0001) between bull calves (6.7 %) and heifer calves (3.8 %) in terms of the probability of PCM. Interestingly, stillborn possibility the calves issued from nulliparous dams (9.9 %) differ significantly (p = 0.019) from the calves from multiparous (6.7 %) and primiparous dams (3.8 %).

The results of the main effects plot of THI values at conception and late gestation were summarized in Figure 3. Hence, no existence of a significant connection was found between the incidence of SB and THI at the 7th month of gestation (p = 0.771), at the 8th month of gestation (p = 0.317) and at the parturition (p = 0.293). Nevertheless, with THI at breeding, there is a significant difference (p = 0.025) in the probability occurrence of PCM, with different THI values.

It can be seen from the data in Table 2 that none of these differences were statistically significant concerning THI values at the 7th (p = 0.771), and at the 8th (p = 0.317) month of gestation, at the parturition (p = 0.293), dam's birth, conception and calving year (p = 0.617; p = 0.879; p = 0.868) with the stillborn calves. Interestingly, the THI [79–83] value at breeding was 3.04 times higher compared to THI < 74 in the manifestation of PCM.

Table 2. Odds ratios (OR) and 95 % confidence intervals (CI) of the environmental factors associated with the incidence of stillbirth.

Variable	N	PCM (%)	OR	95 % CI	p-value
THI at breeding					
<74	39	52.70	Referent		
[75–78]	14	18.92	0.93	(0.22; 3.91)	0.025
[79–83]	16	21.62	3.04	(0.79; 11.62)	
>84	5	6.76	0.1	(0.01; 0.98)	
THI at 7th					
<74	31	41.89	Referent		
[75–78]	18	24.32	0.52	(0.10; 2.70)	0.771
[79–83]	9	12.16	1.25	(0.15; 9.91)	
>84	16	21.62	0.66	(0.04; 9.09)	
THI at 8th					
<74	32	43.24	Referent		
[75–78]	9	12.16	0.14	(0.01; 1.22)	0.317
[79–83]	17	22.97	0.44	(0.02; 6.93)	
>84	16	21.62	0.25	(0.01; 9.98)	
THI at parturition					
<74	30	40.54	Referent		
[75–78]	10	13.51	2.81	(0.52; 15.04)	0.293
[79–83]	16	21.62	4.87	(0.66; 35.84)	
>84	18	24.32	1.79	(0.11; 29.10)	
Year of birth	74	100	0.91	(0.64; 1.30)	0.617
Year of breeding	74	100	0.94	(0.42; 2.09)	0.879
Year of calving	74	100	1.07	(0.47; 2.44)	0.868

N = number of cases; OR = Odds ratios; CI = 95% confidence intervals; PCM = perinatal calf mortality; THI = temperature-humidity index

Twinning birth occurrence

Analyzing the gender ratio in multiple calving, the opposite sex twins were the most common, with the proportion being 45.45 %. Heifer calves were the least frequent (18.18 %) and the proportion of bull calves was around 36 %.

From the Table 3 below, we can see that no significant differences were found between THI values at conception (p = 0.416), BT (p = 0.437), dam's AFC (p = 0.638), dam's birth, conception and calving year (p = 0.678; p = 0.099; p = 0.172) and the appearance of twins, respectively.

Whereas, dam's parity had a significant relationship (p = 0.013) with incidence of twinning birth, especially in multiparous dairy cows, which were more susceptible to having twin calves, 10.02 times more than heifers. Following the addition of the link with THI values at parturition, a significant increase (p = 0.034) in the occurrence of twins was recorded. The susceptibility to getting the twins were 4.10 and 2.32 times higher in heat stress conditions at calving (from 75 to > 84), respectively.

The results of the probability analysis of the main variables affecting the event of twinning birth were summarized in Figure 4. None of these differences were statistically significant excepting the dams' parity, hence, the probability of having multiple calves was 3.05 %; 1.42 %; 0.31 % in multiparous, primiparous and nulliparous cows, respectively. Similarly, concerning the heat stress conditions at the calving (THI-P), the possibilities of twins were 1.72 %; 6.72 %; 3.93 % and 3.93 % following the variation of THI values.

DISCUSSION

The current study found that stillborn calves were represented by 7.07 % of total calving events. This finding is consistent with that of Mellado et al. [45] in North-eastern Mexico (7.3 %). Also, El–Tarabany [9] under subtropical conditions, showed that the prevalence rate for SB was 15.4 %. This differs from the findings presented here.

Table 3. Odds ratios and 95 % confidence intervals (CI) of the independent factors associated with the incidence of twinning birth.

Variable	N	Twinning birth (%)	OR	95 % CI	p-value
Parity of dam					
Nulliparous	2	6.06	Referent		
Primiparous	6	18.18	4.61	(0.88; 24.01)	0.013
Multiparous	25	75.76	10.02	(1.77; 56.60)	
THI at breeding					
<74	22	66.67	Referent		
[75–78]	4	12.12	0.45	(0.15; 1.38)	0.416
[79–83]	6	18.18	1.01	(0.38; 2.65)	
>84	1	3.03	0.45	(0.05; 3.62)	
THI at parturition					
<74	9	27.27	Referent		
[75–78]	8	24.24	4.10	(1.54; 10.86)	0.034
[79–83]	7	21.21	2.32	(0.85; 6.34)	
>84	9	27.27	2.32	(0.91; 5.94)	
Breeding type					
EDAI	32	96.97	Referent		0.437
TAI	1	3.03	0.48	(0.06; 3.71)	
Age at first calving	33	100	0.99	(0.99; 1.00)	0.638
Year of birth	33	100	1.05	(0.80; 1.39)	0.678
Year of breeding	33	100	0.59	(0.30; 1.15)	0.099
Year of calving	33	100	1.58	(0.79; 3.16)	0.172

N = number of cases; OR = Odds ratios; CI = 95% confidence intervals; THI = temperature-humidity index; EDAI = artificial insemination by estrus detection; TAI = timed artificial insemination.

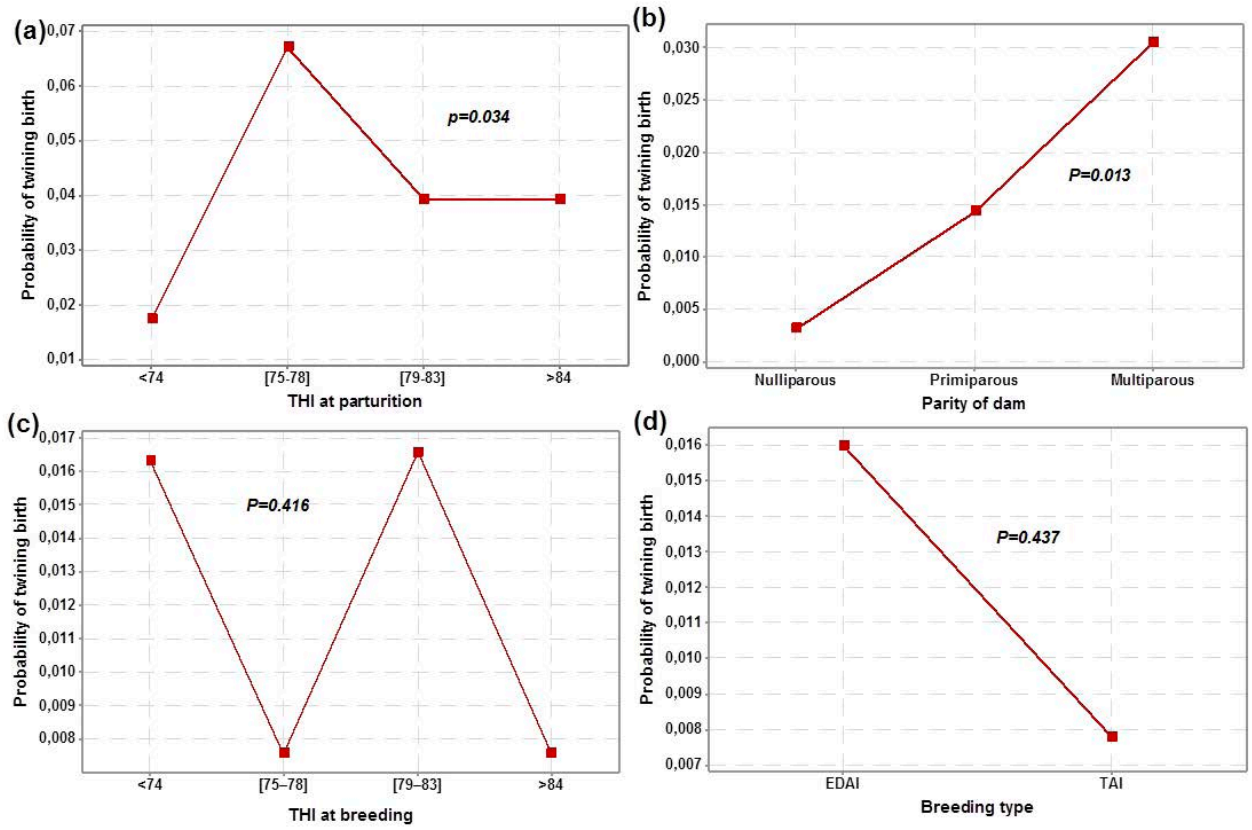

Fig 4. Main effects plot of THI at parturition (a), parity of dam (b), THI at breeding (c) and breeding type (d) for the incidence of twinning birth.

The average incidence of PCM in cows and heifers varies between 2 to 20 % across dairy industries internationally with the majority of countries between 5 to 8 % [37]. In fact, the PCM are related multifactorial complexes [24].

Dystocia

This study supports evidence from previous observations by e.g. H o h n h o l z et al. [24], M e l l a d o et al. [45], H o e d e m a k e r et al. [23], L o m b a r d et al. [31] indicating that the greater odds of SB was in assisted calving. This consistency may be due to the dystocial stillbirths that usually result from internal and external trauma [2], but also from deprivation of adequate oxygen supply (prolonged hypoxia) [42]. Another possible explanation for this is the timing of the movement of pregnant animals to the calving unit that can influence the risk of PCM [37]. Furthermore, K r i s t u l a and S m i t h [29] showed that the lower rates of stillbirth were in cows moved to the maternity unit during stage 2 (presence of feet at the vulva) compared to stage 1 of calving. Whereas, the parturition in tie stalls has been associated with a lower stillbirth rate than in free stalls [18]. According to C a r r i e r [5], who demonstrated that every additional hour in stage 2 of calving increases the odds of stillbirth by 30 %. Although this may reflect an interaction with calving supervision [23]. Nonetheless, V e r n o o y et al. [58] found no association between individual or group maternity accommodation and the risk of SB. Anyway, relatively simple interventions have the potential to significantly reduce the impact of dystocia on calf mortality and morbidity on dairy farms [31].

Environmental conditions

Our outcome is contrary to that of M e l l a d o et al. [45] who found the cows in a severe state of heat stress prenatally and at birth (THI > 83 units) had 1.3 higher risk of SB than cows suffering reduced heat stress. As a matter of fact, the occurrence of SB on this dairy farm was associated with either moderate or extreme heat stress during the peripartum period, with negative impacts from the seventh month of pregnancy until calving [45]. M e y e r et al. [47] showed that the prevalence of SB in summer were 27 % higher than in winter. These differences can be explained in part by increasing of ambient temperature, especially

in calves housed outdoors proved to be a risk factor for early calf mortality in veal calves [54]. As others showed, mortality of 1 to 21 days old Holstein calves was higher in moderate conditions than in the hot season [46]. Yet, the acute brief heat stress during late gestation did not alter passive antibody transfer capacity in calves [56]. However, D a h l et al. [6] concluded that calves experiencing heat stress *in utero* are prone to develop a smaller mature body size and more fat reserves than counterparts in thermoneutrality. All the same, M i s a k a et al. [48] reported that was no effect of interactions between herd size and calving season on the SB rate.

Calf sex and twin pregnancies

A strong relationship between calf gender and PCM has been reported in the literature. Hence, many studies show that male calves have a higher risk of perinatal mortality than females [18, 23, 3]. In fact, male calves have twice the odds of being a stillborn calf than female calves [31]. Whereas, when calves experienced a dystocic calving, female calves were more likely to die than male calves within 24 hours after calving [31]. Also, female calves born from a multiparous cow had 12 % higher odds of being stillborn than male calves [47]. Despite this, the higher percentage of female calves led to reduced dystocia for both heifers and cows, especially for twin births [51]. Provided where perinatal mortality occurs following a normal parturition, there was very little difference between genders of calves [52].

Otherwise, twin pregnancies have a higher risk of fetal death or abortion in the final months of gestation [7, 8, 25]. The limited energy reserves and vitality of twin calves during and after pregnancy are seen as the primary reasons for the elevated proportion of stillbirths [53].

Gestation length

Prior studies have noted the importance of gestation length as the third factor affecting the prevalence of SB, of which GL was the most influential factor for SB in multiparous dams [47]. As a result, extreme birth weight and GL increase PCM rates, implying that premature calves should be given special care at birth [45]. Besides, the both short and prolonged GL were associated with an increased risk of perinatal mortality, when were deviated from the mean [3]. According to N o g a l s k i and P i w c z y ń s k i [50], the lowest PCM rate (3.23%) was observed in respect to GL values in the range of 275–277 days, whereas the share of calves that were born dead and died within 24 h after birth increased three-fold (to 10.48 %), when GL exceeded 283 days. On the whole, the optimal gestation length in Holstein cows was determined in the range of 275–277 days based on calving ease and SB values. In other words, the average GL values optimized lifetime productivity, calving ease, SB rates and calving-to-first-service interval [25]. Overall, the relative risk is much higher for calves born after a short gestation than a prolonged gestation, probably due to organ immaturity [26, 3]. To conclude, GL values should be analyzed in conjunction with calving ease, SB rates and placental expulsion [21].

Placenta retention

In the cow the fetal membranes are normally expelled between 30 minutes and 8 hours after stage 2 of calving. Premature placental separation has been associated with "weak calf syndrome" in heifers [40]. It has been associated with premature birth [1] and maldisposition in multiparous cows [37]. Due to the significant relationship between SB and placental retention may be attributed to the fact that deceased births are often from dystocia, leading to the occurrence of retention of the placenta in dams [8]. Furthermore, E n d l e r et al. [10] found an increased association between placental under perfusion disorders (such as preeclampsia, small for gestational age, and SB) and retained placenta. Besides, S o r g e et al. [55] reported that the level of serum estradiol-17β was lesser in cows with stillbirths, which might be an indicator of placental malfunction or abnormal hormone signals from the calf to the placenta prior to parturition. These relationships may be partly explained by the homeokinetic changes that regulate body temperature of cows suffering heat stress and provoke a redistribution of blood flow from the body core to the periphery, which reduces perfusion of the placental blood vessels [22]. In this respect, heat stress during gestation can compromise placental development, which results in fetal hypoxia, malnutrition, and eventually fetal growth impedance [57]. The condition is adversely affected by hyperthermia-induced placental insufficiency, which results in a reduction in both placental size and functional capacity. This impairment restricts the exchange of oxygen and nutrients between the maternal and fetal systems. Furthermore, even a modest reduction in gestational length, frequently observed during periods of heat stress, exacerbates these detrimental effects [48].

Dams' parity and age at first calving

This also accords with our earlier observations, which showed that calves from first-parity dams had a higher risk ($p < 0.01$) of being stillborn than calves from dams in later parities [24]. In fact, H o h n h o l z et al. [42] and M e e et al. [40] reported that PCM were more frequent for heifers than primiparous and multiparous, especially males, with sexed semen use [51]. Due to the interaction between type of dystocia and parity that has been postulated, whereby a prolonged second stage of calving is a significant risk factor for perinatal mortality in primiparae, whereas maldisposition and twins are more common risk factors in pluriparae [41, 19].

Surprisingly, no differences were found in AFC's of dams and PCM in the current study. Conversely to M e e et al. [40] found that in primiparous cattle, a younger age of calving is associated with an increased risk of perinatal mortality [40]. This includes that the highest risk seen in cattle calving at less than 24 months old is inadequate pelvic size [21, 40]. Stillbirths during the first calving had detrimental effects on subsequent performance of the dam under subtropical conditions [9]. Another point is that prepartum maternal dietary energy content can alter calf birth weight and hence the risk of fetal oversize [13]. Relative fetal oversize is a risk factor for SB [26].

Risk Factors for twinning

Several reports have shown that the incidences of multiple ovulations are much more frequently observed in Holstein Friesian cows than in other dairy cattle breeds [15]. The most interesting finding was that the parity of dam and season of calving were potential risk factors for twinning in dairy cows [25]. Besides, the increase in incidence of double ovulations is approximately linear with increasing calving numbers [12]. J o h a n s o n et al. [27] reported that increasing parity does the chance of twinning, with the largest increase happening between the first and second parities.

Contrary to expectations, this study did not find a significant difference between the incidence of twinning birth when following some FTAI protocols [35, 16]. As mentioned in the literature review, although the heat stress at AI (maximum temperature-humidity index > 72) had not effects on follicular dynamics, cows with two unilateral follicles showed a higher double ovulation rate (48.6 %) than cows with two bilateral follicles (34.8 %) [34]. Furthermore, increased energy content in the diet and increased metabolism of steroid hormones by dairy cows may lead to a higher number of doubled ovulations in lactating dairy cows [59]. Otherwise, the estrus during the warm season decreased the likelihood of double ovulation by a factor of 0.86 [35]. As a matter of fact, K o m i s a r e k and D o r y n e k [28] found the percentage of twin pregnancies were 2.41 % in summer, 2.04 % in spring, and falling to 1.79 % in autumn.

Generally, twinning in dairy cattle is associated with higher milk yields, an increased incidence of abortion, dystocia, retained fetal membranes, freemartinism and an extended calving to conception interval [49]. Over 95 % of female calves twinned to a male are sterile [17].

CONCLUSION

This paper has argued that the PCM is a multifactorial complex, at the same time, twinning births are affected by some factors. Consequently, the binary regression analysis revealed that the rates of stillbirth were significantly higher in nulliparous cows (42 %) than multiparous cows, especially at short and long length of gestation. Besides, dystocia was the most important variable affecting the PCM rate (> 80 %), with the large occurrence (88 %) of the retained placenta cases.

In fact, under the Saharan conditions of the current study, in which prolonged heat loads prevail for most of the year, these data did not support the concept that sustained high ambient temperatures increases PCM of Holstein calves. Otherwise, the overall average twinning rate was 3.15 %. Dams' parity and heat stress at calving were identified as important factors and had significant effects on the incidence of twinning birth.

These results suggest that, in general, possible preventive measures should include improving the peripartum environment and maternal ration, improving genetic selection for calving ease and optimising heifer rearing. The study has contributed to a better understanding of the factors responsible for twin pregnancy in Holstein cattle and the establishment of effective management strategies for pregnant cows. Further research and experimentation on the effects of perinatal mortality and reproductive disorders associated with twins under warm environmental conditions are strongly recommended.

DATA AVAILABILITY STATEMENT

The original datasets used in this research and, if applicable, supporting information files are available.

ETHICAL STATEMENT

No ethical approval was obtained because this study only involved data collection from the farm manager.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influence the work reported in this paper.

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GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

AUTHORS CONTRIBUTIONS

Conceptualization: L. Ouarfli and A. Chehma. Data curation: L. Ouarfli. Formal analysis: L. Ouarfli. Funding acquisition: L. Ouarfli. Investigation: L. Ouarfli. Methodology: L. Ouarfli. Project administration: L. Ouarfli and A. Chehma. Resources: L. Ouarfli and A. Chehma. Software: L. Ouarfli and A. Chehma. Supervision: L. Ouarfli and A. Chehma. Validation: L. Ouarfli. Visualization: L. Ouarfli and A. Chehma. Writing – original draft: L. Ouarfli. Writing – review and editing: L. Ouarfli and A. Chehma.

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ORIGINAL ARTICLE

DEGRADATION OF MILK PROTEIN FRACTIONS DUE TO *PSEUDOMONAS* SPP. IN THE DAIRY INDUSTRY

Mariana Kováčová*, Jana Výrostková

Department of food hygiene, technology and safety, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81, Košice, Slovakia

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***Correspondence:** mariana.kovacova@.uvlf.sk

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Milk is a nutritionally important food, particularly due to its milk protein content. The representation and degradation of individual protein fractions in raw cow's milk were monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with a focus on the presence of undesirable milk microbiota and somatic cell count (SCC). Raw cow's milk samples (n = 240) were collected from three farms in accordance with the principles of the international standard STN EN ISO 707 during the autumn 2022/winter 2022/spring 2023/summer 2023. After cultivating microorganisms (ISO/TS 11059), 73 isolates of *Pseudomonas* spp. were identified using the polymerase chain reaction (PCR). Subsequently, qualitative parameters, including somatic cell count, protein content, and protein profile, were analyzed. The results showed statistically significant differences ($p < 0.001$) in relation to seasonal changes. The findings indicate significant interactions between the presence of *Pseudomonas* spp., somatic cell count, seasonality, and the content and composition of milk proteins ($p < 0.001$). The presence of *Pseudomonas* spp. and a high somatic cell count contributed to a decrease in the α_{s2}-casein, α_{s1}-casein, and ß-casein fractions, accompanied by an increase in serum proteins β-lactoglobulin and α-lactalbumin ($p < 0.001$). The negative impact of these changes in protein composition affects the further processing of milk in the dairy industry, particularly in cheese production. The aim of this work was to determine the level of the protein fractions degradation caused by the presence of undesirable dairy *Pseudomonas* spp. using proteomic methods, considering SCC.

Keywords: electrophoresis; microorganisms; milk; proteins; somatic cells

INTRODUCTION

Milk is not only an important source of protein in the human diet but also serves as a crucial source of protein for the food industry [1]. Milk proteins make up approximately 3.2–3.8 % of the total milk composition. They are categorized into two groups based on their reaction during the acidification of milk to a pH value of 4.6. The first group consists of stable micelles that form a complex of caseins α_{s1}-casein (α_{s1}-CN); α_{s2}-casein (αs2-CN); β-casein (β-CN); and κ-casein (κ-CN) [2]. The second group consists of whey proteins, which, unlike caseins, are soluble. Key whey proteins include α-lactoalbumin (α-LA) and β-lactoglobulin (β-LG) [3]. All these protein fractions can be separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After treatment with SDS, regardless of their native charge, all proteins acquire a high negative charge. Denaturation of proteins is accomplished by heating them in a buffer containing a soluble thiol reducing agent (2-mercaptoethanol; dithiothreitol) and SDS. Mercaptoethanol reduces all disulfide bonds of cysteine residues to free sulfhydryl groups, and heating in SDS disrupts all intramolecular and intermolecular protein interactions. Denatured proteins are subsequently separated electrophoretically strictly according to their molecular size in a polyacrylamide gel [4].

The composition of milk significantly influences its physicochemical properties, yield, and the quality of dairy products. Casein is the primary protein needed for cheese production. During the technological process of cheese making, most whey proteins transition into liquid form of whey [5]. The casein content, along with factors such as somatic cell count (SCC), temperature, the amount of soluble Ca^{2+} ions, and titration acidity (up to 8 °SH), greatly influences milk coagulation, forming the curd. The yield and composition of the main proteins in cow's milk determine the value of the milk depending on its intended use. High-quality milk with a high casein content, especially κ-CN, is necessary for dairy production especially cheese production. Processing such high-quality milk results in increased yield, stronger curd formation, and minimal casein loss in the whey [1,6].

Psychrotrophic bacteria are ubiquitous and can grow at low temperatures (< 7 °C), often multiplying during the storage of raw milk at refrigerated temperatures [7]. The group of psychrotrophic microorganisms consists of Gram-positive microorganisms, such as *Aeromonas hydrophila* a Yersinia enterocolitica and Gram-negative microorganisms, such as *Pseudomonas* spp., *Listeria monocytogenes* and *Bacillus cereus* that is particularly important from a food point of view, since the storage of many foods at low temperatures is a common practice during production, processing and food transport [8].

The genus *Pseudomonas*, dominate the microbial community in stored, refrigerated raw cow milk, and may also be a contaminant of pasteurised milk. The proteolytic activity of *Pseudomonas* spp. in raw cow's milk is an important factor affecting the final quality of dairy products. Milk contains microbial proteases that can cause casein degradation and thus affect the processing of dairy products. The breakdown of milk proteins by proteases negatively affects milk coagulation (coagulation, gelatinization), cheese ripening, ultra-high temperature (UHT) milk shelf life, flavor formation and the texture of dairy products. Microbial proteases cleave milk protein fractions with varying intensity. Whey proteins are resistant to proteases, as indicated by their minimal degradation [9, 10, 11]. Determining the microbiological quality of food provides important information for assessing food safety and serves as an essential indicator for studying protein quality in milk and dairy products [12].

The increase in the presence of *Pseudomonas* spp. is also accompanied by an increase in the number of somatic cells, which indicate the health status of dairy cows. Somatic cells are naturally present in small amounts in milk. Their primary function is to fight disease and help repair damaged tissue. Any intramammary infection (mastitis) leads to an increase in these cells in the milk [13]. The quality of raw cow's milk intended for processing for human consumption is very important, and therefore raw cow's milk must meet certain minimum criteria for the number of somatic cells and the total number of microorganisms. The number of somatic cells at 30 °C in 1 cm^3 may not exceed 400,000 colony forming units (CFU). The criteria for the total number of microorganisms determined at 30 °C in 1 cm^3 may not exceed 100,000 CFU [14].

The aim of this work was to determine the protein fractions degradation using proteomic methods caused by the presence of *Pseudomonas* spp., taking SCC into account.

MATERIALS AND METHODS

Collection of samples

Raw cow's milk samples were collected from three farms (FMa, FMb, FMc) located in the Slovak Republic regions Abov, Spiš and Zemplín. The experimental period spanned from autumn 2022/ winter 2022/ spring 2023/ summer 2023, with a total of 240 samples taken. On each farm an experimental group of 20 Slovakian spotted dairy cows was selected. Samples were collected quarterly, during each season (80 raw cow's milk samples from each farm in total), obtained from morning milking. The farms were selected based on identical housing, husbandry practices, and feeding methods. Sample collection adhered to the principles of STN EN ISO 707 [15], conducted by the farm's zootechnician. The milk samples were transported to the Department of Hygiene, Technology and Food Safety of the Faculty of Veterinary Medicine in Košice. During transport and until the time of analysis, the raw cow's milk samples were stored at a temperature of 6 °C. The samples were subjected to microbiological analysis within three hours and subsequently to physicochemical determination (SCC, protein content and SDS-PAGE). Microbiological analysis was aimed at determining the presence of bacteria of the genus *Pseudomonas*, which are an indicator of the hygienic quality of milk and can also contribute to the development of inflammation of the mammary gland.

Cultivation and isolation of microorganisms of the genus *Pseudomonas*

According to the principles of STN EN ISO 6887-5 [16], suitable tenfold dilutions were prepared from the raw cow's milk samples, which were inoculated in an amount of 0.1 ml onto the surface of the pre-dried selective diagnostic medium Pseudomonas Agar Base (Hi-Media, India) supplemented with penicillin (100,000 IU/l, Dr. Ehrenstorfer GmbH, Augsburg, Germany) and pimaricin (0.01 g/l, Dr. Ehrenstorfer GmbH), which is used for selective isolation of *Pseudomonas* bacteria. After the end of incubation for 48 hours at 25 °C by ISO/TS 11059 [17], 5 identical solitary colonies from each plate were used for further identification.

Identification of isolates of *Pseudomonas* spp.

The isolated strains were primarily identified based on their phenotypic characteristics. Strains that were positive for oxidase and catalase tests, and partially negative for Gram staining and glucose fermentation were evaluated and identified as *Pseudomonas* spp. [18,19]. These strains were subsequently subjected to identification using the polymerase chain reaction (PCR) method [20]. The reference strain *Pseudomonas aeruginosa* CCM 1960T (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a positive control.

Determination of somatic cell count by fluorescence microscopy

A milk sample of 50 ml was transferred to a glass tube, at room temperature, which was then mixed using Vortex. Next, 100 µl of milk sample was taken and transferred to a microtube with lyophilization dye (Sofia Green). Subsequently, the sample was mixed, left to rest, mixed again and an amount of 8 µl of the prepared sample was transferred to the LACTOCHIPx4 (Milkotronic Ltd., Nova Zogora, Bulgaria) [21]. The results of SCC determination are given in log units.

Determination of protein content

The milk protein content (%) was determined using the Lactoscan MCCW device (Milkotronic Ltd., Nova Zogora, Bulgaria). [21].

Detection of protein fractions by SDS-PAGE electrophoretic method

Milk samples were stored at -18 °C until SDS-PAGE electrophoresis was performed. Before use, milk samples were thawed, diluted, and adjusted for protein content (5 µl) using a NanoDrop 2000 Spectrophotometer [22]. Electrophoretic detection of protein fractions was performed using a vertical electrophoretic system (Bio-Rad, California, USA). A combination of two gels (14 % separation gel and 4 % focusing gel) with a thickness of 0.75 mm was used to separate the protein fractions. After the polymerization time, the tested raw cow's milk samples (20 µl) were placed in the formed wells. 180 V, 100 mA for 1.5 h was used for focus of the samples. Subsequently, the gel was fixed, stained and destained. Using GelAnalyzer 19.1 software, the relative amounts of individual protein fractions were analysed and determined [23, 24, 25]. Two molecular weight markers from 6.5 to 66 kDa and one molecular weight marker from 14 to 66 kDa (Serva, iBioTech) were used to determine the molecular weight of pro-

teins. The following protein fraction standards were used: bovine milk lactoferrin, casein α_{s1}, casein α_{s2}, casein-β, casein-κ, β-lactoglobulin, α-lactalbumin, and serum albumin (Sigma-Aldrich, Germany).

Statistical evaluation of experiment results

One-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons with a 95 % confidence interval were used for evaluation using GraphPad Prism 8.3.0.538 statistical software (GraphPad Software, San Diego, CA, USA) [21].

RESULTS

Raw cow's milk samples were collected quarterly during the experimental period (autumn 2022/ winter 2022/ spring 2023/ summer 2023) from three farms. From total of 240 milk samples taken, 151 isolates were confirmed based on phenotypic characteristics which showed a positive oxidase and catalase reaction, and at the same time were negative for Gram staining and glucose fermentation. Isolates (n = 151) obtained from milk samples originating from FMa, FMb and FMc were subsequently subjected to identification using the PCR method, which was aimed at determining the presence of the 16S rRNA gene characteristic of the genus Pseudomonas, which was confirmed in 73 isolates.

The quantitative representation of isolates on individual farms also showed differences in their presence in relation to the season. On FM*a*, we captured a total of 17 isolates (23.29 %), of which the following amount was collected: spring (6 isolates; 35.29 %), summer (8 isolates; 47.06 %), autumn (1 strain; 5.88 %), winter (2 isolates; 11.76 %). On FM*b*, we obtained a total of 21 isolates (29 %), which were isolated during the following seasons: spring (7 isolates; 33.33 %), summer (9 isolates; 42.86 %), autumn (3 isolates; 14.29 %), winter (2 isolates; 9.52 %). Despite the identical management of dairy cattle breeding on farms, we noted an increased number of monitored bacteria of the genus *Pseudomonas* on FM*c*, where we obtained a total of 35 isolates (47.95 %), of which: spring (12 isolates; 34.29 %), summer (15 isolates; 42.86 %), autumn (4 isolates; 11.43%), winter (4 isolates; 11.43 %).

Using the Lactoscan SCC device, quantitative determination of SCC was performed in samples of raw cow's milk originating from FM*a*, FM*b* and FM*c* (Table 1). The results of the determination of SCC indicate significant differences in the milk quality of the sampled farms and also indicate a statistically significant effect of the change in the season $p < 0.0001$.

The protein content was determined on the farms quarterly during the entire experimental period, on the basis of which we were able to record one of the important factors affecting the protein content, i.e. change of seasons (autumn, winter, spring, summer) and associated SCC. Despite identical characteristics (breeding management, breed of cattle, feed ration), different protein content values were determined on the farms (Table 2). These differences were most pronounced ($p < 0.0001$) during the spring season between FMa (3.44 ± 0.10 %) and FMc (3.59 ± 0.11 %) and also between FMb (3.43 ± 0.14 %) and FMc (3.59 ± 0.11 %). Dairy cows with SCC more than 5.60 log exceed the maximum permitted values set by Regulation (EC) 853/2004.

Based on the results of determining the somatic cell count, the samples of raw cow's milk were divided into four experimental groups (ExG): ExG-1: SCC < 5.3 log (n = 20), ExG-2: SCC > 5.3 log < 5.6 log (n = 20), ExG-3: SCC > 5.6 log (n = 20), ExG-4: SCC > 5.3 log with the presence *Pseudomonas* spp. (n = 20). In the investigated milk samples (n = 80), considerable differences were noted in the presence and amount of individual protein (casein and whey) fractions (Table 3). Experimental group InG-4 contained samples of naturally contaminated milk with the presence of *Pseudomonas* spp. confirmed by PCR method. Samples in this group were selected from all farms we investigated, with the SCC in this group being higher than 5.6 log in n = 7 milk samples.

A detailed analysis of the protein fractions: SA, α_{s2}-CN, α_{s1}-CN, β-CN, κ-CN, β-LG, α-LA, which was carried out by SDS-PAGE electrophoresis, showed us the relationship between the relative amount of protein fractions, protein content (%), SCC (log) and, above all, the microbiological (presence of *Pseudomonas* spp.) milk quality. As can be seen from Fig. 1, the content of milk proteins varied from 3.18 % to 3.59 %, but we observed more significant changes in the representation of individual protein fractions.

In the experimental group ExG-1 and ExG-2, where SCC was below 5.6 log, the relative amount of protein fractions of caseins SA, α_{s2}-CN, α_{s1}-CN, β-CN was significantly dominant. We noted a significant decrease and

Table 1. SCC (log ± sd) determined on farms (FM*a*, FM*b*, FM*c*) during the experimental period (autumn, winter, spring, summer)

Farm	SCC [log]				
	Autum	Winter	Spring	Summer	p value
FM*a*	5.11 ± 2.21^{b,2}	5.07 ± 2.21^{b,2}	5.52 ± 2.89^{ab,2}	5.71 ± 3.03^{a}	< 0.001
FM*b*	6.01 ± 3.08^{1}	5.98 ± 2.91^{1}	5.92 ± 3.05^{1}	6.00 ± 3.13	> 0.05
FM*c*	5.91 ± 3.32^{1}	5.79 ± 3.23^{1}	5.65 ± 2.99^{1,2}	5.80 ± 3.17	> 0.05
p value	< 0.001	< 0.0001	< 0.05	> 0.05	

$^{a, b, c}$ - values in rows with different superscripts are statistically different (p < 0.05)
^{1,2,3} - values in columns with different superscripts are statistically different (p < 0.05)
sd - standard deviation

Table 2. Percentage protein content (mean ± sd) determined on farms (FM*a*, FM*b*, FM*c*) during the experimental period (autumn, winter, spring, summer)

Farm	Protein content [%]				
	Autumn	Winter	Spring	Summer	p value
FM*a*	3.38 ± 0.08^{a,b}	3.45 ± 0.11^{a,2}	3.44 ± 0.10^{a,2}	3.30 ± 0.12^{b,c}	< 0.0001
FM*b*	3.36 ± 0.15^{a,b}	3.36 ± 0.18^{a,b,1}	3.43 ± 0.14^{b,2}	3.30 ± 0.11^{a}	< 0.05
FM*c*	3.40 ± 0.15^{b}	3.53 ± 0.21^{a,b,2}	3.59 ± 0.11^{a,1}	3.18 ± 0.25^{c}	< 0.0001
p value	> 0.05	< 0.001	< 0.0001	> 0.05	

$^{a, b, c}$ - values in rows with different superscripts are statistically different (p < 0.05)
^{1,2,3} - values in columns with different superscripts are statistically different (p < 0.05)
sd - standard deviation

Table 3. Representation of relative amounts of protein fractions (mean ± sd) on farms (FM*a*, FM*b*, FM*c*)

ExG	SA	α_{s2}-CN	α_{s1}-CN	β-CN	κ-CN	β-LG	α-LA
ExG-1	5.70 ± 0.45^{a}	15.56 ± 1.13^{a}	9.23 ± 0.69^{a}	39.73 ± 0.83^{a}	10.38 ± 0.51^{a}	15.50 ± 0.55^{a}	7.56 ± 0.42^{a}
EXG-2	4.97 ± 0.62^{b}	14.10 ± 2.85^{a}	8.80 ± 0.42^{a}	42.03 ± 1.33^{b}	13.79 ± 2.02^{b}	22.13 ± 1.21^{b}	11.57 ± 1.06^{b}
exg-3	3.62 ± 0.40^{c}	9.91 ± 1.16^{b}	7.47 ± 0.56^{b}	30.77 ± 1.17^{c}	12.97 ± 0.78^{b}	32.60 ± 1.70^{c}	16.17 ± 0.88^{c}
exg-4	3.61 ± 0.65^{c}	8.71 ± 0.65^{b}	6.15 ± 0.85^{c}	32.11 ± 1.50^{c}	12.15 ± 0.88^{b}	33.15 ± 1.35^{c}	16.65 ± 0.87^{c}
p value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

$^{a, b, c}$ - values in columns with different superscripts are statistically different (*p* < 0.05).
SA = serum albumine; α_{s2}-CN = α_{s2}-casein; α_{s1}-CN = α_{s1}-casein; β-CN = β-casein; κ-CN = κ-casein; β-LG = β-lactoglobulin; α-LA = α-lactoalbumin
sd - standard deviation.

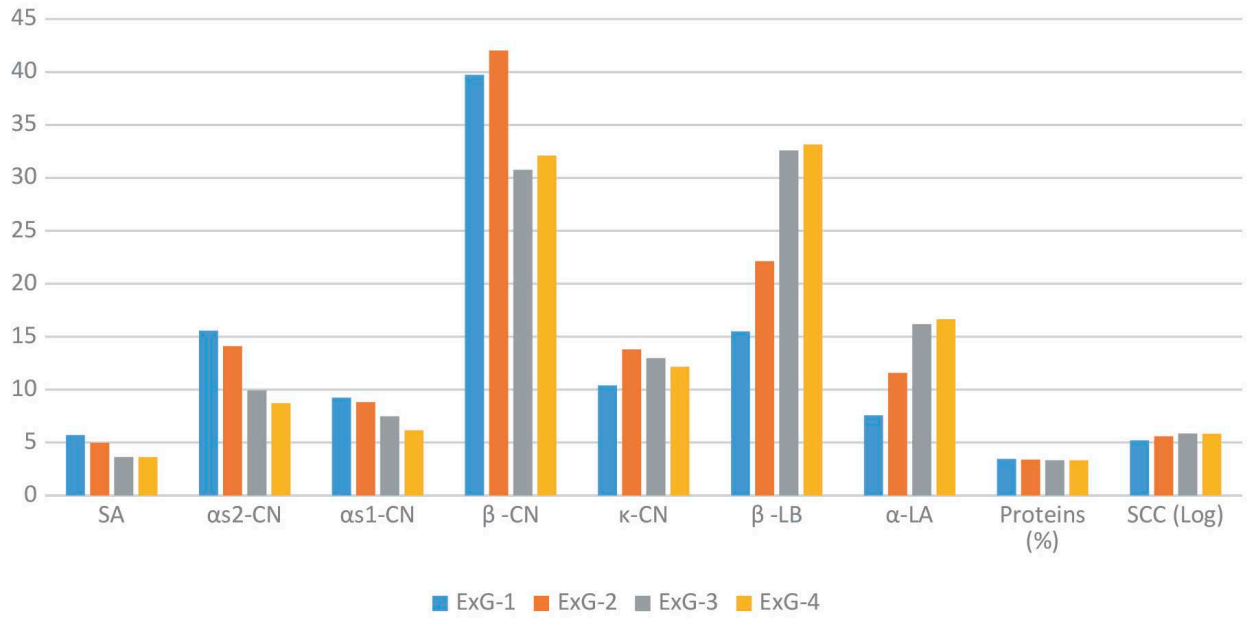

Fig. 1. The effect of *Pseudomonas* spp. and SCC (log) on milk protein content (%) and the relative amount of protein fractions determined in experimental groups (ExG-1, ExG-2, ExG-3 and ExG-4)

increase ($p < 0.001$) of β-LG and α-LA in groups ExG-3 and ExG-4, which contained high SCC. In the experimental group ExG-4, which contained *Pseudomonas* spp., the lowest relative amount of SA, α_{s2}-CN and α_{s1}-CN was detected, as well as the highest representation of β-LG and α-LA, which clearly points to the negative impact of undesirable microorganisms in the dairy industry and their impact on the quality of milk as well as its further processing.

DISCUSSION

Dairy products are extremely diverse due to the nutritionally rich composition of milk and the presence of species of microorganisms that can grow in it and affect its physicochemical and sensory properties [26].

The microbiological quality of milk is linked to the hygiene of milk collection and the health status of cattle. With insufficient hygiene during milking and poor health (inflammation of the mammary gland), there is a prevalence of undesirable dairy microorganisms that degrade the quality of milk and its suitability for further technological processing. The somatic cell count in milk is one of the main criteria used to assess the intramammary health status, both in individual animals and in milk tanks, because there is a direct correlation between the technological quality of milk (protein fractions), the microbiological quality and SCC [27].

The aim of this work was to determine the undesirable degradation of protein fractions caused by the presence of *Pseudomonas* spp., considering SCC and using proteomic methods. Cheese is a globally consumed dairy product produced by various technological processes that provide specific properties of individual types of cheese. The quality of cheese is mainly linked to the quality of the raw milk used for its production. However, when using milk containing undesirable dairy bacteria (*Pseudomonas* spp.) and high SCC, cheeses with changed properties can be produced. The use of milk with a high somatic cell count can negatively affect coagulation, ripening, final yield, chemical composition, overall structure and development of undesirable cheese flavors [28].

B o m b a d e et al. [29] found a slight seasonal variation in SCC from bovine milk, reporting higher SCC in summer than in winter or spring, which correlates with results of this study, where maximum SCC values were detected in farms FM*a* and FM*b* during the summer season. FM*c* farm showed the highest SCC during autumn. Milk with high SCC adversely affects cheese yield and cheese production efficiency, mainly due to the loss of casein to the whey [28].

Undesirable dairy microorganisms of the genus *Pseudomonas* producing heat-stable enzymes, the so-called proteases, cause spoilage of milk stored at refrigerated temperatures. In this study 240 samples of raw cow's milk from farms FM*a* (n = 80), FM*b* (n = 80) and FM*c* (n = 80) were analyzed, from which 73 isolates of *Pseudomonas* spp. were obtained. The results of the experimental work carried out by Z h a n g et al. [30] point to the preservation of 55-100 % of the activity of proteolytic enzymes in milk heated to 141 °C/10 s., which contained the presence of *Pseudomonas* spp., while heat treatment at 160 °C/20 s. saw a decrease in residual proteolytic activity to 9 % [31]. M e n g et al. [32] confirmed that 143 isolates of *Pseudomonas* spp. were found to be randomly distributed among the different farms.

N a r v h u s et al. [9] in their study analysed isolates of Pseudomonas spp. from cold-stored raw milk. The variation in proteolytic and lipolytic properties was observed and they also observed that, in general, the caseins were hydrolysed faster, and to a greater extent, than the whey proteins.

A high level of proteolysis can negatively affect the production of dairy products and the related sensory properties of the final product. In the case of cheese production, the activity of bacterial proteases can affect the casein content, which negatively causes low yield, off-flavor (i.e. bitterness) and textural defects in the cheese. P a l u d e t t i et al. [33] analysed the effect of proteases on milk coagulation in the cheese production process. The results of their experimental work show a negative correlation between the concentration of α_{S2}-CN and β-CN and the storage time of milk. Proteolytic hydrolysis of the α_{S1}-CN casein fraction was determined to be minimal.

The SDS-PAGE electrophoretic method performed in our study determined that in the experimental group ExG-4, (group of raw cow's milk with the presence of *Pseudomonas* spp.) the lowest relative amount of SA, α_{s2}-CN and α_{s1}-CN, and the highest representation of β-LG and α-LA were detected at the same time By this, we demonstrated the negative impact of undesirable microorganisms

in the dairy industry. β-CN together with α_S-CN form the basic microstructure of cheese, and their reduced concentrations in milk could affect milk coagulation and curd formation [34]. As reported by Lamichhane et al. [35], the hydrolysis of α_S-CN and β-CN affects the consistency and related deformation of cheeses.

The composition and interactions of proteins in cow's milk and the modifications resulting from storage, milk processing and above all the presence of microorganisms represent a complicated complex issue [36]. As our results show, even if the husbandry conditions and the type of cattle (dairy cows) are the same, it is important to focus on each production farm individually due to the different management practices implemented at the farm level.

CONCLUSIONS

The presence and enzymatic activity of undesirable microorganisms in dairy products significantly affect the suitability of milk for further processing, as well as its technological, nutritional, and sensory properties. The aim of this study was to investigate the degradation of protein fractions, as observed through SDS-PAGE, caused by the presence of undesirable dairy microbiota *Pseudomonas* spp., while considering somatic cell count levels. The results of this experiment demonstrate that increased SCC and the presence of *Pseudomonas* spp. have a significantly negative impact on the casein protein fractions of milk, with caseins (α_{S1}-CN, α_{S2}-CN, and β-CN) being degraded more than individual whey proteins. Controlling the potential influences on protein fraction degradation, such as the presence of undesirable microbiota and increased SCC, has significant implications for the further processing of raw cow's milk.

DATA AVAILABILITY STATEMENT

The raw data of this article will be made available by the authors, without undue reservation.

ETHICAL STATEMENT

No ethical approval was needed for this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

AUTHORS CONTRIBUTIONS

Conceptualization, M.K.; Methodology, J.V..; Software, M.K.; Formal analysis, J.V.; Investigation and data curation, J.V., M.K.; Writing—original draft preparation, M.K.; Writing—review and editing, M.K.; Supervision, J.V.; Project administration, J.V.

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