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Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 6, Year: 2024

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Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 6, Year: 2024

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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Kafkas Univ Vet Fak Derg
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Review Article

The Use of Computer Assisted Sperm Analysis (CASA) in Domestic Animal Reproduction: A Review

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Abstract

Computer Assisted Sperm Analysis (CASA) was developed in the late 1980s to study the sperm movement characteristics or kinematics. Since then, CASA is becoming among the most important tools in reproductive biotechnologies laboratories and research centers related to animal breeding although the doubt about its effectiveness. This review aimed to investigate the different studies and reports performed by the scientific community recently regarding CASA system, as well as to suggest new areas of use and improvement for this automated device to better interpret the complexity surrounding the sperm sample. The main problem is related to the standardization and optimization of the equipment and procedures. CASA system has evolved dramatically over the past two decades to become powerful tool for the rapid and objective assessment of sperm concentration, motility and kinematics, as well as morphology, in almost all mammals, including humans. Despite the lack of full universal standardization, the various CASA instruments have all currently demonstrated high levels of accuracy and reliability.

Keywords: Computer assisted sperm analysis, CASA, Domestic animal, Sperm, Clinical applications, Standardization

Introduction

Sperm analysis is of great importance when investigating male infertility [1]. A variety of cells enters into sperm composition, in particular, spermatozoa (spz). The latter are composed of several membrane-bound sections, consisting of the plasma membrane, acrosome membrane, and mitochondrial membrane, that must be intact to ensure the viability of the spz to fertilize the oocyte [2].

Andrology laboratories around the world assess semen quantity (volume, sperm concentration, color, density, and viscosity) and quality parameters (total and progressive motility, morphological abnormalities, oxidative status, mitochondrial Activity and DNA fragmentation) [3-12]. Different studies have been performed in pigs [3], rams [4-6], dogs [7], goats [8,9], roosters [10], horses [11], and bulls [12] to examine relationship between such *in vitro* laboratory standards of fertile semen and their relationship with *in vivo* field fertility. The most important objective is to ensure the successful packaging of a straw with fertile spz that meets semen laboratory standards.

Computer Assisted Sperm Analysis (CASA) was developed to study sperm movement characteristics or kinematics and has proven to be very effective in research [1]. CASA has also been used with great success to record sperm characteristics such as concentration and proportions of total and progressive motility in many animal species, including wide application in domestic animal breeding laboratories and research centers [13]. The results obtained allow a great advance in the field of reproduction of domestic animals, especially providing solutions to the threat of animal disappearance by extinction of the breed [14].

This review aimed to document the recent contribution of the scientific community who used and benefited from CASA system in different experiments. Furthermore, the authors tend to suggest new areas of use and improvements

for CASA to better interpret the complexity of the sperm sample. Specifically in this literature, the authors focused on the use, the advantages, and the limits of CASA system.

Presentation and History

CASA is an automated system consisting of hardware and software that is used to view and digitize a sequence of sperm images. It enables clear, precise results to be obtained, which inform about different qualities of sperm parameters, including concentration, motility, morphology, and morphometry [15]. After spz scanning and placing the coordinates (x, y) on the observation field, the same procedure is repeated during the following successive captures. Then an algorithm is used to analyze all the data and reconstruct the individual trajectories of the sperm [16].

The development of computer-assisted sperm analysis (CASA) during the 1980s aroused great excitement, although the systems were only black boxes capable of tracking sperm movement, without being able to generate verifiable data, with a frame rate limited to 30 frames per second [17,18]. In the 1990s and early 2000s, advances in computer science led to several improvements to CASA systems. During this time, CASA has gained in value, especially in research area.

In the early stages of development of the CASA system, various problems arose; initially, CASA could not differentiate sperm from particles of the same size. To solve this problem, several approaches have been tried through modifications and improvements of the software, for example, the presence of the flagellum as a prerequisite [19] or the use of dyes for sperm DNA staining [20]. Currently, more than twelve different CASA systems brands are available for semen quality evaluation in laboratories and AI centres [17]. We cite IVOS and CEROS by Hamliton-Thorn and Motion Analysis (USA) *(Fig. 1)*, the Sperm Class Analyzer by Microptics SL (Spain), Hobson Tracker (UK), Sperm Motility Quantifier (South Africa), Sperm Vision (Germany) and Proiser/ISAS (Spain) and many others. Therefore, it is necessary to update the standardization of the methods for the objective evaluation of sperm quality [21].

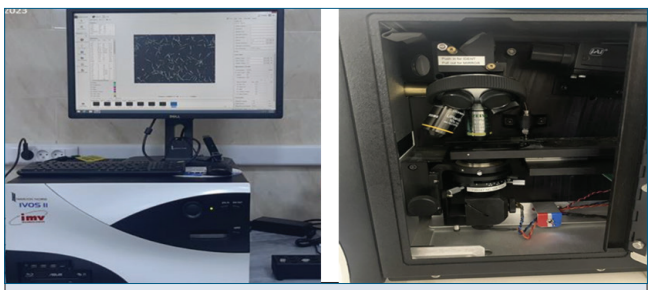


Fig 1. CASA system (Hamilton Thorne, HT-IVOS II)

Parameters Assessed Using Casa System

CASA allows to assess various Sperm parameters.

Sperm Concentration

Concentration is certainly one of the most important parameters when evaluating sperm, as infertility has been associated with low sperm count in many species [22]. Accurate assessment of sperm concentration using CASA systems remains a problem for all species in which this parameter has been studied [23]. If the CASA system proves to be reliable for motility analysis compared to the conventional technique, there is not yet a consensus on its reliability in measuring concentration in the different species [24]. Indeed, the values obtained were often overestimated or underestimated compared to the reference method [25]. The spz detected, because of analytical errors [26], are no longer representative of the microscopic fields in which they were analyzed "the principle of spz homogeneous distribution in a cell suspension is no longer respected". This results in concentration and motility evaluation errors. If the system detects non-spz particles (with the size of a spz head), it will probably overestimate the population of static spz [27]. Agarwal et al.[28] reported in Humans that CASA systems can have difficulties in distinguishing between immotile sperm, non-sperm cells, and debris. This lack of distinction causes inaccurate evaluation of sperm motility as well as counting of spermatozoa, which also affects the evaluation of sperm concentration.

Sperm Motility

Sperm motility evaluation provides essential information on its functional competence and fertilizing potential [12]. CASA analyzer is considered the Gold Standard in Motility assessment [26]. CASA-derived motility parameters include total motility, progressive motility, non-progressive, static, progressive fast [type a], slow progressive [type b], non-progressive [type c] and immobile [type d] [24]. Additionally, this system provides verifiable data, as previously analyzed video images can be re-examined for periodic internal and external quality control evaluations.

Current CASA-Mot systems focus almost exclusively on scanning and tracking the sperm head, with some newer systems starting to look for the presence of the flagellum to help exclude debris and other foreign objects from scans. Mechanically, however, it is the flagellum that propels the sperm and, as such, the behavior of the flagellum is the fundamental characteristic governing sperm motility. Taking this into consideration, it is believed that the most effective way to develop CASA-Mot, and thus enlarge the circle of use of computer-assisted analyzes in clinical

diagnosis is the introduction of flagellar monitoring, as has been done the pioneers Hiramoto and Baba [29].

Sperm Kinematic

In addition to being powerful analytical tools for assessing sperm motility, CASA systems provide additional details on sperm motion via determining their kinematic characteristics. Each individual sperm in the field of view is identified and a series of digital images of the spz head movement is captured. This allows for the reconstruction of their individual trajectories [18]. CASA-based kinematics include the following measurements: straight line velocity (VSL, µ/ms), corresponding to the straight line from the beginning to the end of the track; curvilinear velocity (VCL, µm/s), measured over the actual point-to-point track followed by the cell; average path velocity (VAP, µm/s), the average velocity over the smoothed cell path; amplitude of lateral head displacement (ALH, µm), defined as the maximum of the measured width of the head oscillation as the sperm cells swim; beat cross-frequency (BCF, Hz), defined as the frequency with which the actual track crossed the smoothed track in either direction; motility (%), the percentage of the total motile cells; and progressive motility (%), corresponding to spermatozoa swimming forward quickly in a straight line, (STR ≥45%; VAP ≥25 µm/s). Three progression ratios, expressed as percentages, were calculated from the velocity measurements described above: linearity of forward progression

(LIN = VSL/VCL·100), straightness (STR = VSL/VAP·100) and wobble (WOB = VAP/VCL·100) [21]. These kinematic parameters provide valuable information on sperm quality, which cannot be obtained by the subjective evaluation using manual microscopic examination, and could become important components of male fertility evaluation, thereby enhancing the routine semen analysis [5]. Indeed, using CASA, it was revealed that sperm cells with the highest velocity and progressive motion were positively correlated with their resilience post-cryopreservation [2].

The 3 individual sperm velocity assessed by CASA are presented in *Fig. 2*.

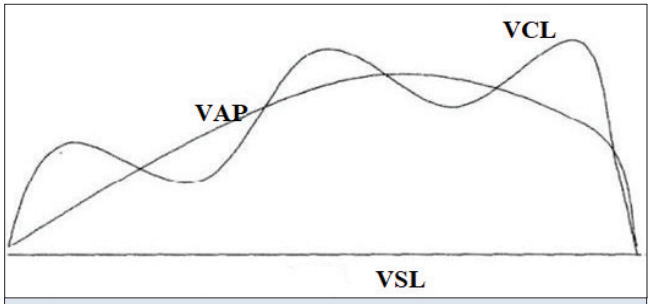
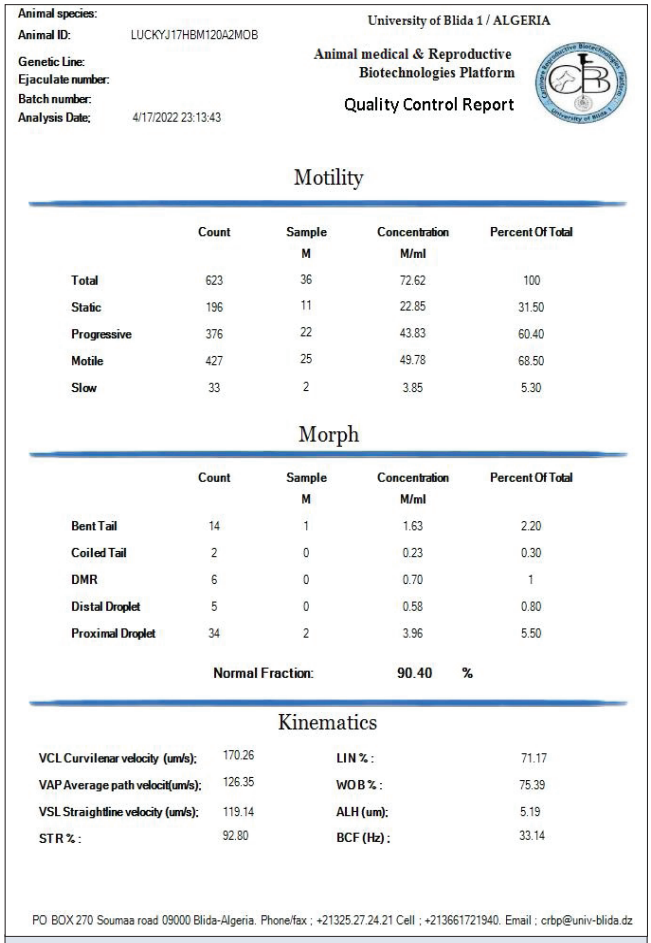


Fig 2. The main kinematic measurements involved in sperm tracking (Velocity)

Sperm Morphology and Morphometrics

Spermatozoa morphology constitutes an important parameter in the exploration of sperm quality and its effectiveness in fertilizing the egg [30]. The competitiveness of spz in the female reproductive organs and their ability to penetrate an ovum may result from their dimensions and shape which they often show a relationship with ejaculate traits [31]. In fact, fertilization process is affected more by morphological defects of spz than motility [10]. However, the predictive value of sperm morphology in male fertility has always been a controversial issue mainly due to the great variability of manual assessment of morphology within and between laboratories, which then limited its practical application [14]. Investigators reported that the changes of classification and standards in successive editions of WHO classification participated in keeping this parameter in a conflictual cercle. The standard values have evolved considerably in 30 years and the doubts about this parameter have been more raised [13,14].

Antoni van Leewenhoek reported microscopic sperm morphology in 1678 for human and dog sperm using an early single-lens microscope [32]. This time-consuming


Animal species:
Animal ID: LUCKYJ17HBM120A2MOB
Genetic Line:
Ejaculate number:
Batch number:
Analysis Date: 4/17/2022 23:13:43

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Quality Control Report

Motility

	Count	Sample M	Concentration M/ml	Percent Of Total
Total	623	36	72.62	100
Static	196	11	22.85	31.50
Progressive	376	22	43.83	60.40
Motile	427	25	49.78	68.50
Slow	33	2	3.85	5.30

Morph

	Count	Sample M	Concentration M/ml	Percent Of Total
Bent Tail	14	1	1.63	2.20
Coiled Tail	2	0	0.23	0.30
DMR	6	0	0.70	1
Distal Droplet	5	0	0.58	0.80
Proximal Droplet	34	2	3.96	5.50

Normal Fraction: 90.40 %

Kinematics

VCL Curvilenar velocity (um/s):	170.26	LIN % :	71.17
VAP Average path velocit(um/s):	126.35	WOB % :	75.39
VSL Straightline velocity (um/s):	119.14	ALH (um):	5.19
STR % :	92.80	BCF (Hz) :	33.14

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Fig 3. Sperm parameters assessed by HT-IVOS II analyzer (Report of canine semen evaluation)

technique is quite precise depending on the experience of the operator, but requires specialized microscopes or stains and can induce visual fatigue which is a probable source of error. Besides, it can also suffer from subjectivity when it is not carried out by highly experienced investigators [33].

To avoid the limits of conventional methods, computer-assisted sperm morphometry analysis systems (CASMA, generically CASA) have been developed and have first appeared on the market since 1990s. CASMA has been widely evaluated and validated for several systems and in different animal species [14]. Compared to morphometry on stained slides, automatic morphometry could thus offer the advantage of speed, lower analysis cost and the large number of spz analyzed [14,34]. It also helps avoid fixation and staining already identified as error factors in morphometry on stained slides [13].

Fig. 3 showed the different parameters assessed by a CASA analyzer.

Casa System Benefits

CASA plays an important and growing role in ensuring the quality of seed products for use in the AI industry at large, accelerating a trend that started a decade ago in many species [35].

Currently, CASA is a very useful laboratory tool, the simpler and more affordable option for the objective evaluation of semen in farm animals. It allows quick and repeatable sperm motility and morphometry assessment [1]. Examining large numbers of samples and acquiring reliable results in a short period of time can support the control of reproductive problems in male animals and lead to maintaining high rates of embryo production within laboratories [36]. The precondition is the existence of specialized and well-trained operators with appropriate functionality of the system.

Casa and Manual Analysis

Unlike manual counting, CASA uses hardware and software to visualize and to evaluate consecutive images of viable sperm in order to obtain accurate and valid information on the kinematics of individual sperm. Both methods (manual and CASA) have their advantages and disadvantages but the most promising technique is CASA. One of the drawbacks of manual method is that efficient use of the hemocytometer is highly dependent on precise pipetting, dilution and careful calculation, all of which are common sources of error [37]. CASA allows to obtain fast results with detailed objective analysis combined to high reproducibility [35].

The sperm parameters obtained in accordance with WHO recommendations remain a reference for clinical examinations. However, they do not always reflect the male fertilizing potential. Manual semen analysis is a very cost-effective and straightforward procedure, but it requires analysis by well-trained laboratory investigator. To date, no procedure has been validated by the WHO as a gold standard for semen analysis [38].

When using frozen sperm, it is interesting to notice that CASA compared to manual methods often overestimates sperm counts before freezing and underestimates this parameter after thawing [25]. It could be due to the variable agglutination rate to which immotile cells after thawing are subject. Fresh sperm settings of the analyzer parameters may not be optimal for counting cryopreserved sperm, where the freezing medium often contains egg yolk and/or a cryoprotectant. In humans, washed sperm samples require different CASA parameters than seminal sperm for semen analysis; similarly, samples after thawing with freezing medium may require different settings when the viscosity of the freezing medium is different from that of the ejaculate. Manual microscopic evaluation of sperm motility is subjective and strongly associated with inter- and intra-laboratory variations. Within the same semen sample, variations of 30-60% have been reported in manually assessed human and animal ejaculates [39].

Unfortunately, although there are general and important guidelines for the measurement of sperm motility analysis and the use of CASA, detailed and generally accepted guidelines for specific internal parameters of CASA and important laboratory species adjustments are lacking [27]. Obviously, due to specific software algorithms for specific CASA devices (which are able to modify the results obtained from particular parameters such as the amplitude of lateral head displacement (ALH)), it is difficult but possible to generate a general directive with specific CASA parameters. Using CASA, significant inter-laboratory variations were observed, multi-centric studies on male topics demonstrated that one of the least subjective parameters is the sperm concentration with a coefficient of variation of 21% to 80% [27]. Therefore, the standardization of assessment methods is becoming more and more necessary with the increasing worldwide export of semen straws and communication between animal breeding centers. Such standardization requires a large amount of organization and involves several laboratories working on the same samples [40].

CASA is very sensitive to small changes in internal parameters that can lead to a considerable modification in the results. For example, a small tick in the box "Slow sperm are counted as motile/static" can lead to completely different results, e.g. an observation of 49% motile cells instead of 80%. Although valid arguments exist for both possibilities - 1: slow cells are motile; or 2: slow cells will never reach the oocyte. There is a high risk of misinterpretation of the results for each of the choices;

a possibility of overinterpretation of the respective information due to missing background knowledge. To which groups do the hyperestimated slow cells belong?

Another example of an important piece of information is the frame rate; e.g. 50 Hz and 60 Hz. Different rates can have a significant impact on the results obtained [41]. Those parameters should be considered before purchasing a specific CASA device.

Lammers et al.[42] performed a prospective double-blind study comparing two automated semen analyzers with manual sperm assessment. Statistical analysis revealed no significant differences for most of the measured sperm parameters. Komori et al.[43] found high agreement between automated and microscopic methods in assessing sperm motility. In the same context, Fuse et al.[44] showed that the measurement of total sperm concentration and percentage of progressive motility by IIB sperm quality analyzer revealed high correlations with those of the conventional manual method recommended by WHO [24] "Enhanced Neubauer cell type". On the contrary, Vested et al.[45] reported significant differences when comparing the results obtained by using CRISMAS CASA and those performed by conventional method regarding sperm concentration and motility analysis.

Both CASA and manual method presented acceptable agreement. However, CASA is a better tool to avoid subjective variations and for better standardization. Moreover, the latter is undoubtedly the best regarding the evaluation of kinematic parameters [38] as well as spz concentration [25].

For many years, the CASA analysis has been one standard in the laboratory for motility and kinetic parameters [5], with no clear description of a species-specific setup [26]. Besides, although the recent advances in automation technology, CASA systems still require manual intervention to rectify errors and provide reliable results [28]. According to Jorge-Neto et al.[26], the correct use of the CASA system, coupled with a detailed description of the setup and procedure employed, facilitates the replication of methodologies and comparisons of studies. Recently, O'meara et al.[40] worked to adjust the kinematic and morphometric setup of the HT-IVOS II analyzer used for frozen bovine spz. These authors confirmed concerns and issues on the variability of the results obtained using different CASA settings and would recommend to the Research Centers to validate systems and ideally engage in a standard-setting for the IVOS II CASA system.

Casa Applications in Clinical Diagnosis

Due to the considerable efforts made to improve the technical performance and efficiency of current CASA systems, it is necessary and has become crucial to assess the biological relevance of parameters derived from CASA in the prediction of male fertility potential. CASA-derived measurements have been shown to be very useful in monitoring subtle changes in sperm distribution among different subpopulations of motility and speed in response to various physiological conditions and environmental exposures, which cannot be observed manually by light microscopic analysis [46,47].

Definition of Risk Factors Affecting Motility

Many factors can affect the quality of sperm movements. These include measurement temperature, sperm processing (freezing/thawing), sperm concentration and other technical factors [48].

Some CASA systems (IVOS II) are equipped with heated specimen stage, which allows precise control of temperature during the analysis, constant to within 0.5°C, whereas manual semen analysis is usually conducted under a phase-contrast microscope at room temperature. This represents an unquestionable advantage, as changes in temperature may significantly affect the analysis of sperm parameters, particularly motility assessment [47].

In humans, measurement of sperm concentration and movement characteristics requires different parameter settings for fresh and washed (after removal of seminal plasma) sperm samples. Similarly, in cryopreserved samples, post-thawed sperm samples may require different parameter settings due to the presence of the freezing medium added to the ejaculate.

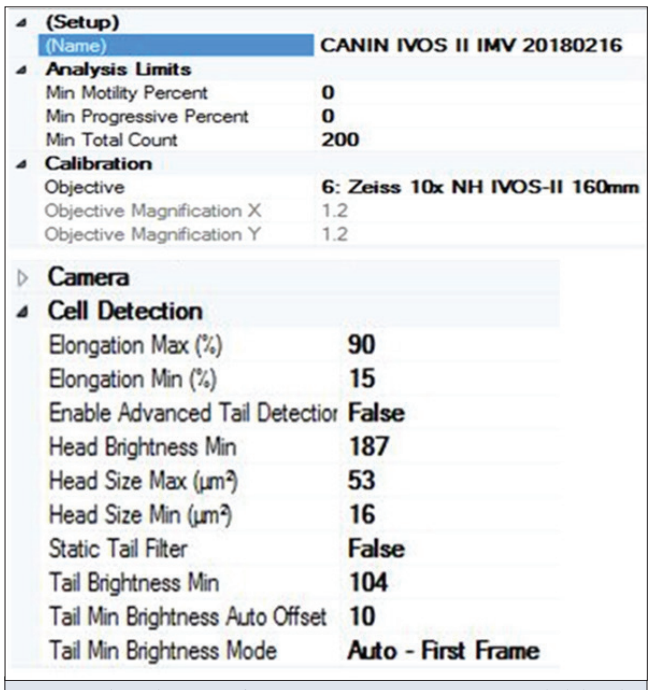

Fig 4. Technical setup of HT-IVOS II system recommended by the manufacturer (IMV Technologies) for canine spz analysis

Accordingly, sperm movement characteristics should not be considered absolute values, but should rather be interpreted in light of the parameter settings. Currently, the potential effects of environmental and occupational hazards on sperm function have been widely studied. External factors like sample temperature, sample dilution factor, mixing, pipetting, time to analysis, technician and the use of counting chambers (Type and depth) caused a large degree of variation or loss of cell motility [14,23,40,49]. Besides, the factors related to the technical configuration *(Fig. 4)* of the analyzer (pre-established setting of the image analysis software) do lead to different final analysis results [40]. These observations indicated that some risk factors may lead to specific changes in sperm movement characteristics.

Prediction of Motility Parameters Increasing the Chances of In-vitro Fertilization

Motility is accepted as the most important parameter in evaluating sperm fertilization capacity [50]. Several scientific laboratories deal with the topic of determining a particular parameter, evaluated by the CASA system, and its close correlation with *in vivo* fertility. However, this parameter or laboratory test, which could accurately report information on the fertilization capacity of the insemination dose, has not yet been established. This cannot be predicted because the reproduction process is very complex [17] and can be affected by different conditions [1,23].

Analysis of Hyperactive Spermatozoa Due to Capacitation Phenomenon

Hyperactivation is a form of non-progressive motility observed in the oocyte at the time of fertilization and is characterized by extremely asymmetrical flagellar undulations with higher amplitudes and lower frequencies leading to highly curved swimming trajectories, like whiplashes [51]. Sperm hyperactivation was initially assessed manually, describing flagellar movement patterns subjectively based on purely visual observations. However, since it is a flagellar phenomenon associated with changes in the amplitudes and frequencies of flagellar waves, manual analysis of the latter remains very laborious, time-consuming and uncontrolled. It has been strongly recommended that CASA systems are a more practical option for the identification of hyperactivated spz [18].

Under normal circumstances, spz that pass through the fallopian tube undergo the capacitation reaction and enter a hyperactive state, which is conducive to pass through the twisted fallopian tube, and fertilize the egg. Although the correlation between the percentage of hyperactive spz and the *in vitro* fertilization rate is not conclusive, but the detection of the percentage of hyperactive spz could predict the capacitation ability of spz *in vivo* and *in vitro,* and guide the treatment of infertility, so it is necessary to evaluate the dynamic characteristics of hyperactive spz by using the CASA system [52].

Dynamic characteristics of hyperactive spz are very different from those of ejaculated and non-capacitated spz. There are two types of sperm hyperactivity: progressive and non-progressive [18].

Factors Affecting Results Provided by Casa

A very important factor for standardizing CASA system data is the pre-analytical part, which includes the sample preparation for analysis [17]. Thus, the frame rate of the CASA system, sample concentration and volume, type of counting chamber, temperature and type of extender are essential for correct interpretation of CASA results.

Frame Rate

The Frame rate (FR) is defined as the number of images acquired per second while obtaining the trajectory of the sperm in the CASA. It depends mainly on the quality of the camera and the imaging standard applied [52]. The FR has a direct effect on the sperm movement trajectories, which can further affect the results of sperm movement parameters. The trajectory of sperm movement varies with the FR. When the path is built with a FR of 30 Hz, it will be relatively simple, while with a 60 Hz FR, it will be relatively complex.

For analysis of sperm movement, images typically represent 0.02 sec exposure at a rate of 50 frames per second (fps). Image exposure can be controlled via a camera shutter or the pulse duration of the strobe light.

The speed at which images are captured and the duration of a "scene" (for example, 60 frames per second for 0.5 sec) affect the distance a sperm can move between successive frames or during an entire scene (i.e. a curvilinear path). These have a direct effect on the shape of the calculated "mean path" for each sperm, deviations from the recorded path of a sperm's center of gravity on successive images, and other output values for motion of sperm. For the analysis of sperm morphology, one or more images are usually evaluated although some systems apparently calculate morphology statistics based on all the images in a scene [16].

As the trajectory of sperm movement changes, the dynamic parameters of the sperm will therefore be altered. The CASA-Mot frame rate used was limited by hardware restrictions from 16 to 60 Hz. Nevertheless, as has been previously indicated, the rate at which images are captured and the length of video recording both affect the distance that spz might move between successive frames [21].

This has a direct effect on the estimated trajectory for each sperm cell, deviations from the recorded path of a spermatozoon's centroid over successive frames, and other output values for sperm motion [17].

It is also stated that grade a and b sperm can be assessed correctly by the commercial CASA system with a FR of 30-60 Hz, while accurate assessment of grade c sperm requires increased FR. Now, it is generally accepted that the CASA system with a FR of 60 Hz can essentially meet the requirements of analyzing the sperm dynamics in mammals [16]. Recently, in boars, using the ISAS® v1 CASA-Mot system, with a video camera working up to 200 Hz while six FRs (25, 50, 75, 100, 150 and 200 fps) were compared, authors concluded that FR affected all the kinematic parameters, with curvilinear velocity (VCL) and BCF the most sensitive ones [21]. These latter reported that high fps values bring significant changes in the value of some sperm kinetic parameters and this must be considered. They mentioned also that the correct determination of sperm tracks results in a fundamental shift in the determination of motility and morphology subpopulation structure (the fact that the ejaculate in many species is constituted of different subpopulations of spermatozoa).

Type of Counting Cell Associated with CASA System

According to the literature, the type of chamber used for analysis could affect the CASA motility results. A variety of analysis chambers with different technical characteristics in terms of shape, size, depth, and charging method of the semen sample are commercially available *(Fig. 5)*. Recent research is focusing on the further standardization of CASA processes in order for data from different laboratories to be comparable [53,54]. Even though the CASA system has very high FR, very good video processing system, and advanced analysis software, if the depth of the sperm counting chamber has a large error, the results of sperm concentration and motility will be directly affected.

For motion analysis, disposable chambers (several manufacturers), capillary charged, with a carefully controlled depth (Z axis; certified depth) of 20 or 10 µm are often recommended. These depths make it less likely that a sperm can move up and/or down outside the useful depth of field of the microscope. However, this does mean that the sperm of some species cannot swim in their normal way; for bull spermatozoa, unlimited helical tail excursions require 12 µm in each direction from the plane of the head when a given sperm reposition itself [55].

Fig 5. Leja® slides with 4 chambers of 20 µm used with HT-IVOS II analyzer

In equine semen, Hoogewijs et al.[56] found that concentration and motility parameters were significantly influenced by the chamber type used. These authors after using the NucleoCounter as the gold standard for determining concentration, revealed that the correlation coefficients were low for all of the various chambers evaluated (Leja chambers of different depths were compared with disposable and reusable ISAS chambers, a Makler chamber and a World Health Organization (WHO) motility slide), with the exception of the 12 µm deep Leja chamber.

For morphology analysis, most systems use a colored dry preparation (on a conventional slide) although some now provide a complete analysis of head and/or tail morphology using a wet preparation [17].

CASA System Algorithms

An irregular path of sperm movement will produce quite a different shape and complexity than the path of sperm movement when FR changes. Therefore, it is necessary to process these tracks using certain principles and algorithms. In general, the values of the kinematic parameters of sperm, such as VAP, ALH, and others, are calculated by the algorithms after smoothing the sperm trajectory [57]. Thus, if the algorithms are different between CASA systems, the results will lack comparability.

Nafisi et al.[58] presented a kind of algorithm for calculating the sperm trace, which was not sensitive to the imaging acquisition conditions, but background and other particles can be successfully removed by an arithmetic method improved in two stages. This arithmetic has been proven to work very well under different imaging acquisition conditions. In addition, the calculation of the percentage of motile sperm is linked to the setting of the threshold between motile and immobile sperm. Threshold settings can be arbitrary, which currently lacks a uniform standard.

Sperm Concentration

Sample concentration effect on sperm quality parameters was very obvious when it was evaluated with CASA system. A high sperm concentration can affect the results of sperm count, motility and kinematics [31].

WHO [24] recommended that the concentration of sperm in semen samples detected with CASA should be less than 50 x 10^6 mL^{-1}, and Mortimer [52] recommended even less values than 40 x 10^6 mL^{-1}.

In fact, this is well understandable. When the kinematic parameters of the sperm are analyzed using CASA, the spz

trajectories in the detectable fields must be reconstructed. If there are too many spz in a field, the risk of collisions between sperm increases. Two or more spz trajectories that appear to be too close cannot be accurately reconstructed by CASA. As a result, the dynamic parameters of the sperm will be altered, and the motility categories of the sperm may be affected.

Therefore, when the semen samples were analyzed with the CASA system, samples with high concentrations of spz must be diluted with their corresponding seminal plasma [16]. The diluent used should not contain particles similar in size to the sperm heads (for example, unclarified egg yolk), as they will not be differentiated from non-motile spz [23].

Standardization and Quality Control Measures for CASA

The human factor can also affect the results of the analysis in different ways, so specific requirements must be set. The laboratory technician plays a crucial role in these complex assessment conditions and can influence almost all of the above-mentioned factors, in order to keep the results obtained reliable and reproducible [17,36]. Therefore, CASA, as a sophisticated system, places high demands on qualified operators. In addition, as there is no defined gold standard for animal sperm motility analysis methods, it is therefore recommended to establish a standard operating procedure for a specific laboratory. This should be accompanied by the determination of the repeatability of the assessment, when repeatability greater than 95% is possible. In other words, one of the difficulties in using CASA is the human factor. The purchase and establishment of CASA not only involves the maintenance of the facilities, but the operators must also know the principles of CASA and must be trained periodically [36].

CASA System Limits

While CASA is a reliable tool that has the ability to provide detailed measurements of the dimensions of the sperm head and midpiece, most commercially available CASA systems are not capable of analyzing characteristics of the sperm tail and therefore limit the application of this technology in clinical settings [18]. Moreover, CASA systems are not ready-to-use robots and can be influenced, like any other automated technique, by several artifacts related to inappropriate configurations and technical errors [59].

With recent advancements in CASA software, many of the limitations affecting CASA measurement performance have been partially or fully reversed. For example, when assessed manually, sperm motility is defined and categorized based on its flagellar movement and beat pattern, while CASA is primarily dependent on tracking the movement of the sperm head. It has been argued that the assessment of the percentage of sperm motility using CASA might be unreliable due to the potential misidentification of particulate debris such as immobile sperm [24].

However, the new CASA models are incorporated with smart filters removing some particles similar in size to sperm but mostly using positive phase contrast where most of the background images are now viewed in dark and therefore not represented as part of the white reflective semen. In addition, a function called "drift" can be defined to eliminate not only Brownian motion but also minor flow and even help counter the detection of immotile spz slightly displaced as motile due to a collision with motile spz. These characteristics allow a more accurate and objective assessment of sperm count and motility [18].

Potential Future of CASA

The conditions for studying the movement of sperm are a compromise. The sperm are suspended in an environment different from anything they will encounter *in vivo*. The suspension is seen in a chamber (or droplet) in which the spz accumulate at the interfaces between the suspension and the air, or in the wall of the chamber, where they swim differently than if they were far from the interface [60]. The laws of optics dictate a shallow depth of field in which an object sperm can be detected by a typical matrix chip and even in a 20 µm deep chamber, some sperm may not be detected. Approaches to acquiring images of three-dimensional swimming sperm are emerging. Researchers studying the biology of sperm should push to increase their availability, as the shallow depth of field hinders the free movement of sperm.

The successful collaboration and fruitful cooperation between science and industry has resulted in establishing CASA as a reliable tool that has the capacity to quantitatively assess sperm motility, kinematics as well as morphological and morphometric features, in a rapid and precise manner. The combination of these basic characteristics with advanced functional parameters will enhance the diagnostic value of semen analysis and provide a more accurate as well as quantitative approach for the assessment of idiopathic male infertility. Therefore, for the future development of CASA technology, advanced markers of sperm functionality (i.e. mitochondrial function, DNA status, hyperactivation, cervical mucous penetration) need to be integrated. This will enable a precise objective description of numerous aspects of sperm quality based on automated assessment. It is important also for clinicians in the field (e.g., examining the quality of breeding bulls under field or wildlife conditions) or in a small fixed-site practice to provide a market for a robust, simple, functional CASA system which does not require a specific location to use.

Conclusions

CASA systems have evolved dramatically over the past two decades to become powerful tools for the rapid and objective assessment of sperm concentration, motility and kinematics, as well as morphology, in almost all mammals, including humans. Within seconds, hundreds to thousands of sperm can be analyzed with great precision. In this regard, CASA is far superior to subjective manual assessments; it quantitatively measures different aspects of sperm speed, hyperactivation and morphometry, which cannot be done manually. New generation CASA devices have recently been developed to automatically quantify various aspects of sperm functionality, such as sperm DNA fragmentation, sperm vitality, and acrosome integrity.

This investigation mainly focused on studies relating to the motility and morphology of sperm in domestic animals, as well as in various wildlife; providing parameter settings, in addition to new basic data for predominantly normospermia samples; showing that even the most difficult samples, such as rat and mouse sperm, can now be routinely analyzed for motility and morphology, thus presenting a powerful tool for toxicology studies. As the current literature showed, there are many applications for the routine use of CASA in the research laboratory, wildlife management and clinical fertility assessment (human and veterinary).

Despite the lack of full universal standardization, the various CASA instruments have all currently demonstrated high levels of accuracy and reliability. Although they can sometimes be disappointing, the availability of these tools and the adherence to a strict partial standardization will give the opportunity to objectively compare the movement and the morphology of the sperm. Therefore, CASA provides an efficient, precise and reliable tool to objectively assess fertility, to improve artificial reproduction technologies and to develop physiological or toxicological studies.

Declarations

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References

1. Tanga BM, Qamar AY, Raza S, Bang S, Fang X, Yoon K, Cho J: Semen evaluation: Methodological advancements in sperm quality-specific fertility assessment - A review. *Anim Biosci,* 34 (8): 1253-1270, 2021. DOI: 10.5713/ab.21.0072

2. Gilmore A, Hitit M, Ugur MR, Dinh TTN, Tan W, Jousan FD, Nicodemus M, Kaya A, Memili E: Functional variables of bull sperm associated with cryotolerance. *Kafkas Univ Vet Fak Derg,* 27 (3): 371-379, 2021. DOI: 10.9775/kvfd.2021.25653

3. Xu C, Yang X, Sui H, Tong X, Zhang D, Zheng X, Jiao J, Wang C, Cao Z, Zhang Y: Effects of different ages on frozen semen quality and *in vitro* fertilization efficiency in Wannan black pigs. *Front Vet Sci,* 11:1395718, 2024. DOI: 10.3389/fvets.2024.1395718

4. Alcay S, Aktar A, Koca D, Kilic MA, Akkasoglu M, Yilmaz MM, Sagirkaya H: Autologous platelet rich plasma have positive effect on ram spermatozoa during cryopreservation in non-breeding season. *Kafkas Univ Vet Fak Derg,* 28 (2): 229-234, 2022. DOI: 10.9775/kvfd.2021.26763

5. Şengül E, Dayanıklı C, Alpcan Künyeli B, Çoban İ, Bülbül B, Üstüner B, Nur Z: Effect of equex on ram semen in different freezing extenders. *Kafkas Univ Vet Fak Derg,* 30 (2): 207-214, 2024. DOI: 10.9775/kvfd.2023.30789

6. Öztürkler Y, Ak K, İleri K: Freezing of ram semen in extenders containing high glycerol. *Istanbul Univ Vet Fak Derg,* 25, 399-414, 1999.

7. Belala R, Briand-Amirat L, Martinot A, Thorin C, Michaud S, Desherces S, Youngs CR, Bencharif D: A comparison of liquid and lyophilized egg yolk plasma to low density lipoproteins for freezing of canine spermatozoa. *Reprod Domest Anim,* 54 (8): 1131-1138, 2019. DOI: 10.1111/rda.13476

8. Kulaksız R, Ari U, Kuru M, Lehimcioğlu N, Yıldız S, Öztürkler Y: Seasonal variations in testicular measurements, fresh sperm quality and post-thaw sperm motility in Gurcu goat bucks. *Slovak J Anim Sci,* 53, 161-167, 2020.

9. Bouchicha AEB, Kalem A, Mimoune N, Djouadi S, Khelef D, Kaidi R: Study of antibiotics and symbiotic effects on sperm quality using the CASA system. *Vet Stanica,* 53 (4): 377-388, 2022. DOI: 10.46419/vs.53.4.2

10. Günay E, Arıcı R, Şenlikçi H, Yağcıoğlu S, Eser A, Demir K, Alkan S, Çeşmeci M: Determination of seminal characteristics in Turkish aseel roosters. *Kafkas Univ Vet Fak Derg*, 29 (1): 1-8, 2023. DOI: 10.9775/kvfd.2022.27920

11. Kelley D, Holyoak R: Stallion sperm concentration measurements: Experience and equipment. *Clinical Theriogenology,* 16:10071, 2024. DOI: 10.58292/ct.v16.10071

12. Önder NT, Gökdemir T, Kılıç MC, Şahin O, Yıldız S, Kaçar C, Demir MC, Öztürkler Y: Insulin and bull sperm interactions during cryopreservation. *Kafkas Univ Vet Fak Derg,* 29 (4): 401-405, 2023. DOI: 10.9775/kvfd.2023.29623

13. Van der Horst G, Maree L, Du Plessis S: Current perspectives of CASA applications in diverse mammalian spermatozoa. *Reprod Fertil Dev,* 30 (6): 875-888, 2018. DOI: 10.1071/RD17468

14. Van der Horst G, Du Plessis S, Maree L: Have we conquered sperm morphology analysis in different mammalian species as analysed by CASMA? **In,** Björndahl L, Flanagan J, Holmberg R, Kvist U (Eds): *XIII*th *International Symposium on Spermatology,* Springer, Cham, 2021.

15. Talarczyk-Desole J, Berger A, Taszarek-Hauke G, Hauke J, Pawelczyk L, Jedrzejczak P: Manual vs. computer-assisted sperm analysis: Can CASA replace manual assessment of human semen in clinical practice? *Ginekol Pol,* 88 (2): 56-60, 2017. DOI: 10.5603/GP.a2017.0012

16. Lu JC, Huang YF, Lü NQ: Computer-aided sperm analysis: Past, present and future. *Andrologia,* 46 (4): 329-338, 2014. DOI: 10.1111/and.12093

17. Amann RP, Waberski D: Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology,* 81:5-17.e3, 2014. DOI: 10.1016/J.THERIOGENOLOGY.2013.09.004

18. Mortimer ST, Van der Horst G, Mortimer D: The future of computer-aided sperm analysis. *Asian J Androl,* 17 (4): 545-553, 2015. DOI: 10.4103/1008-682X.154312

19. Wijchman JG, De Wolf BTHM, Jager S: Evaluation of a computer-aided semen analysis system with sperm tail detection. *Hum Reprod,* 10:2090-2095, 1995. DOI: 10.1093/oxfordjournals.humrep.a136241

20. Zinaman MJ, Uhler ML, Vertuno E, Fisher SG, Clegg ED: Evaluation

of computer-assisted semen analysis (CASA) with IDENT stain to determine sperm concentration. *J Androl,* 17:288-292, 1996. DOI: 10.1002/j.1939-4640.1996.tb01784.x

21. Valverde A, Madrigal M, Caldeira C, Bompart D, de Murga JN, Arnau S, Soler C: Effect of frame rate capture frequency on sperm kinematic parameters and subpopulation structure definition in boars, analysed with a CASA-Mot system. *Reprod Domest Anim,* 54 (2): 167-175, 2019. DOI: 10.1111/rda.13320

22. Bielsa MA, Andolz P, Grtis JM: Which semen parameters have a predicitive value for pregnancy in infertile couples. *Hum Reprod,* 9, 1887-l890, 1994. DOI: 10.1093/oxfordjournals.humrep.a138353

23. Iguer-Ouada M, Verstegen J: Evaluation of the Hamilthon Thorn Computer Based automated system for dog semen analysis. *Theriogenology,* 55, 733-749, 2001. DOI: 10.1016/s0093-691x(01)00440-x

24. World Health Organization: WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed., World Health Organization, 2010. https://iris.who.int/handle/10665/44261; *Accessed:* 03/06/2024.

25. Schubert B, Badiou M, Force A: Computer-aided sperm analysis, the new key player in routine sperm assessment. *Andrologia,* 51:e13417, 2019. DOI: 10.1111/and.13417

26. Jorge Neto PN, Fabio F, Mario Davi C, Santos S, Requena L, Dressel Ramos S, Felix de Goés M, Franco Valle R, Lucia André Padilha F, Colbachini, Rafael Caprioli Gutierrez H, Schneider Brandão Souza L, Takatsuka Manoel V, Chrispim Reisfeld L, Deco-Souza T, Ferreira Leite R, Schilbach Pizzutto C: Specific setup and methodology for Computer-Assisted Sperm Analysis (CASA) in evaluating elasmobranch sperm. *Theriogenology Wild,* 4:100091, 2024. DOI: 10.1016/j.therwi.2024.100091

27. Brito LFC, Althouse GC, Aurich C, Chenoweth PJ, Eilts BE, Love CC, Luvoni GC, Mitchell JR, Peter AT, Pugh DG, Waberski D: Andrology laboratory review: Evaluation of sperm concentration. *Theriogenology,* 85 (9): 1507-1527, 2016. DOI: 10.1016/j.theriogenology.2016.01.002

28. Agarwal A, Panner Selvam MK, Ambar RF: Validation of LensHooke® X1 PRO and computer-assisted semen analyzer compared with laboratory-based manual semen analysis. *World J Mens Health,* 39 (3): 496-505, 2021. DOI: 10.5534/wjmh.200185

29. Hiramoto Y, Baba SA: A quantitative analysis of flagellar movement in echinoderm spermatozoa. *J Exp Biol,* 76, 85-104, 1978. DOI: 10.1242/jeb.76.1.85

30. Wysokińska A, Wójcik E, Chłopik A: Evaluation of the morphometry of sperm from the epididymides of dogs using different staining methods. *Animals,* 11 (1):227, 2021. DOI: 10.3390/ani11010227

31. Kondracki S, Krzysztof G, Maria I: Impact of sperm concentration on sperm morphology of large white and landrace boars. *Livest Sci,* 241:104214, 2020. DOI: 10.1016/j.livsci.2020.104214

32. Bulkeley E, Christine C, Azarene F, Kris G, Heather P, Stuart M: Assessment of an iPad-based sperm motility analyzer for determination of canine sperm motility. *Transl Anim Sci,* 5 (2):txab066, 2021. DOI: 10.1093/tas/txab066

33. Hussein RS, Mohamed EEM, Mohamed RR, Abdelbasset WK, Morsy WE, Elsayed SH: Assessment of sperm morphometry in evaluating male infertility. *Int J Biomed,* 14 (1): 93-98, 2024. DOI: 10.21103/Article14(1)_OA14

34. Agustinus A, Pakpahan C: Computer assisted sperm analysis: A review. *Indonesian Androl Biomed J,* 1, 60-66, 2020. DOI: 10.20473/iabj.v1i2.35

35. Zuidema D, Kerns K, Sutovsky P: An exploration of current and perspective semen analysis and sperm selection for livestock artificial insemination. *Animals,* 11 (12):3563, 2021. DOI: 10.3390/ani11123563

36. Michos IA, Basioura AG, Boscos CM, Tsakmakidis IA: Proper use and impact of 'Computer Assisted Semen Analysis' technique on semen evaluation of farm animals. *J Hellenic Vet Med Soc,* 64 (4): 267-74, 2013. DOI: 10.12681/jhvms.15506

37. Zhang M, Gu L, Zheng P, Chen Z, Dou X, Qin Q, Cai X: Improvement of cell counting method for Neubauer counting chamber. *J Clin Lab Anal,* 34 (1):e23024, 2020. DOI: 10.1002/jcla.23024

38. Baig AS, Shoebuddin M, Ahmed M: Comparison of manual sperm analysis with computer-assisted sperm analysis: A comparative cross-sectional study. *Natl J Physiol Pharm Pharmacol,* 9 (9): 862-864, 2019. DOI: 10.5455/njppp.2019.9.0621817062019

39. Verstegen J, Iguer-Ouada M, Onclin K: Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology,* 57, 149-179, 2002. DOI: 10.1016/S0093-691X(01)00664-1

40. O'Meara C, Henrotte E, Kupisiewicz K, Latour C, Broekhuijse M, Camus A, Gavin-Plagne L, Sellem E: The effect of adjusting settings within a Computer-Assisted Sperm Analysis (CASA) system on bovine sperm motility and morphology results. *Anim Reprod,* 19 (1):e20210077, 2022. DOI: 10.1590/1984-3143-AR2021-0077

41. Abaigar T, Barbero J, Holt WV: Trajectory variance and autocorrelations within single sperm tracks as population-level descriptors of sperm track complexity, predictability, and energy-generating ability. *J Androl,* 33 (2): 216-228, 2012. DOI: 10.2164/jandrol.110.012302

42. Lammers J, Splingart C, Barrière P, Jean M, Fréour T: Double-blind prospective study comparing two automated sperm analyzers versus manual semen assessment. *J Assis Reprod Genet,* 31 (1): 35-43, 2014. DOI: 10.1007/s10815-013-0139-2

43. Komori K, Tsujimura A, Ishijima S, Tanjapatkul P, Fujita K, Matsuoka Y, Takao T, Miyagawa Y, Takada S, Okuyama A: Comparative study of sperm motility analysis system and conventional microscopic semen analysis. *Reprod Med Biol,* 5 (3): 195-200, 2006. DOI: 10.1111/j.1447-0578.2006.00141.x

44. Fuse H, Akashi T, Nozaki T, Nishio R, Mizuno I: Assessment of sperm quality analyzer II B: Comparison with manual semen analysis and CASA. *Syst Biol Reprod Med,* 51 (1): 65-67, 2005. DOI: 10.1080/014850190513012

45. Vested A, Ramlau-Hansen CH, Bonde JP, Thulstrup AM, Kristensen SL, Toft G: A comparison of conventional and computer-assisted semen analysis (CRISMAS software) using samples from 166 young danish men. *Asian J Androl,* 13 (3): 453-458, 2011. DOI: 10.1038/aja.2011.14

46. Choi Jw, Alkhoury L, Urbano LF, Masson P, VerMilyea M, Kam M: An assessment tool for computer-assisted semen analysis (CASA) algorithms. *Sci Rep,* 12:16830, 2022. DOI: 10.1038/s41598-022-20943-9

47. Finelli R, Leisegang K, Tumallapalli S, Henkel R, Agarwal A: The validity and reliability of computer-aided semen analyzers in performing semen analysis: A systematic review. *Transl Androl Urol,* 10 (7): 3069-3079, 2021. DOI: 10.21037/tau-21-276

48. Yánez-Ortiz I, Catalán J, Rodríguez-Gil JE, Miró J, Yeste M: Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. *Anim Reprod Sci,* 246:106904, 2022. DOI: 10.1016/j.anireprosci.2021.106904

49. Ozimic S, Ban-Frangez H, Stimpfel M: Sperm cryopreservation today: Approaches, efficiency, and pitfalls. *Curr Issues Mol Biol,* 45 (6): 4716-4734, 2023. DOI: 10.3390/cimb45060300

50. Waberski D, Suarez SS, Henning H: Assessment of sperm motility in livestock: Perspectives based on sperm swimming conditions *in vivo. Anim Reprod Sci,* 246:106849, 2022. DOI: 10.1016/j.anireprosci.2021.106849

51. Demott RP, Suarez SS: Hyperactivated sperm progress in the mouse oviduct. *Biol Reprod,* 46 (5): 779-785, 1992. DOI: 10.1095/biolreprod46.5.779

52. Mortimer ST: CASA-practical aspects. *J Androl,* 21(4): 515-524, 2000. DOI: 10.1002/j.1939-4640.2000.tb02116.x

53. Hu YA, Lu JC, Lu NQ, Shao Y, Huang YF: Comparison of four methods for sperm counting. *Zhonghua Nan Ke Xue*, 12, 222-224, 227, 2006.

54. Basioura A, Tsousis G, Boscos C, Lymberopoulos A, Tsakmakidis I: Method agreement between three different chambers for comparative bull semen computer assisted sperm motility analysis. *Polish J Vet Sci,* 23, 325-331, 2020. DOI: 10.24425/pjvs.2020.133649

55. Rikmenspoel R: Movements and active moments of bull sperm flagella as a function of temperature and viscosity. *J Exp Biol,* 108, 205-230, 1984. DOI: 10.1242/jeb.108.1.205

56. Hoogewijs M, De Vliegher S, Govaere J, De Schauwer C, Kruif A, Soom A: Influence of counting chamber type on CASA outcomes of equine semen analysis. *Equine Vet J,* 44, 542-549, 2011. DOI: 10.1111/j.2042-3306.2011.00523.x

57. Mortimer ST, Swan MA: The development of smoothing-independent kinematic measures of capacitating human sperm movement. *Hum Reprod,* 14 (4): 986-996, 1999. DOI: 10.1093/humrep/14.4.986

58. Nafisi VR, Moradi MH, Nasr-Esfahani MH: A template matching algorithm for sperm tracking and classification. *Physiol Meas,* 26 (5): 639-651, 2005. DOI: 10.1088/0967-3334/26/5/006

59. Singh AK, Kumar A, Bisla A: Computer-assisted sperm analysis (CASA) in veterinary science: A review. *Indian J Anim Sci,* 91 (6): 419-429, 2021.

60. Su TW, Choi I, Feng J, Huang K, McLeod E, Ozcan A: Sperm trajectories form chiral ribbons. *Sci Rep,* 3:1664, 2013. DOI: 10.1038/srep01664

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Review Article

Role of Probiotics in Increasing Meat and Egg Production in Poultry: A Review

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Abstract

Antibiotics have been used as growth promotors to enhance poultry's growth performance and feed efficiency. However, their use leads to various side effects like antimicrobial resistance (AMR), destruction of beneficial bacteria, and microbes in the gut, and dysbiosis with time. Various alternatives have been used over the years to overcome the poultry problems and these are necessarily required to replace antibiotics to prevent these side effects. Among alternatives, probiotics are the best option because of their unique pharmacological and therapeutic properties. They are living and non-pathogenic microbes that have health benefits for the host. In poultry diets, they are used as feed additives. They help in proper feed digestion to make beneficial nutrients available for more feed intake and faster growth. They play a significant role in the host by modulation of the host's immune response thus protecting the host from many infections. They contribute to health by offering anti-mutagenic, anti-carcinogenic, anti-hypertensive, hypocholesterolemic, and immune-modulating effects. They help to enhance the production and quality of poultry meat and eggs. The selection of suitable probiotic strains is of great concern to achieve all optimal effects of probiotics. Therefore, this review explores probiotics, their properties, mode of action, and effects on commercial egg and meat production.

Keywords: Alternatives, Growth promotors, Immune-modulator, Hypocholesterolemic effects, Quality eggs, Meat

Introduction

The poultry industry plays a key role in economic progress and fulfilling the food requirements of the human population in the country [1]. There is a huge demand for food products from the poultry industry with time across the globe. Feed additives and nutritional supplements have become a crucial need in the poultry field because these have a wide range of useful activities, like increasing growth, production, and health protection by improving the immune system [2]. There is a huge progress in the poultry production system after obtaining advancements in genetic makeup, good management, and nutrition. They are commonly used as growth promotors and enhance the growth of broilers and egg production in layers [3]. This increase in production and growth rate is related to the productive role of antibiotics. Yet they have led to an imbalance in the intestinal bacterial population, resulting in alterations of the gastrointestinal tract (GIT) and the immune response. Multiple undesirable effects have been noticed, especially affecting the determination of antimicrobial resistance (AMR) genes. AMR appears due to the misuse and overuse of antibiotics during disease control and it is a predominant issue, currently prevailing around the globe [4].

With the emergence of AMR, the focus on searching out substitutes for antibiotics has increased [5]. Feed additives like probiotics have the potential to minimize enteric

disorders and contamination of poultry products. The term "probiotic" is derived from two Greek words 'pro' which means 'for' and 'biotic' which means 'life' [6]. It was used very first time in 1965 as a growth promotor produced from a ciliate protozoan that enhances the growth of another ciliate [7]. Elie Metchnikoff was the first person who worked on probiotics at the Pasteur Institute in Paris. He observed the existence of an increased survival rate by consuming a large amount of soured milk in Bulgarian peasants [2]. Many scientists have worked during the period of this discovery, from 1953 to this date. Probiotic microbes have led to valuable effects like immunomodulation and modulation of local microbiota during in-vivo trials followed by high success in the field as developed by *in-vitro* testation. They include microorganisms that have positive effects on the host by improving the properties of intestinal microflora. They are live non-pathogenic microorganisms mixed into the diet of both humans and animals. They include microorganisms like bacteria, yeast, or fungi. *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Bacillus* are bacterial strains used in manufacturing probiotics [8]. They are obtained by isolation from milk, fermented foods, gut microbes, and feces of different animals.

The addition of probiotics in the poultry diet results in improving the performance and some egg quality traits [9]. Yeast probiotics enhance egg weight, eggshell-breaking strength, nutrition utilization, and feed conversion ratio (FCR) [10]. They play a critical role in maintaining the natural balance in the digestive system as they are involved in the regulation of gut microbes and increase the activity of digestive enzymes [11]. They increase poultry production by reducing the risk of illness and enhancing the efficiency of poultry birds. The efficiency of probiotics is affected by different ways like selecting efficient strains, gene modification, the interaction between strains, and the synergistically acting components of probiotics. Therefore, the main objective of this review is to explain the role of probiotics in raising meat and egg production in poultry due to their beneficial effects along with their limitations and future use

Probiotics

Probiotics were first introduced by Lilly and Stillwell in 1965 to explain factors required for improving growth and performance [7,12]. As described earlier probiotics mean microorganisms for life. Different scientists gave different definitions for them. Parker defined it as microbes contributing to intestinal microbial balance and Crawford defined it as a culture of specific living microbes that are involved in setting up the intestinal population of useful microorganisms [13]. In 1989, the FDA (Food and Drug Administration) needed producers to operate the term direct-fed microbial (DFM) instead of probiotics. The FDA described DFM as a source of naturally present live animals like bacteria *(Lactobacillus, Streptococcus),* yeast, and fungi [14].

Microorganisms that are used as probiotics are mostly bacteria except some yeast and fungus *(Aspergillus oryzae* and *Candida pintolopesii).* Bacterial probiotics include many species of *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and *Enterococcus* as shown in *Fig. 1* [15]. However, the properties, benefits, and aims of these microbes as probiotics are not similar but rather specific to each one as shown in *Table 1* which is given below after the properties of probiotics.

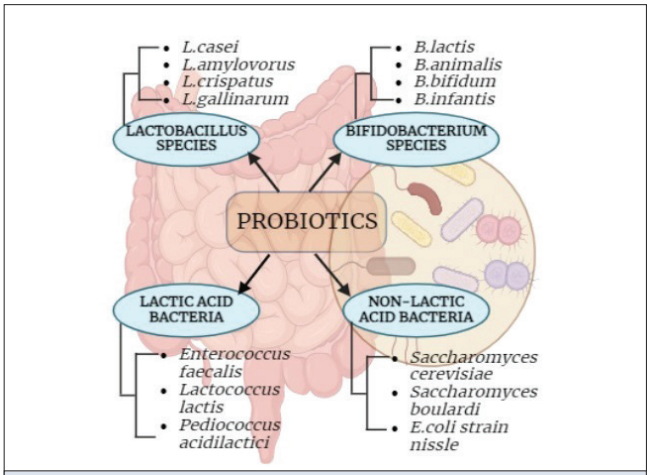

Fig 1. Various strains of bacteria and fungi used as probiotics (Retrieved from Biorender)

Properties of Probiotics Regarding Meat and Egg Production

Assessment of the quality of eggs and meat is mostly dependent on the diet's nutritional makeup. A diet that has a balanced proportion of vital elements promotes better nutrition utilization and overall quality [4]. An imbalanced diet causes more protein breakdown by acquiring extra energy in the form of ATP which in turn leads to a reduction of fat deposition in the live poultry birds [16]. To overcome the situation, probiotics have been used as an alternative in the poultry sector to enhance meat and health rates by decreasing disease load. Probiotics can be used alone or in combination with other additives, generally regarded as a safe alternative. The fundamental aim of using probiotics in poultry feed is to reduce intestinal bacterial pathogens and infections. Probiotics maintain the intestinal microbial population and improve the feed intake [16]. Probiotics may improve digestion, promote general health, and provide defense

against harmful bacteria and pathogens by maximizing the microbial balance in the gastrointestinal tract of chicken hosts when given in appropriate quantities. Additionally, it can help reduce environmental contamination and improve food safety in general. Furthermore, they can increase the activity of glutathione peroxidase, superoxide dismutase, and other chemicals in the animal body [17]. As so, they successfully reduce reactive oxygen species (ROS) levels. This decrease in ROS level is essential for reducing the harmful effects on phospholipid-containing muscle cell membranes [18]. Consequently, it helps to maintain the freshness of myoglobin in fresh meat for a longer time, which results in notable improvements in meat color [17].

The use of probiotics also leads to the modulation of the immune system to protect poultry birds from fatal diseases. Thus, these are safe to use as they are non-pathogenic. They do not lead to AMR by preventing the transfer of antibiotic resistance genes and regulating genetic stability [5]. Different properties of probiotics are described below and also labeled in *Fig. 2*.

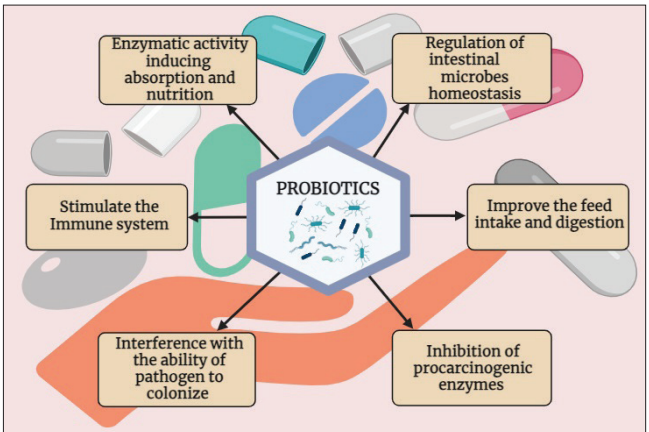

Fig 2. Properties of probiotics regarding meat and egg production (Retrieved from Biorender)

Enzymatic Activity Inducing Absorption and Secretion

Probiotics produce certain enzymes to improve digestion in the host. These enzymes such as protease and lipase are involved in improving and enhancing the absorption of proteins and lipids in the digestive system respectively, which leads to better growth and development of chick [17]. They increase the surface area of the intestinal villi thus causing an increase in the absorption of food material in the digestive tract [18]. Protease enzyme causes hydrolysis of peptide bonds of proteins and lipase causes the breakdown of ester linkages present in lipids. In this way, these enzymes break these compounds into smaller sub-units to improve their absorption and play an important role in increasing the growth rate and production of meat.

Stimulation of Immune Response

Probiotics stimulate the immune system of the host against pathogens. Exposure of the intestine to various kinds of antigens like bacteria, proteins, and other components of probiotics in feed additives causes an immune response by the host immune system [19]. They cause the growth of certain useful microbes that lower the risk level of gastrointestinal diseases resulting in healthy growth of birds with more meat and egg production [20]. They also have beneficial effects like anti-mutagenic, anti-hypertensive, and anti-osteoporosis.

Interference with the ability of the Pathogen to Colonize

Probiotics reduce the colonization of infectious pathogens like *Salmonella* and *Campylobacter* in the intestines of poultry birds [21]. These pathogens are zoonotic and lead to zoonotic diseases like necrotic enteritis and coccidiosis in developing countries. These microbes decrease the infection rate in this way and lead to more production of meat and eggs by improving the growth of poultry birds [22]. The healthy and useful microorganisms supplied by probiotics colonize the intestinal mucosa and stop the colonization of pathogens in the intestinal mucosa.

Regulation of Intestinal Microbial Homeostasis

Probiotics maintain the homeostasis of intestinal micro-organisms. These useful microbes decrease the number of harmful organisms and reduce the risk level of many diseases in the host causing healthy chickens. Probiotic microbes are part of the natural microbiota and maintain the population of useful microbes in the intestines [23]. The probiotic microbes improve intestinal health by regulating intestinal epithelial function [24]. This leads to intestinal mucosal homeostasis resulting in better growth and more meat production [25].

Improve the Feed Intake and Digestion

As poultry products are the major source of food so high production in the poultry is needed. In this regard, probiotics increase the growth rate of chickens and control different diseases. Probiotics cause an increase in feed and water intake by chickens and improve the digestion of feed by the production of useful enzymes like lipase and protease. Thus, leads to the effective digestion of lipids and protein ingredients in poultry feed and improves the overall health of poultry birds [26].

Inhibition of Pro-carcinogenic Enzymes

An imbalance in the population of intestinal microbes results in disruption of homeostasis and this imbalance leads to a pro-inflammatory immune response along with several other diseases in poultry. Moreover, such a type of response leads to different diseases including cancer [27].

Table 1. *Probiotics and their effects on meat and egg production*

Probiotics	Dose	Bacterial/ Fungal	Properties	Action	Effect on Meat Production	Effect on Egg Production	References
Lactobacillus casei	1 million-1 billion CFU	Bacterial	Acts as a growth promotor and anti-carcinogenic agent	Improves feed intake and feed conversion ratio	Increases meat production	N/A	[29]
Lactobacillus amylovorus	1-10 billion CFU	Bacterial	Promotes the growth	Improves meat quality by promoting the immune system, FCR, and gastrointestinal tract	Increases meat production and quality	Improves egg quality and production	[30,31]
Lactobacillus gallinarum	1-5 billion CFU	Bacterial	Increases immune response, weight gain, and organ health. It has hypocholesterolemic effects	Increases immune function to improve immune response and overall health.	Results in better meat production.	Increases egg production.	[32,33]
Lactobacillus crispatus	1-5 billion CFU	Bacterial	Acts as a growth enhancer and improves overall health.	Performs its action by improving and balancing gut microbiota	Enhances the meat quality	Shows positive effects on eggshell production and egg quality but up to a limit	[34,35]
Lactobacillus lactis	1-10 billion CFU	Bacterial	Acts as a growth promotor	Balances the gut microbiota and improves feed intake	N/A	Potentially enhances egg production	[36]
Bifidobacterium lactis	1-3 billion CFU	Bacterial	Acts as a growth promotor.	Maintains and improves the gut microbiota.	N/A	Enhances the egg quality and production	[37]
Bifidobacterium animalis	1-10 billion CFU	Bacterial	Accelerates the FCR and improves growth	Performs its action by improving gut microbiota and increasing FCR	Enhances the meat quality and meat production	Increases the egg production and improves quality	[38]
Bifidobacterium bifidum	1-5 billion CFU	Bacterial	Improves feed intake and has hypocholesterolemic effects on poultry products	Increases growth by improving feed intake and growth performance	Gives more meat production with good quality	Results in more egg production with good characteristics	[39]
Bifidobacterium infantis	0.5-2 billion CFU	Bacterial	Acts as a growth promotor	Improves the gut microbes which enhances nutrient absorption and utilization	Enhances growth performance and better meat production	Results in more egg production with better quality	[40]
Enterococcus faecalis	10^8-10^{10} CFU	Bacterial	Useful for growth and feed efficiency	Increases growth performance by improving feed efficiency	Increases meat production with better characteristics	Some strains like *E. faecalis* UGRA 10 maintain egg production while some other strains can decrease egg production	[41]
Lactococcus lactis	1-10 billion CFU	Bacterial	Lowers the cholesterol level in poultry products, improves gut health, and reduces mortality	Improves gut microbiota and increases growth performance to increase poultry production	Results in more meat production with good quality	N/A	[42]

Table 1. *Probiotics and their effects on meat and egg production (continued)*

Probiotics	Dose	Bacterial/ Fungal	Properties	Action	Effect on Meat Production	Effect on Egg Production	References
Pedicoccus acidilactici	0.5-1 g per bird per day	Bacterial	Enhances the growth of poultry birds	Shows its effects by improving FCR	N/A	Significantly increases the egg-laying rate with good quality	[42]
Saccharomyces cerevisiae	0.5-5 g per kg of feed	Fungal	Acts as a growth promotor and enhances the overall health of poultry birds	Enhances growth performance by improving, feed conversion, gut health, utilizing minerals, and absorbing nutrients	This microbe results in more meat production with better quality	Increases egg production	[43]
*Escherichia coli (*nissle*)*	10^6-10^8 CFU	Bacterial	Exhibits a preventive measure against agents resulting in foodborne diseases and acts as a growth promotor	Maintains the gut microbial population and reduces the *Campylobacter jejuni* colonization inside the caecum of birds to improve growth performance	Causes more meat production by improving growth performance	Decrease in egg production in poultry birds due to inflammatory reaction during *E. coli* infection	[44]
Aspergillus oryzae	0.1-1% of feed	Fungus	Acts as a growth promotor and improves the overall health of hens	Performs its function by improving gut microbiota and modulating the immune system of poultry birds	N/A	Indirectly increases egg production in laying hens by modulation of the immune system and improving gut health	[45]
Cyberlindera jadinii	1-5g per kg of feed	Fungus	Increases the nutritional quality of poultry products	Improves protein quantity in poultry products like meat to enhance its nutritional quality	Produces meat with better quality enriched in proteins and various vitamins	N/A	[46]
Debaromyces hansenii	1-10 billion CFU	Fungus	Stimulates the immune system, digestive system, and cell proliferation	Performs its activities as a probiotic by releasing enzymes known as β-D-glucan	Produces more meat with high free fatty acids and amino acid levels	N/A	[47]

Probiotics deactivate carcinogenic agents like N-nitrous and heterocyclic aromatic amines (HCA) by two major methods known as deactivation and binding [28]. In this way, probiotics protect from cancer by maintaining homeostasis through these methods, resulting in more growth and enhanced production of poultry products (meat and eggs).

Limitations and Future Prospectives

Probiotics can proficiently enhance the beneficial intestinal microorganisms and enhance their effectiveness in terms of health and growth in poultry birds. They also reduced the disease burden by lowering the level of hazardous microbes such as *Staphylococcus aureus* [21]. However, researchers have found varying results about the advantages of using them, with other studies indicating no apparent effect [48]. There are many obstacles to overcome when choosing the best probiotic for improving the quality of poultry meat and eggs. In reality, only a few probiotics fulfill these requirements. For instance, only *Pediococcus pentosaceus* 62781-3, 46035-1, and 46035-4, along with *Mesenteria leuconostoc* 14324-8, showed promising results as probiotics out of 42 isolated strains [26]. Additionally, the expression of virulence genes, antibiotic resistance, the existence of biogenic amines and enterotoxins, and the manufacture of related hazardous compounds should all be taken into account when choosing appropriate probiotic strains. In another study, it was discovered that probiotics extracted from milk samples have been found to have β-hemolytic activity [22].

Probiotics, however, are often found to have poor thermal stability, and heat treatment of meat products may threaten their vitality. The effective concentration of probiotics in birds' gastrointestinal tracts declines as a result of this decline in viability [27]. Additionally, probiotics, such as *Bacillus* species, have been shown to provide inconsistent

outcomes occasionally having no impact at all. For example, it was found that *B. subtilis* supplementation did not affect the hens' growth performance as measured by the average daily feed intake (ADFI), average daily gain (ADG), and FCR indices [42].

Similarly in another study, it was found that addition of probiotic such as *B. coagulans* did not show any growth-promoting and preventative impact on broiler chicks that was attained with antibiotic. It has been demonstrated that, in addition to *B. cereus*, several strains of *Bacillus*, including *B. subtilis, B. pumilus,* and *B. licheniformis*, can produce both entero and emetic toxins [40]. The potential for antibiotic-resistance genes to be passed on to pathogens is another issue. Their use in animal feed may result in a drug-resistant reservoir that people can contract. Furthermore, resistance genes may be transferred to animal pathogens that can infect humans through food products and transcend the species barrier [12].

Studies on the use of probiotics in chicken production are steadily growing, despite adoption barriers [49]. Before probiotics may be regarded as a full replacement for antibiotic growth promoters, much more research needs to be done. It is possible that applying technologies like next-generation sequencing will improve our current comprehension of the underlying mechanisms. More laboratory research that can be verified *in vivo* is required for multiple-mode probiotic development and consortium studies. By doing so, time and money could be saved by identifying biochemical characteristics in the laboratory and choosing strong candidates for field testing. By combining them with nanotechnology, we can improve their form and distribution systems, improve their necessary actions in the body, and shield probiotics from various harms as they travel to the site of action [50]. To determine the best circumstances for probiotics to work better and to generate healthier and higher-quality poultry meat, more studies on the use of probiotics in poultry diets are still needed [51,52]. Furthermore, more investigation is needed on the form, delivery, and mechanisms of action of probiotics as feed additives for poultry.

Additionally, little is known about the formulation of probiotic products and the suitable carriers that should be included in them, even though these aspects affect the stability of probiotic performance and shelf life. The main factors influencing broader industry interest and adoption will be improved cost-benefit ratios, more stringent regulations, and proven effectiveness on an industrial scale. The expense and limits of conventional probiotics continue to be issues for the industry, but future demand for more natural poultry production will make alternatives to AGP more appealing. Because of increased studies on the genus, there seems to be more evidence that probiotics are safe to use for poultry birds.

Conclusion

The increasing world population is alarming and the demand for more meat, eggs, and animal products is increasing day-by-day. Initially, antibiotics were used as growth promotors to increase production but their use stopped with the emergence of AMR and was replaced by probiotics. They have a large number of useful benefits for poultry production. They enhance the growth rate, feed intake, FCR, increase meat and egg production, and have hypocholesterolemic on poultry products. However, few probiotics have significant effects on feed intake, egg production, and growth performance. They are live microbes that affect host animals by maintaining intestinal microbes. Probiotics are of many kinds and they are different in their actions and effects on poultry production. They are used to treat various infections. Although, they are commonly used in poultry farming; still, the determination of the optimal dose remains an issue that needs to be solved. Probiotics are used in different dosages and can be given by mixing them with water. There are various microbial infections such as *Campylobacter*, *C. perfringes*, and *Salmonella*. Many antibiotics are extensively used to enhance poultry production and treat infections. However, this leads to the presence of antibiotic residues in poultry products like meat and eggs, followed by antibiotic resistance. As antibiotic resistance becomes more critical, the use of probiotics and prebiotics gains prominence. Poultry farmers increasingly incorporate probiotics into feed as nutritional supplements, benefiting from their positive effects on growth, egg quality, immune function, and overall health.

Declarations

Competing Interests: The authors have shown no conflict of interest

Declaration of Generative Artificial Intelligence (AI): We Declare that the main manuscript, tables, and figures were not written/created by AI and AI-assisted technologies

Authors Contributions: AR, RZA, OK, AMAK: conceptualization, design, planning, investigation, writing the original draft. AR, MZK, NM, AQ, AH revised and edited. KPK finalized the manuscript. All authors read and approved the final manuscript.

References

1. Bidura IGNG, Siti NW, Wibawa AAPP, Puspani E, Candrawati DPMA: Improving the quality of Tofu waste by mixing it with carrots and probiotics as a feed source of probiotics and β-carotene. *Int J Vet Sci,* 12(3): 407-413, 2023. DOI: 10.47278/journal.ijvs/2022.213

2. Abd El-Hack ME, El-Saadony MT, Shafi ME, Qattan SYA, Batiha GE, Khafaga AF, Abdel-Moneim AME, Alagawany M: Probiotics in poultry feed: A comprehensive review. *J Anim Physiol Anim Nutr,* 104 (6): 1835-1850, 2020. DOI: 10.1111/jpn.13454

3. Ahmad S, Humak F, Ahmad M, Altaf H, Qamar W, Hussain A, Ashraf U, Abbas RZ, Siddique A, Ashraf T, Mughal MAS: Phytochemicals as alternative anthelmintics against poultry parasites: A review. *Agrobiol Rec,*

12, 34-45, 2023. DOI: 10.47278/journal.abr/2023.015

4. Tajudeen H, Ha SH, Hosseindoust A, Mun JY, Park S, Park S, Kim J: Effect of dietary inclusion of Bacillus-based probiotics on performance, egg quality, and the fecal microbiota of laying hen. *Anim Biosci,* 37 (4): 689-696, 2024. DOI: 10.5713/ab.23.0299

5. Çapan B, Bağdatlı A: Usage of probiotics in the poultry industry and effects on meat quality. *Selcuk J Agric Food Sci,* 36 (1): 120-126, 2022. DOI: 10.15316/SJAFS.2022.017

6. Mal-Ganji S, Eivani MJ, Sohrabvandi S, Mortazavian AM: Health-related aspects of probiotics. *Iran J Nutr Sci,* 7 (5): 579-589, 2013.

7. Lilly DM, Stillwell RH: Probiotics: growth-promoting factors produced by microorganisms. *Science,* 147 (3659): 747-748, 1965. DOI: 10.1126/science.147.3659.747

8. Rashid S, Tahir S, Akhtar T, Altaf S, Ashraf R, Qamar W: *Bacillus*-based probiotics: An antibiotic alternative for the treatment of salmonellosis in poultry. *Pak Vet J,* 43 (1): 167-173, 2023. DOI: 10.29261/pakvetj/2023.017

9. Gul ST, Alsayeqh AF: Probiotics as an alternative approach to antibiotics for safe poultry meat production. *Pak Vet J,* 42 (3): 285-291, 2022. DOI: 10.29261/pakvetj/2022.061

10. Aktaş ŞCB, Yaşar S, Yıldırım FM, Din HGQU: Enhanced egg weight, egg production and shell breaking strength in late laying period of hens fed a diet containing a eubiotic mixture. *Int J Agric Sci,* 34 (2): 154-159, 2019. DOI: 10.28955/alinterizbd.665271

11. Saleh AA, Galosi L, Metwally M, Ragab M, Hamza M, Shukry M, Alagawany M: Influence of dietary probiotic and alpha-monolaurin on performance, egg quality, blood constituents, and egg fatty acids' profile in laying hens. *Probiotics Antimicrob Proteins,* 16, 1148-1157, 2024. DOI: 10.1007/s12602-024-10260-0

12. Halder N, Sunder J, De AK, Bhattacharya D, Joardar SN: Probiotics in poultry: A comprehensive review. *J Basic Appl Zool,* 85 (1):23, 2024. DOI: 10.1186/s41936-024-00379-5

13. Gul ST, Alsayeqh AF: Probiotics improve physiological parameters and meat production in broiler chicks. *Int J Vet Sci,* 12 (2): 182-191, 2023. DOI: 10.47278/journal.ijvs/2022.191

14. Bootwalla SM, Miles RD: Development of diluents for domestic fowl semen. *World's Poult Sci J,* 48 (2): 121-128, 2019. DOI: 10.1079/WPS19920012

15. Mia N, Alam AMMN, Rahman MM, Ali MS, Hashem M: Probiotics to enhance animal production performance and meat quality: A review. *Meat Res,* 4 (2): 1-7, 2024. DOI: 10.55002/mr.4.2.85

16. Sher ALI, Nouman A, Hayat M, Bilal RM, Nauman K, Nasir J, Asghar B, Badar I: Omega-3 fatty acids enriched flaxseed oil effects on meat quality and fatty acid profile of broiler chicken. *Kafkas Univ Vet Fak Derg,* 29 (4): 327-334, 2023. DOI: 10.9775/kvfd.2022.27854

17. Saleem M, Rahman HU, Qureshi MHF: Rapid recovery of *Salmonella* from chicken meat and poultry fecal samples by selective pre-enrichment. *Continental Vet J,* 3 (1): 49-53, 2023.

18. Nupur MN, Afroz F, Hossain MK, Harun-ur-Rashid SM, Rahman MG, Kamruzzaman M, Ferdous KA, Haque MA: Prevalence of potential zoonotic bacterial pathogens isolated from household pet birds and their antimicrobial profile in northern Bangladesh. *Agrobio Rec,* 11, 28-38, 2023. DOI: 10.47278/journal.abr/2023.005

19. Raheem A, Liang L, Zhang G, Cui S: Modulatory effects of probiotics during pathogenic infections with emphasis on immune regulation. *Front. Immunol,* 12:616713, 2021. DOI: 10.3389/fimmu.2021.616713

20. Ölmez M, Şahin T, Karadağoğlu Ö, Öğün M, Yörük M, Dalğa S: Effect of probiotic mixture supplementation to drinking water on the growth performance, carcass parameters and serum biochemical parameters in native Turkish geese. *Kafkas Univ Vet Fak Derg,* 28 (1): 131-137, 2022. DOI: 10.9775/kvfd.2021.26633

21. Getabalew M, Alemneh T, Zewdie D: Overview on probiotics and their impact in commercial poultry production. *Microbiol Res Int,* 8, 43-50, 2020.

22. Mehmood A, Nawaz M, Rabbani M, Mushtaq MH: Probiotic effect of *Limosilactobacillus fermentum* on growth performance and competitive exclusion of *Salmonella gallinarum* in poultry. *Pak Vet J,* 43 (4): 659-664, 2023. DOI: 10.29261/pakvetj/2023.103

23. Yan F, Polk DB: Probiotics and probiotic-derived functional factors - Mechanistic insights into applications for intestinal homeostasis. *Front Immunol,* 11:1428, 2020. DOI: 10.3389/fimmu.2020.01428

24. Anwar U, El-Kott AF, Bilal MQ, Riaz M, Khalid MF, Mustafa R, Farooq U, Ashraf M, Chisti MFA, Rahman MAU: Supplementation of xylanase levels in lower energy diets on digesta viscosity, blood metabolites and gut health of broiler. *Pak Vet J,* 43 (2): 351-355. DOI: 10.29261/pakvetj/2023.033

25. Ahmad S, Yousaf MS, Tahir SK, Rashid MA, Majeed KA, Naseem M, Raza M, Hayat Z, Khalid A, Zaneb H: Effects of co-supplementation of β-galacto-oligosaccharides and methionine on breast meat quality, meat oxidative stability and selected meat quality genes in broilers. *Pak Vet J,* 43 (3): 428-434 2023. DOI: 10.29261/pakvetj/2023.043

26. Zhi-Gang T, Naeem M, Chao W, Tian W, Yan-Min Z: Effect of dietary probiotics supplementation with different nutrient density on growth performance, nutrient retention and digestive enzyme activities in broilers. *J Anim Plant Sci,* 24 (5): 1309-1315, 2014.

27. Śliżewska K, Markowiak-Kopeć P, Śliżewska W: The role of probiotics in cancer prevention. *Cancers,* 13 (1):20, 2020. DOI: 10.3390/cancers13010020

28. Davoodvandi A, Fallahi F, Tamtaji OR, Tajiknia V, Banikazemi Z, Fathizadeh H, Abbasi-Kolli M, Aschner M, Ghandali M, Sahebkar A: An update on the effects of probiotics on gastrointestinal cancers. *Front Pharmacol,* 12:680400, 2021. DOI: 10.3389/fphar.2021.680400

29. Lokapirnasari WP, Pribadi TB, Al Arif A, Soeharsono S, Hidanah S, Harijani N, Najwan R, Huda K, Wardhani HCP, Rahman NFN: Potency of probiotics *Bifidobacterium* spp. and *Lactobacillus casei* to improve growth performance and business analysis in organic laying hens. *Vet World,* 12 (6):860, 2019. DOI: 10.14202/vetworld.2019.860-867

30. Al-Shawi SG, Dang DS, Yousif AY, Al-Younis ZK, Najm TA, Matarneh SK: The potential use of probiotics to improve animal health, efficiency, and meat quality: A review. *Agr,* 10 (10):452, 2020. DOI: 10.3390/agriculture10100452

31. Jha R, Das R, Oak S, Mishra P: Probiotics (direct-fed microbials) in poultry nutrition and their effects on nutrient utilization, growth and laying performance, and gut health: A systematic review. *Animals,* 10 (10):1863, 2020. DOI: 10.3390/ani10101863

32. Chen X, Ishfaq M, Wang J: Effects of Lactobacillus salivarius supplementation on the growth performance, liver function, meat quality, immune responses and *Salmonella pullorum* infection resistance of broilers challenged with Aflatoxin B1. *Poult Sci,* 101 (3):101651, 2022. DOI: 10.1016/j.psj.2021.101651

33. Abbas A: Immuno-modulatory effects of *Lactobacillus* in *Salmonella gallinarum* infected broiler chicks. *Pak J Sci,* 73 (1): 77-87, 2021. DOI: 10.57041/pjs.v73i1.645

34. Wu F, Kong Y, Chen W, Liang D, Xiao Q, Hu L, Tan X, Wei J, Liu Y, Deng X: Improvement of vaginal probiotics *Lactobacillus crispatus* on intrauterine adhesion in mice model and in clinical practice. *BMC Microbiol,* 23 (1):78, 2023. DOI: 10.1186/s12866-023-02823-y

35. Lv J, Guo L, Chen B, Hao K, Ma H, Liu Y, Min Y: Effects of different probiotic fermented feeds on production performance and intestinal health of laying hens. *Poult Sci,* 101 (2):101570, 2022. DOI: 10.1016/j.psj.2021.101570

36. Nour MA, El-Hindawy MM, Abou-Kassem DE, Ashour EA, Abd El-Hack ME, Mahgoub S, Aboelenin SM, Soliman MM, El-Tarabily KA, Abdel-Moneim AME: Productive performance, fertility and hatchability, blood indices and gut microbial load in laying quails as affected by two types of probiotic bacteria. *Saudi J Biol Sci,* 28 (11): 6544-6555, 2021. DOI: 10.1016/j.sjbs.2021.07.030

37. Wang W, Dong H, Chang X, Chen Q, Wang L, Chen S, Chen L, Wang R, Ge S, Wang P: *Bifidobacterium lactis* and *Lactobacillus plantarum* enhance immune function and antioxidant capacity in cats through modulation of the gut microbiota. *Antioxidants,* 13 (7):764, 2024. DOI: 10.3390/antiox13070764

38. Balcón-Pacheco CD, Coronel-Gómez FD, Ozuna C, Jiménez-Fernández M, Angel-Sahagún CA, Franco-Robles E: Effect of the probiotic

Bifidobacterium animalis as a substitute to growth-promoting antibiotics on performance and egg quality in laying hens. *Iran J Appl Anim Sci,* 13 (2): 357-365, 2023.

39. Abou-Kassem DE, Elsadek MF, Abdel-Moneim AE, Mahgoub SA, Elaraby GM, Taha AE, Elshafie MM, Alkhawtani DM, Abd El-Hack ME, Ashour EA: Growth, carcass characteristics, meat quality, and microbial aspects of growing quail fed diets enriched with two different types of probiotics *(Bacillus toyonensis* and *Bifidobacterium bifidum). Poult Sci,* 100 (1): 84-93, 2021. DOI: 10.1016/j.psj.2020.04.019

40. Chichlowski M, Shah N, Wampler JL, Wu SS, Vanderhoof JA: *Bifidobacterium longum* subspecies *infantis (B. infantis)* in pediatric nutrition: current state of knowledge. *Nutrients,* 12 (6): 1581, 2020. DOI: 10.3390/nu12061581

41. Akhtar T, Shahid S, Asghar A, Naeem MI, Aziz S, Ameer T: Utilisation of herbal bullets against Newcastle disease in poultry sector of Asia and Africa (2012-2022). *Int J Agri Biosci,* 12 (1): 56-65, 2023. DOI: 10.47278/journal.ijab/2023.044

42. Shanmugam S, Barbé F, Park JH, Chevaux E, Kim IH: Supplemental effect of *Pediococcus acidilactici* CNCM I-4622 probiotic on the laying characteristics and calcium and phosphorus metabolism in laying hens. *Sci Rep,* 14 (1):12489, 2024. DOI: 10.1038/s41598-024-62779-5

43. Chaney WE, McBride H, Girgis G: Effect of a *Saccharomyces cerevisiae* postbiotic feed additive on *Salmonella enteritidis* colonization of cecal and ovarian tissues in directly challenged and horizontally exposed layer pullets. *Animals,* 13 (7):1186, 2023. DOI: 10.3390/ani13071186

44. Gelfat I, Aqeel Y, Tremblay JM, Jaskiewicz JJ, Shrestha A, Lee JN, Hu S, Qian X, Magoun L, Sheoran A: Single domain antibodies against enteric pathogen virulence factors are active as curli fiber fusions on probiotic *E. coli* Nissle 1917. *PLOS Pathog,* 18 (9):e1010713, 2022. DOI: 10.1371/journal.ppat.1010713

45. Yadav S, Jha R: Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. *J Anim Sci Biotechnol,* 10, 1-11, 2019. DOI: 10.1186/s40104-018-0310-9

46. Zhao D, Liu H, Zhang H, Liu K, Zhang X, Liu Q, Wu Y, Zhang T, Zhang Q: Dietary supplementation with *Cyberlindnera jadinii* improved growth performance, serum biochemical indices, antioxidant status, and intestinal health in growing raccoon dogs *(Nyctereutes procyonoides). Front Microbiol,* 13:973384, 2022. DOI: 10.3389/fmicb.2022.973384

47. Coniglio MV, Luna MJ, Provensal P, Watson S, Ortiz ME, Ludueña HR, Cavaglieri L, Magnoli AP: Use of the probiotic *Saccharomyces cerevisiae* var. *boulardii* RC009 in the rearing stage of calves. *Int J Agri Biosci,* 12 (3): 188-192, 2023. DOI: 10.47278/journal.ijab/2023.063

48. Aykut MN, Erdoğan EN, Çelik MN, Gürbüz M: An updated view of the effect of the probiotic supplement on sports performance: A detailed review. *Curr Nutr Rep,* 13 (1): 1-13, 2024. DOI: 10.1007/s13668-024-00527-x

49. El-Hawary AS, Ibrahim OM, Kalaba MH, El-Sehrawy MH, Ismail MKA: *Limosilactobacillus fermentum*-derived silver nanoparticles: Biosynthesis, optimization, and biological activities. *Biomass Convers Biorefin,* 14 (12): 1-15, 2024. DOI: 10.1007/s13399-024-05784-y

50. Dong S, Li L, Hao F, Fang Z, Zhong R, Wu J, Fang X: Improving the quality of poultry and its meat products with probiotics, prebiotics, and phytoextracts. *Poult Sci,* 103 (2):103287, 2024. DOI: 10.1016/j.psj.2023.103287

51. Rashid S, Alsayeqh AF, Akhtar T, Abbas RZ, Ashraf R: Probiotics: An alternative to antibiotics in poultry production.*Int J Vet Sci,* 12 (1): 45-53, 2023. DOI: 10.47278/journal.ijvs/2022.175

52. Kalita R, Pegu, Baruah C: Prospects of probiotics and fish growth-promoting bacteria in aquaculture: A review. *Int J Agri Biosci,* 12 (4): 234-244, 2023. DOI: 10.47278/journal.ijab/2023.070

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Research Article

Cardiac Troponin I Activity Compared with Other Cardiac Markers in the Dry Period, Early, and Peak Lactation in Dairy Cattle

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Abstract

The current study aimed to evaluate the effects of parturition and different stages of milk production on Cardiac Troponin I activity (CTnI) and other cardiac markers. A total of 42 healthy dairy cattle with an average body weight of 520±17 kg and an average parity of 2.4±0.7 were randomly selected from a commercial dairy farm. Through a clinical examination and ECG recording conducted by a veterinarian, blood samples were collected from all cattle's coccygeal vein one month before parturition (dry period), one week after parturition (early lactation), and 10 weeks after parturition (peak of the lactation). Dairy cows in the experimental group were observed closely before and during parturition and in the postpartum period. Biochemical parameters, including Cardiac Troponin I activity (CTnI), Creatine kinase (CK), Aspartate aminotransferase (AST), and Lactate dehydrogenase (LDH) were analyzed at three-time intervals. Regarding time intervals, there were no significant differences among the cattle in terms of CTnI, CK, LDH, AST, parturition, and different stages of milk production. It was found that the effect of late-stage gestation and different lactation periods on cardiac biomarkers in dairy cows was not significant.

Keywords: Creatine kinase, Dairy farm, Lactate dehydrogenase, Parturition

Introduction

Clinicians face significant challenges when dealing with cardiac diseases in cattle, specifically in the advanced stage. When the primary heart disease advances to the point in which compensatory mechanisms, including neural and hormonal responses, are no longer effective, heart failure occurs [1]. The early diagnosis of heart diseases in dairy cattle is crucial and can be life-saving. One effective method for diagnosing these conditions involves the evaluation of cardiac markers [2,3]. Early and prompt diagnosis of heart disease in cows can contribute to treating animals successfully. In addition, identifying and removing less productive cows from the herd can prevent further economic losses [4-6]. In the case of heart disease, many blood findings are nonspecific, which can challenge accurate diagnosis [7,8]. Troponin, creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) are regarded as biomarkers for assessing cardiac injuries among various mammals [9]. Creatine kinase and lactate dehydrogenase are specific isoenzymes associated with cardiac function that exhibit elevated levels in a range of cardiac conditions. Nevertheless, in large animals, the sensitivity and specificity of these biomarkers are inferior to that of troponin [7]. Troponin is a globular protein that exists within the thin filaments of all striated muscles and has a crucial function in the contraction and relaxation of these muscles. It is a component of the myofibrillar proteins found in the cardiac contractile system, which regulates the interactions between actin and myosin in controlling the contractions of muscle cells [3,10]. These proteins exist in three forms, namely Cardiac Troponin C (TNNCI), Cardiac Troponin T (cTnT), and Cardiac Troponin I (cTnI). The cTnI and cTnT are specific isoforms uniquely

produced by cardiac myocytes, and they have proven to be valuable biomarkers for assessing myocardial damage. Notably, the amino acid sequence of cTnI exhibits over 96% homology between bovine and human species [2]. Studies conducted on laboratory animals and humans have confirmed that cardiac troponins, particularly cTnI, are typically found at very low concentrations or even below the detection limit in most measurements. However, when myocytes are damaged, these proteins are released into the bloodstream [10,11]. The presence of local or systemic hypoperfusion and myocardial lesions due to high metabolism and production in cows could potentially lead to an increase in blood concentrations of markers of hypoperfusion, such as L-lactate (LAC) and cardiac biomarkers, such as cTnI [2].

Consequently, the measurement of cardiac troponins is currently recognized and widely used as an available biochemical marker for diagnosing myocardial injuries in humans [12]. Some studies have demonstrated that the level of cardiac troponins in the blood is elevated in both primary and secondary cardiac disorders in dogs and cats [13]. The increased serum concentration is a valuable indicator of cardiac abnormalities in these animals [10,11].

Furthermore, a rise in troponin serum concentration is proposed as a prognostic indicator of disease. In other words, the continued elevation of troponin levels in the blood may provide valuable information about the progression and severe condition [11]. Measuring cardiac troponins has proven to be highly sensitive and specific in detecting cardiac damage, and it is considered the gold standard for evaluating myocardial injury in animals [14]. While the troponin protein complex has been identified in animals, limited research has been conducted on its clinical utility. Most animal studies have focused on laboratory animals that mimic human heart infarction [15]. Consequently, limited information on the diagnostic application of cTnI in cattle is available.

Early detection of myocardial damage could be determined by evaluating myocardial markers in primary and secondary myocardial diseases. Utilizing cardiac troponin tests (cTnI and cTnT) has facilitated the early diagnosis of secondary myocardial degeneration from foot-and-mouth disease [16]. In a study by Mellanby et al. on cows with pericarditis, an increased level of cTnI was observed in 4 out of 5 cases of cows with traumatic pericarditis [17]. However, upon necropsy, none of these cows showed evidence of myocardial penetration by foreign bodies, indicating a predominance of chronic disease. Peek et al.[18] found that endotoxemia caused an elevation in cTnI concentration in calves, while the level of cTnT remained unchanged. Measurement of cTnI and cTnT serum levels could be used as a diagnostic test for assessing cardiac cell damage in traumatic pericarditis [19].

Cardiac troponin kits have been utilized to quantitatively assess myocardial cell damage resulting from traumatic reticuloperitonitis in cattle [19]. High-producing cows are particularly susceptible to various metabolic and infectious diseases during the peripartum period, especially at the production peak. These cows experience a negative energy balance, metabolic disturbances, and electrolyte deficiencies [3]. Furthermore, the proximity to calving makes them increasingly vulnerable to infectious diseases and other associated health issues. The combination of these factors increases the risk of diseases in high-producing cows [20].

The current study aimed to investigate the possibility of increasing CTnI and its association with other cardiac markers of dairy cows in different production periods.

Material and Methods

Ethical Statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the School of Veterinary Medicine, Shiraz University, Shiraz, Iran with approval code number: 47317.

Animals, Sampling Process, and Study Design

The present study investigated a dairy cattle unit with 120 heads, operating within an industrial setting in Shiraz, Iran (spring 2012). For the research, 42 dairy Holstein cows were randomly selected, with an average body weight of 520±17 kg, an average parity of 2.4±0.7, and an average age of 3.2±0.6. The inclusion criteria were body weight, parity, and milk production in animals with healthy evaluated cardia and the exclusion criteria were animals that have diseases such as heart disease, laminitis, and metritis. Throughout the study, the cows were maintained under proper and consistent management and appeared to be in good health. The feeding regimen followed the NRC 2000 guidelines for dairy cows during parturition. The diet consisted of alfalfa, silage, straw, concentrate, mineral, and vitamin supplements. All cows underwent general clinical examinations. The average body temperature, respiratory rate, and heart rate were 37.4, 15, and 70 respectively. The specific cardiac evaluations, including heart auscultation, observation of vein condition, and ECG were recorded and all animals were in normal condition [9]. Considering the predicted calving time, a 10 mL blood sample was collected from the coccygeal vein in the tube without anticoagulant for all cows one month before parturition (dry period). Additional samples were collected one week after parturition (early lactation) and 10 weeks after parturition (peak of the lactation).

Study Parameters

Cardiac troponin levels were measured using the CTnI

AccuBind Elisa assay kit (Monobind Inc, USA) based on an immunoenzymometric test. The test had a sensitivity (detectable limit) of 0.05 ng/mL and a specificity of 25 ng/mL. Levels of AST were measured using a commercial biochemical kit (Zistshimi, Iran). The CK levels were measured using a commercial biochemical kit (Zistshimi, Iran). A commercial biochemical kit (Zistshimi, Iran) and the Caboud-Wroblewski calorimetric method were utilized to measure LDH levels.

Statistical Analysis

All statistical analyses were performed using SPSS version 24 software (USA). The variables were examined for normality using the Kolmograph-Smirto test. The one-way repeated measure ANOVA was used to analyze the variables in this longitudinal study. The Bonferroni test was used as a confident interval adjustment to compare the main effects in three sampling times. In all cases, $P<0.05$ was considered statistically significant. All data is presented as mean ± standard deviation (SD).

Results

The obtained results related to the biochemical parameters of dairy cows' blood serum in different periods, including one month before parturition, one week after calving, and 10 weeks after calving are shown in *Fig 1*, *Fig 2*,

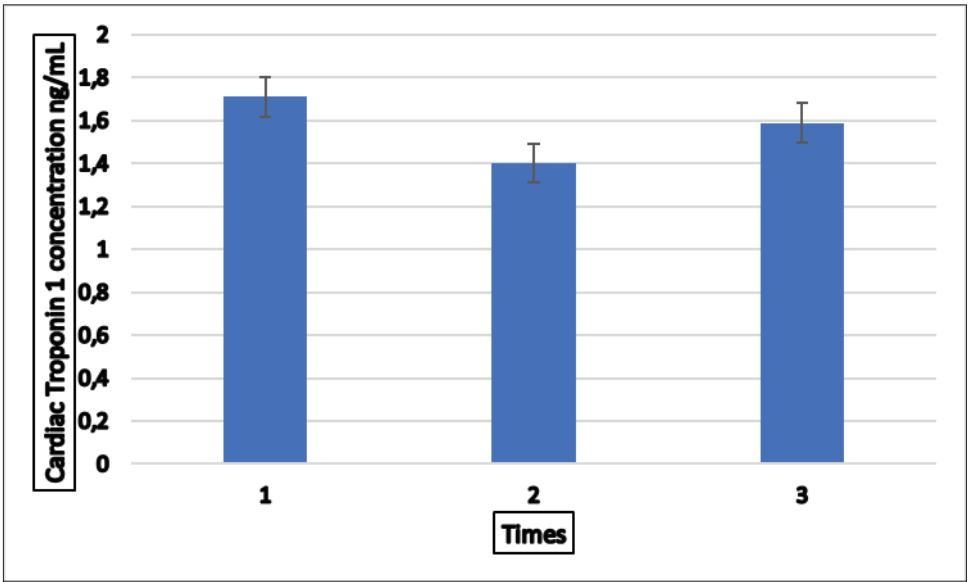

Fig 1. The troponin 1 activity in dairy Holstein cows in three sampling times, including one month before parturition, one week after parturition, and 10 weeks after parturition

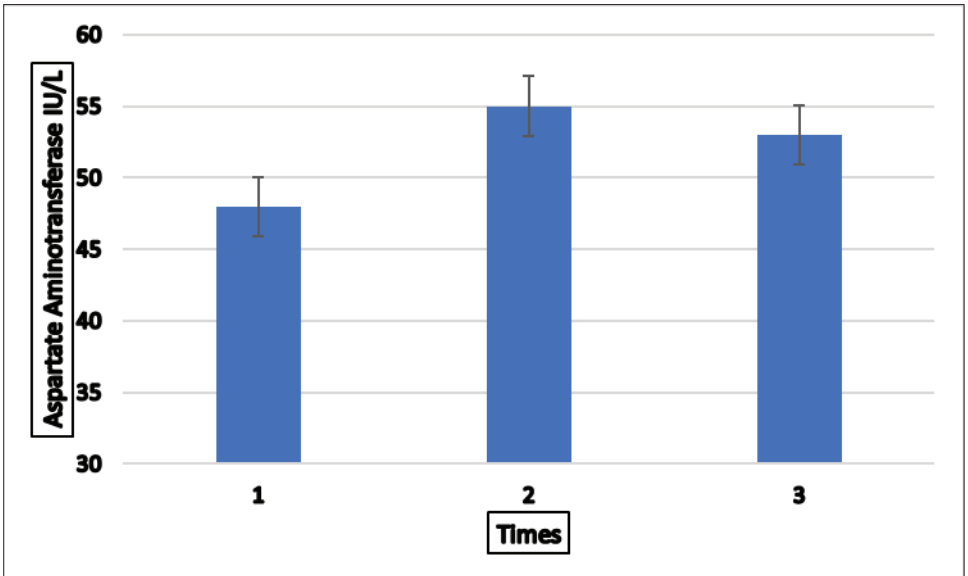

Fig 2. The Aspartate aminotransferase activity in dairy Holstein cows in three sampling times, including one month before parturition, one week after parturition, and 10 weeks after parturition

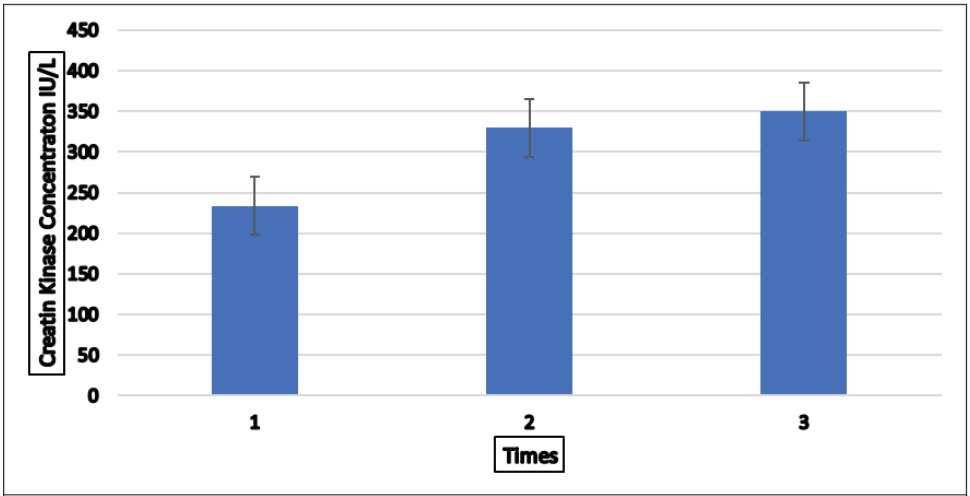

Fig 3. The creatine kinase activity in dairy Holstein cows in three sampling times, including one month before parturition, one week after parturition, and 10 weeks after parturition

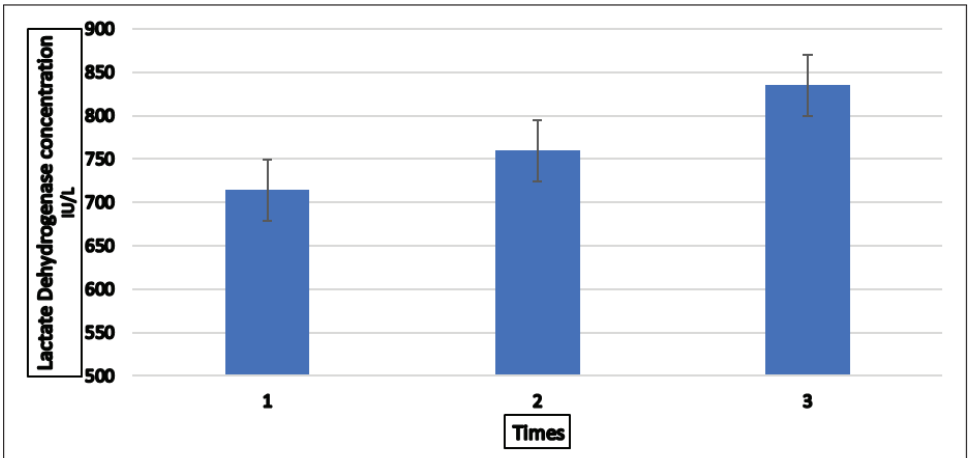

Fig 4. The Lactate dehydrogenase activity in dairy Holstein cows in three sampling times, including one month before parturition, one week after parturition, and 10 weeks after parturition

Fig 3, and *Fig 4*. *Fig 1*, *Fig 2*, *Fig 3*, and *Fig 4* includes data after measuring the serum enzymes CK, LDH, AST, and troponin. Regarding the CTnI parameter, the results indicated insignificant differences in the amount of this enzyme in the whole trial *(Fig 1*, P>0.01).

The analysis of AST showed no significant statistical difference in the amount of this enzyme in the study animals *(Fig 2*, P>0.01). No significant changes were observed during the study for LDH *(Fig 3*, P>0.01). In addition, CK concentrations in dairy cows were insignificant in three sampling time intervals *(Fig 4*, P>0.01).

Discussion

High-productive cows are prone to various metabolic and infectious diseases around the calving time, which is more evident at the production peak [21,22]. The present study aimed to examine potential variations in the levels of CK, LDH, CTn-I, and AST in dairy cows across different lactation stages, and to compare these levels with those observed during the dry period of these cows. It is widely recognized that high-yielding dairy cows may experience varying degrees of negative energy balance, compared to low-yielding cows [21]. Negative energy balance may occur in three situations, including at the end of pregnancy, in the first weeks of lactation, and during diseases [5,23].

Metabolic diseases predispose animals to various infectious diseases around parturition; therefore, the priority in milk production should be prevented. Heart damage, including endocarditis, pericarditis, and myocarditis, is one of the diseases with a high incidence rate in this period [9,22]. The timely diagnosis of a disease can lead to effective treatment of valuable cows to prevent economic losses [6]. The obtained results indicated no significant difference in cardiac biomarkers in the dry period, early, and peak lactation in dairy cattle in this study. Most blood findings in the case of heart disease are nonspecific [7,24]. However, cardiac troponin has been used in animals. A few studies are addressing its clinical use, partly due

to the lack of sufficient information on their natural values. Animal studies, which mostly include laboratory animals, are designed to diagnose myocardial infarction in humans [15,25]. Insufficient information is available regarding the diagnostic use of CTnI in cattle. Early diagnosis of myocardial damage may be useful in treating primary and secondary myocardial diseases. Using tests to measure cardiac troponin enables veterinarians to make an early diagnosis of myocardial degeneration caused by foot and mouth diseases [16]. This is particularly crucial as animals in such cases may be killed before sudden death, making a cardiac index, such as troponin important for diagnosing animal myocarditis [16]. Hajimohammadi et al.[26] pointed out that secondary heart disease caused by salinomycin poisoning could increase CTnI levels in the bloodstream. In all the poisoned groups with different doses of salinomycin, there was a statistically significant difference in the CTnI levels, compared to the control group. Mellanby et al. conducted a study on cows with pericarditis [17]. They observed that a small number of cows with pericarditis had CTnI concentration within the source range. In the necropsy study, the foreign body did not penetrate any of these cows, and most had chronic diseases. The findings of the present study demonstrated that the animals did not exhibit any significant changes in cardiac markers, during the different production stages in this study.

The assessment of the blood concentration of cTnI in healthy cows was below 0.02 ng/mL in a cohort of 30 cows representing various breeds, specifically Jersey and Holstein, in differing physiological conditions, which included 20 lactating cows and 10 dry cows [2]. Additionally, in a group of 28 healthy Holstein calves that were 2.5 months old, the measured values varied from 0.00 to 0.04 ng/mL, although no reference interval was provided for comparison [18].

For many years, the increased blood concentration of CK indicated muscle damage [9]. The CK is presented in both skeletal tissue and cardiac muscles. Changes in the blood CK concentration are associated with acute necrosis of the heart muscle following very intense activity or diseases. In the present study, no statistically significant difference was observed in the amount of CK enzyme. Cozzi et al. indicated that the serum concentration of CK changes significantly in different stages of lactation [27]. The CTnI remains in the blood longer than CK (4-14 days vs. 24-36 hours), making it a valuable tool for diagnosing myocarditis a few days after its onset [28]. This is while serum cardiac troponin is the first biochemical indicator during myocardial injury [29,30]. The increase in CTnI concentration is related to the severity of histopathological lesions and myocardial necrosis. According to Lim et al.[31], after myocardial damage following viral infection, serum cardiac troponin concentration changes earlier than the histological findings of inflammation. In the immunohistochemistry study conducted by Tunca et al. on calves suffering from foot and mouth disease, some points had a clear reduction and absence of CTnI levels, indicating myocardial cell degeneration [32]. In a study by Jesty et al.[33], the concentration of serum CTnI in a cow with pericarditis of unknown origin was 0.49 ng/mL, while the CTnI concentration in a healthy cow was 0.04 ng/mL. In another study by Tunca et al.[32], serum CTnI concentration in calves with foot and mouth disease was 11.7-16.4 ng/mL, and the mean of CTnI in healthy calves was 0.24 ng/mL. A study indicated that the measurement of serum troponin I levels is beneficial for diagnosing traumatic pericarditis and traumatic reticuloperitonitis in cattle [34]. Normal values of CTnI in healthy dairy cows have been reported to be less or equal to 0.03 ng/mL in all studies [17,18,33]. In the present study, the mean serum CTnI for cows in the middle of the dry period was 2.16±1.29 ng/mL, one week after calving was 1.55±0.87 ng/mL, and at the peak of production was 1.33±041/ng/mL.

Aspartate aminotransferase exists in almost all tissues of the body. The muscles and liver are the main sources of this enzyme activity [35]. Regarding the AST enzyme, the present study revealed no significant statistical difference in the amount of this enzyme in cows during three sampling times.

In the current study, the mean serum LDH was 684.36±355.50 U/L in the middle of the dry period and one week after calving was 806.18±501.33 U/L, and at the peak of production was 853.5±276.84 U/L.

Lactate dehydrogenase is a widely distributed enzyme in the body, with high activity in the heart, liver, skeletal muscle, kidney, and erythrocytes. In contrast, a lower amount is found in the lung, smooth muscle, and brain [36]. The average serum LDH concentration was 825.35±434.22 U/L 10 weeks after calving, U/L 330.69±712 one week after calving, and 391.49±49 in the middle of the dry period according to this study.

In conclusion, there is a need to continuously evaluate the mentioned parameters in the production period to investigate animals' health. It is vital to have normal values of these parameters in different stages of production, especially CTnI, which has recently been proposed as a useful side test to diagnose myocardial diseases. More studies are suggested to evaluate the diagnostic benefits of CTnI analysis in cows that naturally suffer from heart diseases.

Declarations

Availability of Data and Materials: The data that support the findings ofthis study are available on request from the corresponding author (D.B.).

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Ethical Statement

All procedures performed in studies involving animals were in accordance with the ethical standards of the School of Veterinary Medicine, Shiraz University, Shiraz, Iran with approval code number: 47317.

Competing Interests: The authors declare that there is no competing of interest regarding the publication of this article.

Declaration of Generative Artificial Intelligence: The authors of the current study declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Authors' Contributions: Ali HAJIMOHAMMADI conceived and designed research. Mojtaba BARMAKI conducted experiments. Saeed NAZIFI contributed to new reagents and analytical tools. Seyed Amin RAZAVI and Mojtaba DANESHI analyzed the data. Seyed Amin RAZAVI and Mojtaba BARMAKI prepared the draft of the manuscript. Daryoush BABAZADEH revised the final draft of the manuscript. All authors read and approved the final version of the manuscript and also agreed to the submission of the article to this journal.

References

1. Labonté J, Roy JP, Dubuc J, Buczinski S: Measurement of cardiac troponin I in healthy lactating dairy cows using a point of care analyzer (i-STAT-1). *J Vet Cardiol,* 17 (2): 129-133, 2015. DOI: 10.1016/j.jvc.2015.02.003

2. Varga A, Schober KE, Walker WL, Lakritz J, Michael Rings D: Validation of a commercially available immunoassay for the measurement of bovine cardiac troponin I. *J Vet Intern Med,* 23 (2): 359-365. 2009. DOI: 10.1111/j.1939-1676.2009.0256.x

3. Le Page T, Buczinski S, Dubuc J, Labonté J, Roy JP: Development of a nomogram to estimate the 60-day probability of death or culling due to severe clinical mastitis in dairy cows at first veterinary clinical evaluation. *Vet Sci,* 10 (4):268, 2023. DOI: 10.3390/vetsci10040268

4. Razavi SA, Pourjafar M, Hajimohammadi A, Valizadeh R, NaserianAA, Laven R, Mueller RK: Effects of dietary supplementation of bentonite and yeast cell wall on serum blood urea nitrogen, triglyceride, alkaline phosphatase, and calcium in high-producing dairy cattle during the transition period. *Comp Clin Pathol,* 28, 419-425, 2019. DOI: 10.1007/s00580-018-2849-4

5. Mirzaei A, Hajimohammadi A, Badiei K, Pourjafar M, Naserian AA, Razavi SA: Effect of dietary supplementation of bentonite and yeast cell wall on serum endotoxin, inflammatory parameters, serum and milk aflatoxin in high-producing dairy cows during the transition period. *Comp Clin Pathol,* 29, 433-440, 2020. DOI: 10.1007/s00580-019-03074-y

6. Rahmani Shahraki A, Mirzaei A, Badiei A, Bittar JHJ, Ruth Mueller K, Naserian A, Razavi SA: Impact of COVID-19 on dairy herd profitability: A review at the global level. *Farm Anim Health Nutr,* 2 (1): 1-7, 2023. DOI: 10.58803/Fahn.2023.2.1.01

7. Bexiga R, Mateus A, Philbey AW, Ellis K, Barrett DC, Mellor D: Clinicopathological presentation of cardiac disease in cattle and its impact on decision making. *Vet Rec,* 162 (18): 575-580, 2008. DOI: 10.1136/vr.162.18.575

8. Mohammadi M, Taslimian Fasaii A, Kiafar P, Razavi SA, Hajimohammai A, Chalmeh A: Evaluating oxidative stress parameters, pepsinogen, and insulin-like growth factor-1 in dairy cows with displacement of abomasum. *Farm Anim Health Nutr,* 1 (1): 6-9, 2022. DOI: 10.58803/fahn.v1i1.6

9. Buczinski S, Rezakhani A, Boerboom D: Heart disease in cattle: Diagnosis, therapeutic approaches and prognosis. *Vet J,* 184 (3): 258-263, 2010b. DOI: 10.1016/j.tvjl.2009.05.005

10. Smith JS, Varga A, Schober KE: Comparison of two commercially available immunoassays for the measurement of bovine cardiac troponin I in cattle with induced myocardial injury. *Front Vet Sci,* 7:531, 2020. DOI: 10.3389/fvets.2020.00531

11. Spratt DR, Mellanby RJ, Drury N, Archer J: Cardiac troponin I: Evaluation of a biomarker for the diagnosis of heart disease in the dog. *J Small Anim Pract,* 46 (3): 139-145, 2005. DOI: 10.1111/j.1748-5827.2005.tb00304.x

12. Panteghini M: The new definition of myocardial infarction and the impact of troponin determination on clinical practice. *Intern J Cardiol,* 106, 298-306, 2006. DOI: 10.1016/j.ijcard.2005.01.046

13. Linde A, Summerfield NJ, Sleeper MM, Wright FB, Clifford CA, Melgarejo T, Knight DH: Pilot study on cardiac troponin I levels in dogs with pericardial effiusion. *J Vet Cardiol,* 8 (1): 19-23, 2006. DOI: 10.1016/j.jvc.2005.12.002

14. Labonté J, Dubuc J, Roy JP, Buczinski S: Prognostic value of cardiac troponin I and L-lactate in blood of dairy cows affected by downer cow syndrome. *J Vet Intern Med,* 32 (1): 484-490, 2018. DOI: 10.1111/jvim.14874

15. Fredericks S, Merton GK, Lerena MJ, Heining P, Carter ND, Holt DW: Cardiac troponins and creatine kinase content of striated muscle in common laboratory animals. *Clin Chim Acta,* 304 (1-2): 65-74, 2001. DOI: 10.1016/S0009-8981(00)00409-5

16. Güneş V, Erdoğan HM, Çitil M, Özcan K: Assay of cardiac troponins in the diagnosis of myocardial degeneration due to foot and mouth disease in a calf. *Vet Rec,* 156 (22): 714-715, 2005. DOI: 10.1136/vr.156.22.714

17. Mellanby RJ, Henry JP, Cash R, Ricketts SW, Dias Bexiga JR, Mellor DJ: Serum cardiac troponin I concentrations in cattle with pericarditis. *Vet Rec,* 161 (13): 454-455, 2007. DOI: 10.1136/vr.161.13.454

18. Peek SF, Apple FS, Murakami MA, Crump PM, Semrad SD: Cardiac isoenzymes in healthy Holstein calves and calves with experimentally induced endotoxemia. *Can J Vet Res,* 72 (4): 356-361, 2008.

19. Güneş V, Atalan G, Citil M, Erdogan HM: Use of cardiac troponin kits for the qualitative determination of myocardial cell damage due to traumatic reticuloperitonitis in cattle. *Vet Rec,* 162 (16): 514-517, 2008. DOI: 10.1136/vr.162.16.514

20. Babazadeh D, Razavi SA, Abd El-Ghany WA, Cotter PF: Vitamin D deficiency in farm animals: A review. *Farm Anim Health Nutr,* 1 (1): 10-16, 2022. 10.58803/fahn.v1i1.7

21. Mirzaei A, Merenda VR, Ferraretto LF, Shaver RD, Peñagaricano F, Chebel RC: Individual animal variability in rumination, activity, and lying behavior during the periparturient period of dairy cattle. *JDS Communications,* 4 (3): 205-209, 2023. DOI: 10.3168/jdsc.2022-0300

22. Fraser BC, Anderson DE, White BJ, Miesner MD, Wheeler C, Amrine D, Lakritz J, Overbay T: Assessment of a commercially available point-of-care assay for the measurement of bovine cardiac troponin I concentration. *Am J Vet Res,* 74(6): 870-873. 2013. DOI: 10.2460/ajvr.74.6.870

23. Howie-Esqivel J, White M: Biomarkers in acute cardiovascular disease. *J Cardiovasc Nurs,* 23 (2): 124-131, 2008. DOI: 10.1097/01.JCN.0000305072.49613.92

24. Buczinski S, Fecteau G, DiFruscia R: Ventricular septal defects in cattle: A retrospective study of 25 cases. *Can Vet J,* 47 (3): 246-252, 2006.

25. Charles CJ, Elliott JM, Nicholls MG, Rademaker MT, Richards M: Myocardial infarction with and without reperfusion in sheep: Early cardiac and neurohumoral changes. *Clin Sci,* 98 (6): 703-711, 2000. DOI: 10.1042/cs0980703

26. Hajimohammadi A, Rajaian H, Khaliji E, Nazifi S, Ansari-Lari M: Serum cardiac troponin I as a biomarker in cardiac degeneration following experimental salinomycin toxicosis in sheep. *Vet Arh,* 84 (1): 41-51, 2014.

27. Cozzi G, Ravarotto L, Gottardo F, Stefani AL, Contiero B, Moro L, Brscic M, Dalvit P: Short communication: Reference values for blood

parameters in holstien dairy cows: Effects of parity, stage of lactation, and season of production. *J Dairy Sci,* 94 (8): 3895-3901, 2011. DOI: 10.3168/jds.2010-3687

28. Collinson PO, Boa FG, Gaze DC: Measurement of cardiac troponins. Annals of Clinical Biochemistry. *J Lab Med,* 38 (5): 423-449, 2001. DOI: 10.1177/000456320103800501

29. Boccara G, Pouzeratte Y, Troncin R, Bonardet A, Boularan AM, Colson P, Mann C: The risk of cardiac injury during laparoscopic fundoplication. Cardiac troponin I and ECG study. *Acta Anaesthesiol Scand,* 44 (4): 398-402, 2000. DOI: 10.1034/j.1399-6576.2000.440407.x

30. Ooi DS, Isotalo PA, Veinot JP: Correlation of antemortem serum creatine kinase-MB, troponin I and troponin T with cardiac pathology. *Clin Chem,* 46 (3): 338-344, 2000. DOI: 10.1093/clinchem/46.3.338

31. Lim BK, Shin JO, Choe SC, Choi SW, Jeong JO, Seong IW, Kim DK, Jeon ES: Myocardial injury occurs earlier than myocardial inflammation in acute experimental viral myocarditis. *Exp Mol Med,* 37, 51-57, 2005. DOI: 10.1038/emm.2005.7

32. Tunca R, Sozmen M, Erdogan HM, Citil M, Uzlu E, Ozen H, Gokce E: Determination of cardiac Troponin I in the blood and heart of calves with foot and mouth disease. *J Vet Diagn Invest,* 20 (5): 598-605, 2008. DOI: 10.1177/104063870802000510

33. Jesty S, Sweeney RW, Dolente BA, Reef VB: Idiopathic pericarditis and cardiac tamponade in two cows. *J Am Vet Med Assoc,* 226 (9): 1555-1558, 2005. DOI: 10.2460/javma.2005.226.1555

34. Nasr HA, Aref NE, Ellah MRA, Abdelhakiem MAH: Cardiac biomarkers as tools in the prediction and diagnosis of traumatic pericarditis and traumatic reticuloperitonitis in cattle and buffaloes. *BMC Vet Res*, 20:329, 2024. DOI: 10.1186/s12917-024-04174-w

35. Nozad Sh, Ramin AG, Moghaddam Gh, Asri-Rezaei S, Kalantary L: Monthly evaluation of blood hematological, biochemical, mineral, and enzyme parameters during the lactation period in Holstein dairy cows. *Comp Clin Pathol,* 23, 275-281, 2014.

36. Klein R, Nagy O, Tóthová C, Chovanová F: Clinical and diagnostic significance of lactate dehydrogenase and its isoenzymes in animals. *Vet Med Int,* 2020:5346483, 2020. DOI: 10.1155/2020/5346483

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Research Article

Suspicion of Feline Infectious Peritonitis in Cats with Uveitis: Diagnostic Value of Coronavirus Antibodies and Blood Parameters

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Abstract

This study aimed to discuss the suspicion of FIP in cats presenting solely with uveitis as a clinical finding but with positive coronavirus antibody tests by evaluating antibody test results, complete blood count and some biochemical parameters. The study consisted of 94 cats of different breeds, ages, and genders with discoloration, opacity, or vision loss in one or both eyes. Coronavirus-specific antibody test results were categorized. Complete blood count, serum total protein, and albumin/globulin tests were carried out. The predominant ocular symptom was iris hyperemia. No significant changes were observed in neutrophil, eosinophil, lymphocyte and monocyte. A positive, statistically significant relationship was found between RDW and the antibody score. A negative, statistically significant correlation was observed between total protein and antibody score. The difference in A/G ratios between antibody titers was statistically significant. In conclusion, no direct correlation was identified between the types or symptoms of uveitis and antibody levels, albumin/globulin ratio, or complete blood count parameters. Stress leukogram, which is used in differential diagnosis by many researchers, was found to be completely ineffective, with even the lowest lymphocyte count observed in animals with S1 antibody titer. The results of RDW parameters obtained in cats suspected of FIP suggest that this simple parameter could be used as a cost-effective and reliable marker for FIP with further studies.

Keywords: Antibody, Blood parameters, Eye, FIP, RDW

Introduction

Feline coronavirus (FCoV) is an RNA virus capable of adapting to various mammalian and avian species, causing digestive and respiratory infections. Its prevalence in the global feline population is high, with viral positivity rates exceeding 90% in multi-cat environments like production farms and shelters [1,2]. FCoV is classified into two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV), which differ in pathogenicity and clinical presentation [3].

FECV typically causes mild and latent infections, and while it primarily replicates in the intestinal tract, the virus can persist in feces for up to 15 weeks, contributing to its highly contagious nature [4-7]. FIPV, a mutated form of FECV, leads to feline infectious peritonitis (FIP), a complex disease with effusive, non-effusive, and mixed forms [8-11]. Ocular inflammation, particularly uveitis, is a prominent feature of the non-effusive form [12,13]. Ocular inflammation results from increased blood vessel permeability caused by disturbances in endothelial cells, leading to the focal or diffuse distribution of macrophages, lymphocytes, plasma cells, and neutrophils [14-16].

Laboratory findings, such as hyperproteinemia, hyperglobulinemia, and hypoalbuminemia, are frequently associated with FIP diagnosis [17-19]. Changes in the albumin-to-globulin ratio and blood profiles, including non-regenerative anemia and lymphopenia, further support diagnosis [20,21]. Despite ongoing research, a definitive diagnostic test and vaccination program for FIP remain elusive, marking it as a significant disease in veterinary medicine that demands further study [3,22].

This study aimed to discuss the suspicion of FIP in cats presenting solely with uveitis as a clinical finding but with positive coronavirus antibody tests by evaluating antibody test results, complete blood count and some biochemical parameters. It is believed that the results obtained will

serve as a supportive resource in interpreting FIP infection in live cats

Material and Methods

Ethical Statement

The required ethics committee report for the study was obtained from Animal Experiments Local Ethics Committee of Ankara University (Approval No: 2024-08-62). An "Informed Consent Form" was obtained from the animal owners before examination of animals.

Animals

The study cohort comprised 94 cats of diverse breeds, ages, and genders, presented at Ankara University Faculty of Veterinary Medicine Animal Hospital between April 2021 and April 2024. These cats exhibited complaints such as color change, opacity, blepharospasm, or vision loss in one or both eyes.

Study Design

A comprehensive clinical examination of the eyes was conducted for all animals, incorporating ophthalmoscopy, slit lamp biomicroscopy, fluorescein staining, and pupillary light reflex examination. During the initial assessment of the overall appearance of the eye in the clinical examination, slit lamp biomicroscopy was employed to evaluate the cornea, anterior chamber, and iris, while ophthalmoscopy was utilized to assess the lens, vitreous, and fundus.

Eye symptoms were classified as acute or chronic based on their duration, and as unilateral or bilateral based on their occurrence. The presence of uveitis was evaluated based on iris hyperemia, aqueous flare, and the formation of keratic precipitates. Additionally, uveitis was classified as granulomatous or non-granulomatous based on type, and as anterior uveitis (inflammation of the anterior chamber, affecting the iris and anterior ciliary body), posterior uveitis (inflammation of the retina or choroid) or panuveitis (inflammation of the anterior chamber, vitreous and retina or choroid) according to anatomical classification.

Ultrasonography was performed to evaluate intraocular structures in all cats. A 7.5 MHz convex probe was used for B-mode ultrasonography, generating detailed images of the lens, vitreous, and retina. In cats with uveitis in both eyes, the eye with the most severe symptoms was included in the study.

A volume of 1 mL of blood was collected from all cats for the measurement of complete blood count, serum total protein, albumin and globulin values. Additionally, an ELISA test (ImmunoComb FCoV Antibody Test Kit, Biogal) was conducted to detect the FCoV antibodies. The results of the test were categorized as S1, S2, S3, S4, S5, and S6 based on the severity of the antibody level, as outlined in *Table 1*.

Table 1. *Evaluation of ImmunoComb FCoV antibody test kit results according to level coding (Biogal Galed Labs)*

Scale (S)	Test Results
S1	Non specific reaction - considered negative
S2	Low positive reaction - FIP unlikely
S3	Medium positive reaction - FIP possible
S4	Positive reaction - FIP possible
S5	High positive reaction - greater likelihood with FIP
S6	Very high positive reaction - significantly increased likelihood with FIP

***FIP:** Feline infectious peritonitis*

Statistical Analysis

Descriptive statistics for the data were calculated. Before proceeding with the significance tests, the data were examined using the Shapiro-Wilk test for normality, one of the assumptions for parametric tests, and the Levene test for homogeneity of variances. Since the assumptions for parametric testing were not met, the Kruskal-Wallis test was employed to assess the statistical significance of FCoV antibody scores in relation to blood parameters. In cases where a significant difference was found, the Dunn-Bonferroni test was used for post-hoc analysis. A criterion of $P<0.05$ was used for all statistical comparisons. Data

Fig 1. Severe iris hyperemia and discoloration were observed in the left eye of an 8-year-old male mixed breed cat. The case exhibited an FCoV antibody level of S5 and an A/G ratio of 0.25

Fig 2. Severe uveitis in the left eye of a 2-year-old male tabby cat. The pupil cannot be seen clearly due to aqueous flare and hyphema in the anterior chamber. This cat was brought with S3 antibody titer and 0.31 A/G ratio

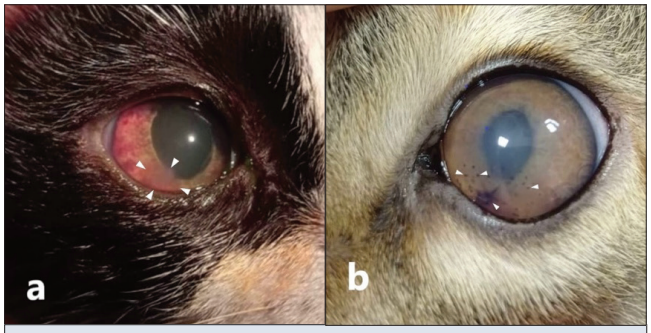

Fig 3. a- Uveitis in the right eye of a 3-year-old mixed breed female cat. Severe hyperemia was noted in the iris and aqueous flare *(arrow heads)* in the anterior chamber. The cat's FCoV antibody level was S1, with an A/G ratio of 0.21; **b-** Keratic precipitates *(arrow heads)* in a 1-year-old male tabby cat with uveitis. The antibody level was S1, and the A/G ratio was 0.39

analysis was conducted using the SPSS 21 software package.

Results

The study included a total of 94 cats, with a breakdown as follows: 32 mixed breeds, 32 tabby cats, 13 British Shorthairs, 8 Scottish Folds, 4 Tuxedos, 2 Van cats, 2 Persians, and 1 Russian Blue cat. Among these, 43 were male, and 51 were female. The age range varied from 2 months to 12 years, with 71% of the animals being below 1 year of age.

The results indicated a higher prevalence of acute eye symptoms (62 cases) compared to chronic symptoms (32 cases). Unilateral eye symptoms were found in 39 cats, while bilateral symptoms were present in 55 cats. During

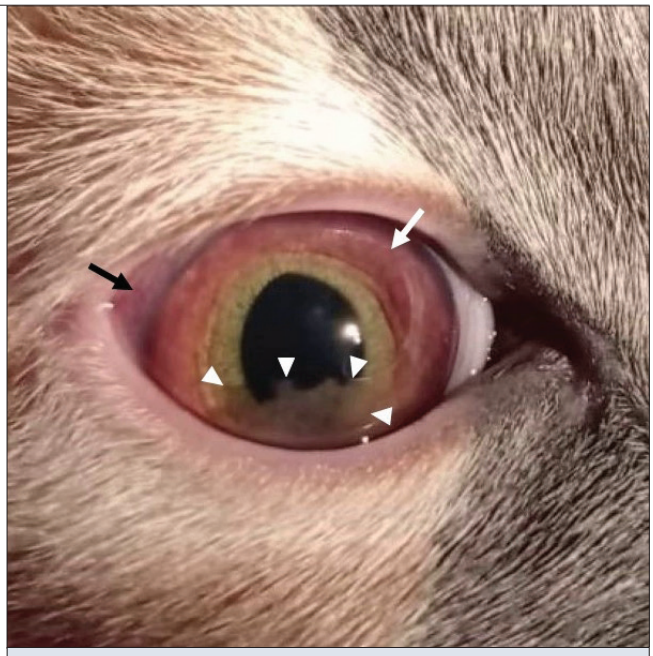

Fig 4. Granulomatous uveitis. A 4-month-old female mixed-breed cat exhibited severe inflammation in the right eye, affecting the iris extensively up to the vicinity of the pupil *(white arrow)*. Conjunctival hyperemia associated with corneal flush was observed *(black arrow)*. Concurrently, a hazy aqueous flare was notable in the ventral aspect of the anterior chamber *(arrow heads)*. This cat presented with an antibody titer of S3, an A/G ratio of 0.43, and severe lymphopenia

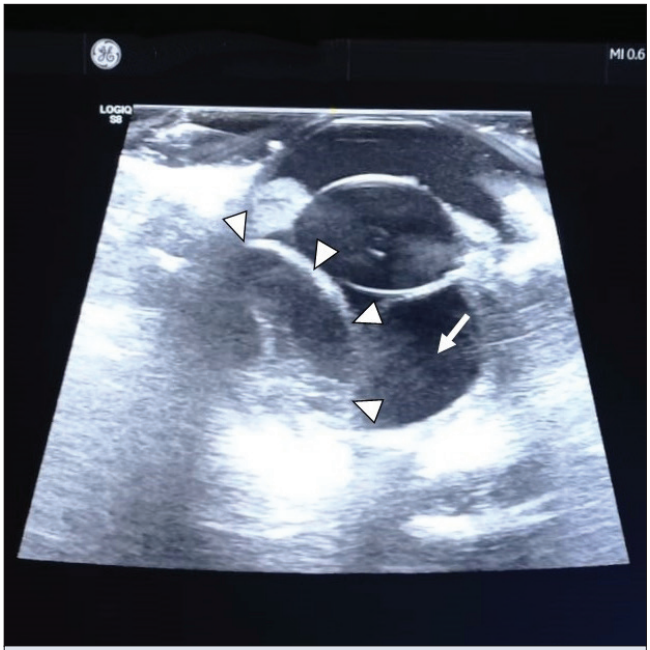

Fig 5. Ultrasonographic image of retinal detachment *(arrow heads)* and vitreal degeneration *(arrow)* in a 3-year-old male mixed-breed cat with uveitis and an FCoV titer of S5

the clinical examination, hyperemia of the iris emerged as the most frequently observed ocular manifestation, noted in 79 out of 94 cases *(Fig. 1)*. Additionally, 49 out of the 94 evaluated cats exhibited the presence of aqueous flare, while 30 cats presented keratic precipitates, both of which were common ocular findings *(Fig. 2, Fig. 3)*.

Table 2. *Comparison of FCoV scores according to blood parameters in cats. Normal reference ranges provided with Mindray BC5000 Hematology Analyzer and Randox Monaco*

Parameters	Reference Interval	FCoV Antibody Titers	Animals Examined (n)	Mean ± SEM	Median (Min-Max)	P-value
Total Protein (g/dL)	6.0-7.9	S1	6	11.92±0.26	11.79 (11.27-12.97) a	0.003
		S2	4	10.07±0.12	10.05 (9.85-10.35) b	
		S3	15	8.64± 0.23	8.78 (6.39-10.62) b	
		S4	17	9.03±0.38	8.76 (5.62-12.41) b	
		S5	21	8.96±0.32	8.92 (6.86-11.56) b	
		S6	31	8.95±0.26	8.72 (6.62-12.74) b	
Albumin (A) (g/dL)	2.8-3.9	S1	6	2.68±0.12	2.6 (2.33-3.2) c	<0.001
		S2	4	3.95±0.27	3.93 (3.4-4.56) a	
		S3	15	3.35±0.12	3.5 (2.4-3.85) ab	
		S4	17	3.52±0.13	3.6 (2.1-4.25) ab	
		S5	21	3.19±0.13	3.19 (2.2-4.68) bc	
		S6	31	2.96±0.08	3 (2.1-3.8) c	
Globulin (G) (g/dL)	2.6-5.1	S1	6	9.24±0.37	9.23 (8.18-10.64) a	0.002
		S2	4	6.12±0.27	6.03 (5.62-6.81) b	
		S3	15	5.29±0.22	5.07 (3.99-7.42) b	
		S4	17	5.51±0.29	5.16 (3.52-8.51) b	
		S5	21	5.77±0.32	5.34 (4.16-9.18) b	
		S6	31	5.99±0.28	6.03 (3.93-10.64) b	
A/G	-	S1	6	0.29±0.03	0.28 (0.21-0.39) c	<0.001
		S2	4	0.65±0.07	0.66 (0.49-0.78) a	
		S3	15	0.64±0.03	0.66 (0.37-0.79) ab	
		S4	17	0.65±0.02	0.66 (0.45-0.79) a	
		S5	21	0.56±0.04	0.62 (0.17-0.78) ab	
		S6	31	0.51±0.03	0.52 (0.08-0.78) b	
White blood cell count (WBC) (10^9/L)	5.5-19.5	S1	6	11.08±2.91	9.5 (4.2-24.6)	0.269
		S2	4	15.55±6.08	13.9 (2.5-31.9)	
		S3	15	8.28±1.34	6.3 (2.1-22)	
		S4	17	7.86±1.32	6.5 (1.1-22.7)	
		S5	21	10.16±1.37	8.3 (2.8-27.85)	
		S6	31	10.09±0.92	8.6 (1.8-29.1)	
Lymphocytes (10^9/L)	1.5-7.0	S1	6	1.5±0.67	0.6 (0.2-4)	0.351
		S2	4	1.6±0.37	1.9 (0.5-2.1)	
		S3	15	1.88±0.34	1.76 (0.3-4.8)	
		S4	17	2.29±0.46	1.4 (0.6-7.56)	
		S5	21	2.73±0.43	2 (1-8.3)	
		S6	31	2.03±0.26	1.4 (0.2-4.7)	
Monocytes (10^9/L)	0.2-0.9	S1	6	1.05±0.51	0.8 (0.1-3.5)	0.831
		S2	4	1.13±0.32	1 (0.5-2)	
		S3	15	0.97±0.23	0.6 (0.1-3.3)	
		S4	17	0.79±0.12	0.7 (0.1-2)	
		S5	21	0.71±0.1	0.6 (0.17-2)	
		S6	31	0.78±0.1	0.7 (0-2.69)	

Table 2. *Comparison of FCoV scores according to blood parameters in cats. Normal reference ranges provided with Mindray BC5000 Hematology Analyzer and Randox Monaco (continued)*

Parameters	Reference Interval	FCoV Antibody Titers	Animals Examined (n)	Mean ± SEM	Median (Min-Max)	P-value
Neutrophils (10^9/L)	2.8-13.0	S1	6	6.55±2.04	5.25 (2.6-16.4)	0.448
		S2	4	12.03±5.46	9.7 (1.4-27.3)	
		S3	15	5.11±0.95	4.3 (1.06-14.3)	
		S4	17	5.03±1.1	4 (0.3-19.6)	
		S5	21	6.31±1.19	5.04 (1-23.73)	
		S6	31	6.15±0.67	5.6 (0.39-16.3)	
Eosinophils (10^9/L)	0-0.8	S1	6	0.52±0.09	0.5 (0.2-0.8)	0.075
		S2	4	0.8±0.29	0.8 (0.1-1.5)	
		S3	15	0.32±0.06	0.3 (0-0.73)	
		S4	17	0.26±0.08	0.1 (0-1.31)	
		S5	21	0.36±0.06	0.3 (0-1.2)	
		S6	31	0.39±0.06	0.3 (0-1.5)	
Red Cell Distribution Width (RDW) (%)	10.6-14.3	S1	6	15.7±0.9	15.1 (13.9-20.2) b	0.004
		S2	4	16.1±0.6	16 (14.6-17.6) ab	
		S3	15	16.3±0.4	15.9 (14.3-18.4) ab	
		S4	17	16±1.1	14.8 (11.9-32.6) b	
		S5	21	19.9±2	16.7 (13.9-53) ab	
		S6	31	18.8±0.8	17.5 (15-32.7) a	

a,b,c: *Different letters in the same column indicate statistically significant difference (P<0.05)*

Granulomatous uveitis was identified in 8 cases, while the remaining cases were characterized as non-granulomatous *(Fig. 4)*. Among the evaluated eyes, 63 were diagnosed with anterior uveitis, 29 with panuveitis, and 2 with posterior uveitis. Fluorescein staining was negative, and pupillary light reflex was slow to absent in all eyes. Ultrasonographic examination revealed vitreous degeneration as the most typical finding in the panuveitis cases *(Fig. 5)*. Upon evaluating the ocular findings, it was observed that uveitis, which presented with varying types and symptoms, showed no correlation with blood parameters and antibody titers in the animals.

Evaluation of the antibody titer levels revealed that among the 94 cats, 6 were classified as S1, 4 as S2, 15 as S3, 17 as S4, 21 as S5, and 31 as S6. When complete blood count parameters were examined, no significant changes were observed in neutrophil, eosinophil, lymphocyte and monocyte. However, it was noted that the mean and median lymphocyte counts in animals with the S1 antibody titer were lower than those with other titers. A positive, statistically significant relationship was found between RDW and the antibody score (P<0.05). As the antibody titer level increased, the most pronounced elevation in RDW value was observed in cats with S6 level antibodies. RDW was found to be above the normal range at all antibody titer levels *(Table 2)*.

A negative, weak but statistically significant correlation was observed between total protein and antibody score. Accordingly, the decrease in total protein was notable as antibody intensity increased. A negative, significant correlation was also detected between albumin value and antibody score. Total protein and globulin values of animals with S1 antibodies, and the albumin values of cats with S2 antibodies, were significantly higher than those in other groups. While the evaluations revealed the presence of hyperproteinemia and hyperglobulinemia, albumin levels were found to be within normal range at all antibody titer levels *(Table 2)*.

The difference in A/G ratios between antibody titers was statistically significant (P<0.05). The highest A/G values were observed in animals with S2 and S4 antibody scores. The lowest mean A/G ratio (below 0.4) occurred at the lowest antibody titer level, S1. This value was below 0.7 in other titers. A negative, weak, and statistically insignificant correlation was found between the A/G ratio and antibody score (P>0.05) *(Table 2)*.

Discussion

FECV establishes itself and manifests symptoms in the intestines post-infection. However, if it transforms into FIPV, the virus initiates replication in monocytes and macrophages, activating these cells and inducing inflammatory reactions. In cats with FIPV, infected macrophages transport the virus to targets such as

the kidney, pleura, uvea, and the nervous system. The role of blood monocytes, precursors of macrophages, in FIP pathogenesis remains unclear. Nevertheless, it is noteworthy that monocyte-associated viremia is also observed in healthy cats infected with FCoV [23]. The number of macrophages in FIP-related ocular inflammations is relatively low compared to other tissues, considering the eye's immune system. Studies have demonstrated the presence of viral antigen-infected macrophages in the inflammatory infiltrate around the choroidal vessels, the connective tissue of the third eyelid, and the conjunctiva following infection. Furthermore, B cells and plasma cells indicate an effective humoral response in ocular inflammation [24].

The occurrence of these reactions results in the disruption of the blood-aqueous humor barrier in the eye. With the breakdown of the barrier, the virus reacts in the vascular-rich layer of the eye. Destruction in the region causes ocular symptoms such as fibrinous exudation accumulation in the anterior chamber, pyogranulomatous uveitis, dilation of perivascular vessels, exudative retinal detachment, retinal vasculitis, and optic neuritis [24]. In the present study, the most frequently observed finding in cases was iris hyperemia, which occurred in 83% of cats (12% of which had a granulomatous character), followed by an aqueous flare in 51% of cats. Additionally, keratic precipitates were observed in 29% of cats, while retinal detachment was observed in 12%.

Diagnosing FIP in cats without effusion is extremely challenging during the antemortem period [2]. To the extent that, even in aqueous humor samples taken from cats with FIP-related uveitis, no FCoV RNA was detected, including a case with a confirmed FIP diagnosis [25]. Currently, the gold standard for diagnosing FIP is the detection of intracellular FCoV antigen in macrophages in biopsy or necropsy samples [12,26]. Additionally, the presence of intracellular FCoV might be detected in samples taken from skin lesions in some cats with dermatological problems [27]. Merely the presence of antibodies is not indicative of FIP, and conversely, their absence doesn't necessarily negate it [28]. Although serological tests are insufficient for the diagnosis of FIP, in this study, the use of serological tests as a preliminary diagnostic tool for low, possible, or probable FIP was provided by looking for FCoV antibodies in all cats with uveitis. Thus, while animals with low antibody titers were approached with suspicion of FIP, further diagnostic support was obtained considering the possible presence of FIP in cats with high titers. Another notable finding pertains to the correlation between cats presenting with varying symptoms and classifications of uveitis and their antibody levels. When evaluating the symptoms of uveitis in the study cases, there was an inconsistency noted in the antibody levels. Remarkably, a cat with S6 antibody level (high positive reaction) exhibited mild symptoms of uveitis such as mild aqueous flare or iris hyperemia, while a cat with an S1 level (non-specific reaction) presented severe symptoms such as severe iris hyperemia or hyphema. It was concluded that ocular symptoms were not directly related to antibody density.

To enhance the diagnosis of FIP in cats, numerous studies have been conducted to identify various laboratory alterations. This research continues to progress unabated today. In their study, Wegg et al.[29] employed clinical symptoms in animals, elevated coronavirus titers, and high alpha-1 acid glycoprotein values for the diagnosis of FIP, subsequently confirming their diagnoses in two cases. The changes, particularly observed in protein values, can be reflected in the blood panel as hyperproteinemia, hypoalbuminemia, and hyperglobulinemia, consequently resulting in a proportional decrease in the values of albumin and globulin. One of the most crucial biochemical abnormalities in FIP is hyperglobulinemia, believed to result from non-specific immune responses [17]. Studies have shown that hyperglobulinemia has a positive correlation with virus antibody titers. In these animals, whether effusion is present or not, hyperglobulinemia is significantly elevated [20,30]. Upon examination of the present study data, a statistically significant difference was found between globulin values and FCoV antibody titers, with hyperglobulinemia present at all titer levels. However, it is noteworthy that the significant increase in globulin was observed specifically in animals with S1 antibody levels. This finding contradicts the expected positive correlation between hyperglobulinemia and increasing antibody titer levels.

Although the presence of hyperglobulinemia and hypoalbuminemia together raises suspicion about FIP, the most important biochemical abnormality was thought to be the ratio of A/G [17,18,31]. In numerous studies on FIP, a pronounced reduction in the A/G ratio is observed in the vast majority of cats. An A/G ratio of 0.5 or lower is deemed indicative of a definitive antemortem period diagnosis of FIP. Moreover, some researchers consider a value of 0.4 or lower to be diagnostic [32]. Conversely, values of 0.8 or higher suggest a low probability of FIP [13,19,33]. In the present study, A/G ratios were found to be below 0.7 across all antibody titer levels, with the differences between antibody scores being statistically significant. However, A/G ratios of 0.4 and below, which some studies consider diagnostic for antemortem FIP, were only observed at the S1 antibody titer in this study. Although a negative relationship was noted between A/G ratio and antibody titer, the highest values within this statistically insignificant relationship were found in animals with S2 and S4 scores. These results indicate that the increase in

FCoV antibody intensity did not correspond to a similar increase in A/G ratios. Indeed, the fact that the S1 titer level, which is regarded as a negative and non-specific reaction, exhibited the lowest A/G ratio supports this finding. Some studies have interpreted this as the high amount of virus binding to the antibody, which may reduce the antibody detection and even mask its presence [5,12,34].

Research indicates that FIP virus induces distinct alterations in hematological parameters. While these alterations are not yet definitive for the virus, they are continually being examined to support the diagnosis of FIP in living animals. Some researchers have emphasized the stress leukogram as a typical finding in FIP, stating that the co-existence of lymphopenia and neutrophilia is an important factor in the diagnosis [20]. When the complete blood parameters of the study were examined, no significant changes were observed in the values of neutrophils, eosinophils, lymphocytes, and monocytes. Although the mean lymphocyte count in animals with S1 antibody titer was lower than in animals with other titers, this statistically insignificant decrease was still within the normal range of leukocyte count. The fact that this decrease in lymphocyte count, like the situation in A/G level, was most pronounced at low antibody titer, suggested that the antibody level could be masked by the high amount of virus binding to the antibody. This suggests that the stress leukogram may not be a contributing factor in the diagnosis of FIP.

A notable observation in the complete blood count changes was the positive, statistically significant correlation between Red Cell Distribution Width (RDW) and antibody score. The increase in RDW was most pronounced at the S6 titer level. RDW is a simple laboratory parameter and biomarker that reflects the variation in erythrocyte size, commonly used in the differential diagnosis of anemia [35]. RDW, utilized as a marker in numerous human diseases, has gained prominence during the global COVID-19 pandemic. It was recognized as a prognostic indicator and has been the focus of multiple studies that directly correlate its elevation in blood with mortality rates [36-38]. Numerous hypotheses have attempted to elucidate the reasons for this alteration in RDW. It has been underscored that hemolytic anemia and intravascular coagulopathy lead to secondary RBC damage, or that the persistent inflammatory response induced by the virus directly damages erythrocytes by impairing iron metabolism [39]. When the study results were examined, it was observed that the RDW value increased with antibody density in cats suspected of FIP, and that the RDW was outside the normal limits at the most severe antibody titer. Contrary to the negative correlation of protein values with antibody titers, the positive correlation of RDW suggested the hypothesis in the covid-19 pandemic and led to the conclusion that the change in this parameter may have occurred as a result of indirect damage to erythrocytes by chronic inflammation induced by the virus in cats. Nevertheless, additional research is necessary to delve deeper into this phenomenon.

In conclusion, a definitive diagnostic tool for FIP in living animals remains elusive. The goal of ongoing research has been to strengthen the ability to suspect the disease using a variety of parameters. The commonly observed alterations in blood parameters are often deemed sufficient to initiate treatment when FIP is diagnosed in cats presenting with systemic and/or ocular signs, along with positive coronavirus antibody detection. However, current research continues to seek more precise markers to improve these uncertain parameters. This study evaluated the correlation between clinical, immunological, and hematological parameters of FIP in cats with uveitis. Surprisingly, the results revealed inconsistencies with many findings in the international literature that support FIP suspicion. Notably, no significant correlation was found between key indicators of dry FIP-such as the severity and types of uveitis-and antibody levels, complete blood counts, or blood protein values. Even more striking, the most pronounced changes in protein values, considered crucial prognostic factors, were observed at low antibody levels. The stress leukogram, widely used by researchers in differential diagnoses, proved to be completely ineffective, with the lowest lymphocyte counts observed in animals with S1 antibody titers. One of the most compelling findings was the RDW value. This simple, cost-effective complete blood count parameter, which has emerged as a prognostic marker in human viral and infectious diseases, showed promise as a potential indicator for FIP. Further research may confirm RDW as a reliable and affordable marker for diagnosing FIP in cats.

Declaration

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (O.O. Şenel).

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Authors' Contributions: The authors confirm a group work for interpretation and preparation of the manuscript.

References

1. Pedersen NC, Sato R, Foley JE, Poland AM: Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus. *J Feline Med Surg,* 6 (2): 83-88, 2004. DOI: 10.1016/j.jfms.2003.08.008

2. Pedersen NC: A review of feline infectious peritonitis virus infection: 1963-2008. *J Feline Med Surg,* 11 (4): 225-258, 2009. DOI: 10.1016/j.jfms.2008.09.008

3. Pedersen NC: An update on feline infectious peritonitis: Virology and immunopathogenesis. *Vet J,* 201 (2): 123-132, 2014. DOI: 10.1016/j.tvjl.2014.04.017

4. Sainkaplan S, Irdem DI, Ergin I: Assessment of ocular lesions in a Persian cat concurrently infected with *Chlamydia felis*, herpesvirus and coronavirus. *Kafkas Univ Vet Fak Derg,* 28 (6): 781-784, 2022. DOI: 10.9775/kvfd.2022.28026

5. Kipar A, Meli ML: Feline infectious peritonitis: Still an enigma? *Vet Pathol,* 51 (2): 505-526, 2014. DOI: 10.1177/0300985814522077

6. Tekes G, Thiel HJ: Feline coronaviruses: Pathogenesis of feline infectious peritonitis. *Adv Virus Res,* 96, 193-218, 2016. DOI: 10.1016/bs.aivir.2016.08.002

7. Ün H: Coronaviridae virus family: An overall assessment. *J Adv VetBio Sci Tech,* 5 (1): 1-12, 2020.

8. Gunn-Moore DA, Gruffydd-Jones TJ, Harbour DA: Detection of feline coronaviruses by culture and reverse transcriptase-polymerase chain reaction of blood samples from healthy cats and cats with clinical feline infectious peritonitis. *Vet Microbiol,* 62 (3): 193- 205, 1998. DOI: 10.1016/s0378-1135(98)00210-7

9. Vennema H, Poland A, Foley J, Pedersen NC: Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronavirus. *Virology,* 243 (1): 150-157, 1998. DOI: 10.1006/viro.1998.9045

10. Lin LN, Su BL, Huang HP, Lee JJ, Hsieh MW, Chueh LL: Field strain feline coronaviruses with small deletions in ORF7b associated with both enteric infection and feline infectious peritonitis. *J Feline Med Surg,* 11 (6): 413-419, 2008. DOI: 10.1016/j.jfms.2008.09.004

11. Sangl L, Matiasek K, Felten S, Gründl S, Bergmann M, Balzer HJ, Pantchev N, Leutenegger CM, Hartmann K: Detection of feline coronavirus mutations in paraffin-embedded tissues in cats with feline infectious peritonitis and controls. *J Feline Med Surg,* 21 (2): 133-142, 2019. DOI: 10.1177/1098612X18762883

12. Hartmann K: Feline infectious peritonitis. *Vet Clin North Am Small Anim Pract,* 35 (1): 39-79, 2005. DOI: 10.1016/j.cvsm.2004.10.011

13. Tsai HY, Chueh LL, Lin CN, Su BL: Clinicopathological findings and disease staging of feline infectious peritonitis: 51 cases from 2003 to 2009 in Taiwan. *J Feline Med Surg,* 13 (2): 74-80, 2011. DOI: 10.1016/j.jfms.2010.09.014

14. Ziółkowska N, Pazdzior-Czapula K, Lewczuk B, Mikulska-Skupien E, Przybylska-Gornowicz B, Kwiecinska K, Ziołkowski H: Feline infectious peritonitis: Immunohistochemical features of ocular inflammation and the distribution of viral antigens in structures of the eye. *Vet Pathol*, 54 (6): 933-944, 2017. DOI: 10.1177/0300985817728557

15. Baydar E, Eröksüz Y, Timurkan MÖ, Eröksüz H: Feline infectious peritonitis with distinct ocular involvement in a cat in Turkey. *Kafkas Univ Vet Fak Derg,* 20 (6): 961-965, 2014. DOI: 10.9775/kvfd.2014.11195

16. Carossino M, Del Piero F, Lee J, Needle DB, Levine JM, Riis RR, Maes R, Wise AG, Mullaney K, Ferracone J, Langohr IM: Relationship between uveal inflammation and viral detection in 30 cats with feline infectious peritonitis. *Pathogens,* 11 (8):883, 2022. DOI: 10.3390/pathogens11080883

17. Felten S, Hartmann K: Diagnosis of feline infectious peritonitis: A review of the current literature. *Viruses,* 11 (11):1068, 2019. DOI: 10.3390/v11111068

18. Hartmann K, Binder C, Hirschberger J, Cole D, Reinacher M, Schroo S, Frost J, Egberink H, Lutz H, Hermanns W: Comparison of different tests to diagnose feline infectious peritonitis. *J Vet Intern Med,* 17 (6): 781-790, 2003. DOI: 10.1111/j.1939-1676.2003.tb02515.x

19. Nururrozi A, Ramandani D, Wasissa M, Artono Y, Indarjulianto S: Serum biochemistry profiles in confirmed effusive feline ınfectious peritonitis Cats. *Adv Anim Vet Sci*, 10 (1): 126-130, 2022. DOI: 10.17582/journal.aavs/2022/10.1.126.130

20. Paltrinieri S, Comazzi S, Spagnolo V, Giordano A: Laboratory changes consistent with feline infectious peritonitis in cats from multicat environments. *J Vet Med A Physiol Pathol Clin Med,* 49 (10): 503-510, 2022. DOI: 10.1046/j.1439-0442.2002.00494.x

21. Sykes JE: Feline coronavirus infection. *Canine Feline Infect Dis,* 2014, 195-208, 2013. DOI: 10.1016/B978-1-4377-0795-3.00020-X

22. Tekelioglu BK, Berriatua E, Turan N, Helps CR, Kocak M, Yilmaz H: A retrospective clinical and epidemiological study on feline coronavirus (FCoV) in cats in Istanbul, Turkey. *Prev Vet Med,* 119 (1-2): 41-47, 2015. DOI: 10.1016/j.prevetmed.2015.01.017

23. Rottier PJM, Nakamura K, Schellen P, Volders H, Haijema BJ: Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. *J Virol,* 79 (22): 14122-14130, 2005. DOI: 10.1128/JVI.79.22.14122-14130.2005

24. Peiffer RLJ: Fundamentals of veterinary ophthalmic pathology. **In**, Gelatt KN (Ed): Veterinary Ophthalmology. 2nd ed., 335-425, Lippincott, Williams & Wilkins, Philadelphia, 1999.

25. Wiggans KT, Wernau W, Lappin MR, Thomasy SM, Maggs DJ: Diagnostic utility of aqueocentesis and aqueous humor analysis in dogs and cats with anterior uveitis. *Vet Ophthalmol,* 17 (3): 212-220, 2014. DOI: 10.1111/vop.12075

26. Poland AM, Vennema H, Foley JE, Pedersen NC: Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. *J Clin Microbiol,* 34 (12): 3180-3184, 1996. DOI: 10.1128/jcm.34.12.3180-3184.1996

27. Bauer BS, Kerr ME, Sandmeyer LS, Grahn BH: Positive immunostaining for feline infectious peritonitis (FIP) in a Sphinx cat with cutaneous lesions and bilateral panuveitis. *Vet Ophthalmol,* 16 (1): 160-163, 2013. DOI: 10.1111/vop.12044

28. Sharif S, Arshad SS, Hair-Bejo M, Omar AR, Zeenathul NA, Alazawy A: Diagnostic methods for feline coronavirus: A review. *Vet Med Int,* 2010:809480, 2010.

29. Wegg ML, Jeanes EC, Pollard D, Fleming L, Dawson C: A multicenter retrospective study into endogenous causes of uveitis in cats in the United Kingdom: Ninety two cases. *Vet Ophthalmol,* 24 (6): 591-598, 2021. DOI: 10.1111/vop.12898

30. Riemer F, Kuehner KA, Ritz S, Sauter Louis C, Hartmann K: Clinical and laboratory features of cats with feline infectious peritonitis - A retrospective study of 231 confirmed cases (2000-2010). *J Feline Med Surg,* 18 (4): 348-356, 2016. DOI: 10.1177/1098612X15586209

31. Addie D, Belak S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, Hosie MJ, Lloret A, Lutz H, Marsilio F, Pennisi MG, Radford AD, Thiry E, Truyen U, Horzinek MC: Feline infectious peritonitis: ABCD guidelines on prevention and management. *J Feline Med Surg,* 11 (7): 594-604, 2009. DOI: 10.1016/j.jfms.2009.05.008

32. Yin Y, Li T, Wang C, Liu X, Ouyang H, Ji W, Liu J, Liao X, Li J, Hu C: A retrospective study of clinical and laboratory features and treatment on cats highly suspected of feline infectious peritonitis in Wuhan, China. *Sci Rep,* 11 (1):5208, 2021. DOI: 10.1038/s41598-021-84754-0

33. Jeffery U, Deitz K Hotetter S: Positive predictive value of albumin: globulin ratio for feline infectious peritonitis in a mid-western referral hospital population. *J Feline Med Surg,* 14 (12): 903-905, 2012. DOI: 10.1177/1098612X12454862

34. Addie DD, Poder S, Burr P, Decaro N, Graham E, Hofmann-Lehmann R, Jarrett O, McDonald M, Meli ML: Utility of feline coronavirus antibody tests. *J Feline Med Surg,* 17 (2): 152-162, 2015. DOI: 10.1177/1098612X14538873

35. Salvagno GL, Sanchis-Gomar F, Picanza A, Lippi G: Red blood cell distribution width: A simple parameter with multiple clinical applications. *Crit Rev Clin Lab Sci,* 52 (2): 86-105, 2015. DOI: 10.3109/10408363.2014.992064

36. Foy BH, Carlson JCT, Reinertsen E, Valls RPI, Lopez RP, Tost EP,

Mow C, Westover MB, Aguirre AD, Higgins JM: Association of red blood cell distribution width with mortality risk in hospitalized adults with SARS-CoV-2 infection. *JAMA Netw Open,* 3 (9):e2022058, 2020. DOI: 10.1001/jamanetworkopen.2020.22058

37. Lorente L, Martín MM, Argueso M, Solé-Violán J, Perez A, Ramos JAMY, Ramos-Gómez L, López S, Franco A, González-Rivero AF, Martín M, Gonzalez V, Alcoba-Flórez J, Meli ML, Burr P, Decaro N, Graham E, Jarrett O, Lutz H, McDonald M, Addie DD: Association between red blood cell distribution width and mortality of COVID-19 patients. *Anaesth Crit Care Pain Med,* 40 (1):100777, 2021. DOI: 10.1016/j.accpm.2020.10.013

38. Owoicho O, Tapela K, Olwal CO, Djomkam Zune AL, Nganyewo NN, Quaye O: Red blood cell distribution width as a prognostic biomarker for viral infections: Prospects and challenges. *Biomark Med,* 16 (1): 41-50, 2022. DOI: 10.2217/bmm-2021-0364

39. Henry BM, Benoit JL, Benoit S, Pulvino C, Berger BA, Olivera MHS, Crutchfield CA, Lippi G: Red blood cell distribution width (RDW) predicts COVID-19 severity: A prospective, observational study from the Cincinnati SARS-CoV-2 emergency department cohort. *Diagnostics (Basel),* 10 (9): 618 (1-9), 2020. DOI: 10.3390/diagnostics10090618

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Research Article

lncRNA NONRATT021477.2 Interference Aggravates H_2O_2-Induced Oxidative Stress in BRL Cells

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Abstract

Long non-coding RNA (lncRNA) plays important biological regulatory functions at different levels. lncRNA NONRATT021477.2 (lncRNA77.2) was identified as a key gene under cold stress conditions in rats. From the literature, lncRNA77.2 may have important roles during antioxidant, but this conjecture requires investigation. To address this knowledge gap, we investigated the effects of lncRNA77.2 interference on H_2O_2-induced oxidative stress in rat liver cells (Buffalo rat liver, BRL).In the current study, H_2O_2 treatment simulated BRL cell oxidative stress. H_2O_2 treatment led to a significant increase in oxidative stress levels in BRL cells, whereas the gene expression levels of antioxidase and lncRNA 77.2 and cell viability were significantly reduced. Additionally, the expression of Nrf2 and Keap1 proteins decreased significantly compared to the control group. BRL cells were transfected with antisense oligonucleotides (ASO) or a negative control ASO (ASO-NC) and then treated with H_2O_2. The results showed lncRNA77.2 interference increased oxidative stress levels, reduced gene expression of antioxidant enzymes, Nrf2 and Keap1 expression levels. The results indicated that lncRNA77.2 interference aggravated H_2O_2-induced oxidative stress in BRL cells, which suggested that lncRNA77.2 is an antioxidant factor that plays an important role in the regulation of oxidative stress in BRL cells.

Keywords: BRL cells, lncRNA, Oxidative stress

Introduction

Long non-coding RNA (lncRNA) mediated gene regulation mechanisms are diverse, which may be attributed to their ability to interact with DNA, RNA, or proteins [1]. Despite the large proportion of lncRNAs in complex transcription processes, their functions remain poorly understood. LncRNA has a non-random small open reading frame, which can interact with ribosomes. LncRNA is involved in the regulation of mRNA prophase splicing, RNA editing, regulation of mRNA stability, translation activation, and miRNA sponge [2]. Additionally, lncRNAs play key regulatory roles in biological processes, including cell differentiation, proliferation, apoptosis, immune response, and in vivo homeostasis by forming RNA-DNA triplets and targeting specific DNA sequences [3]. Recent studies have found that certain lncRNAs can promote protein-protein interactions, such as epigenetic reprograming and signaling, to influence cancer occurrence [4]. LncRNAs can also interact with RNA or DNA, acting as the skeleton of subcellular domains or complexes to regulate protein activity. Abnormalities in lncRNAs are associated with oxidative stress in many human diseases, and there is a strong association between several human diseases and oxidative stress. Recent studies have shown that lncRNA plays an important role in the cellular response to oxidative stress by responding to different genes that regulate the expression of proteins [5].

Oxidative stress refers to an imbalance in the oxidative antioxidant system due to the accumulation of free radicals after internal and external environment stimulation [6]. Generally, the increased production of highly reactive oxygen species (ROS) and highly reactive nitrogen radicals (RNS) inhibits the function of normal substances in cells and destroys the structure of normal cells, leading to a decrease or loss of the activity of various enzymes, as well as damage to the cell structure. This imbalance can cause severe damage to some biomolecules and organs and may have systemic effects. Oxidative stress plays an important role in the pathogenesis of several diseases, including Alzheimer's disease and cancer. A previous study has demonstrated that long downregulation of strand-noncoding RNA SNHG1 can improve oxidative

stress and inflammation in Parkinson's disease models by inhibiting the miR-125b-5p/MAPK 1 axis [4]. Sunwoo et al.[3] evaluated the differential expression profile of lncRNA in Huntington's disease and demonstrated that transfection of NEAT 1 short isoforms into H_2O_2-treated Neuro-2a cells exhibiting oxidative stress caused cell death, demonstrating a neuroprotective role for lncRNA NEAT1 in the pathogenesis of Huntington's disease. Moreover, Kong et al.[7] revealed that the lncRNA LEGLTBC can function as a ceRNA to regulate SIRT 1 in glycolipid toxicity-induced oxidative stress and apoptosis in INS-1 β cells. Recent reports have also revealed that lncRNA plays an important role in response to oxidative stress, which implies that lncRNA, as a key molecule, may participate in oxidative stress [8]. The low conservation and tissue-specific features of most lncRNAs suggest that long non-coding RNAs can serve as specific biomarkers for oxidative stress-related diseases [9].

After preliminary laboratory identification, we selected the newly discovered *lncRNA NONRATT021477.2*, with a length of 746 bp. The subcellular localization of lncRNA77.2 in BRL cells was detected by FISH technology, and it was found to be mainly expressed in the nucleus of BRL cells [10]. Therefore, we interfered with the expression of *lncRNA77.2* to initially explore its regulatory role in the oxidative stress response in rat liver cells and build a foundation for the study of the mechanism of *lncRNA77.2* in regulating oxidative stress in rat hepatocytes.

Materials and Methods

Cell Culture and H_2O_2 Treatment

Buffalo rat liver (BRL) cell from Cell Bank, Chinese Academy of Sciences (Shanghai, China) were maintained in DMEM medium(Gibco, Carlsbad, CA, USA)with 10% fetal bovine serum (FBS, Gibco) and the optimal H_2O_2 treatment concentration using preliminary laboratory screening was 200 μ mol/L at a concentration of 200 μmol/L in a moist CO_2 incubator at 37°C under 5% CO_2 [11].

To construct an *in vitro* oxidative stress cell model and screen the optimal time point for hydrogen peroxide treatment, we stimulated BRL cells for 2h, 4h, and 6h respectively at 37°C using hydrogen peroxide at 200 μmol/L concentration.

Antisense Oligonucleotide(ASO) Interfere with Expression

The antisense oligonucleotide (ASO) of *lncRNA77.2* was designed and synthesized according to the full-length sequence of *lncRNA77.2*, whose sequence was: ASO: 5′-TCTCCTCTCTCAAATATCTG-3′, and a negative control ASO-NC was chemically synthesized. Antisense nucleotide sequences were designed and synthesized by Raybo Bio. The cells were coated on a 24-well plate, and when the cells reached 50% growth, BRL cells were transfected according to the instructions of the ASO kit (Ribo, Guangzhou, China) to construct an *lncRNA77.2* interference expression model. The experiments were divided into negative control group (NC group), H_2O_2 group, ASO interference expression group (H_2O_2+ ASO group), ASO no-load negative control group (H_2O_2 + ASO-NC group), and samples from each group were collected to detect oxidative stress related indicators.

Cell Viability Assays

CCK-8 assay was proceeded to detect cell viability. In brief, cells (100 μL) were seeded into 96-well plates and cultivated in a 5% CO_2 incubator for 48 h at 37°C. BRL cells were stimulated with H_2O_2 for 2 h, 4 h, and 6 h at 37°C, then CCK-8 reagent (Beyotime, China) was added to each well and incubated for 3 h. Finally, the absorbance at 450 nm was determined by a microplate reader (MR-96A, China Mindrary Mindray Medical Company, China).

Malondialdehyde (MDA) detection, Reactive Oxygen Species (ROS) Activity and Superoxide Dismutase (SOD) Activity Assay

The levels of MDA release, ROS and SOD were determined by the MDA Assay Kit (Beyotime, China), ROS Assay Kit (Sigma, Japan) and SOD Assay Ki (Beyotime, China), respectively, referring to the directions of manufacturers. The absorbance of samples at 450 nm, 532 nm and 530 nm was tested to reflect SOD, MDA and ROS activity via a microplate reader, respectively.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted fromBRL cells and cDNA synthesis and qRT fluorescence PCR performed to quantify mRNA expression. β-actin was used to normalize gene

Table 1. *Primer sequences*

Gene	Primer Sequences	
	Forward Primer	**Reverse Prime**
GAPDH	CATCACCATCTTCCAGGAGCG	GAGGGGCCATCCACAGTCTTC
SOD	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT
CAT	CCGACCGTCCGTAAATGCTA	GCTTTTCAGATAGGCTCTTCATGTAA
GSH-Px	GATTCGTTCCAAACTTCCTGCTA	GCTCCCAGAACAGCCTGTTG

expression data. Relative quantification was calculated using 2-ΔΔCT comparison threshold formula. Primer sequences *SOD*, *CAT*, *GSH-Px* and *GAPDH* are listed in *Table 1*.

Western Blot

After cell lysis, the total protein concentration was determined according to the method described in the BCA kit (BioTeke, Beijing, China). Proteins were separated on 12% SDS-PAGE gels and then transferred to a polyvinylidene difluoride membrane. After sealing with 5% skim milk, the membranes were incubated with murine anti-Nrf 2, Keap 1, Bax, and Bcl-2 overnight at 4ºC. After 24 h, the PVDF membrane was washed with TBST and subsequently incubated with TB Green (cat: RR020A, fluorescence-conjugated secondary antibody, TAKARA) for 2 h. Finally, band intensity was quantified using Image J software.

Statistical Analysis

SPSS software was performed to analyze the experimental data, the data were displayed as mean ± standard deviation (SD). The GraphPad Prism 8.0 system was used to perform one-way ANOVA analysis. The t-test was used to reveal the pairwise differences between the samples, and the P less than 0.05 was considered statistically significant.

Results

In order to probe the impact of H_2O_2 on BRL cells, we first explored the level of ROS production of BRL cells. The level of intracellular ROS production at different times of H_2O_2 action (2 h, 4 h, 6 h) were detected, and the results showed that the level of ROS in BRL cells gradually increased with the duration of H_2O_2 stimulation in a time-dependent manner *(Fig. 1-A,B*, P<0.01). Meanwhile, similar results were obtained for the MDA level *(Fig. 1-C*, P<0.01). Moreover, the viability of BRL cells showed a gradual trend with the extension of H_2O_2 stimulation in a time-dependent manner *(Fig. 1-D*, P<0.01). TThe above results showed that ROS and MDA, the key indicators of oxidative stress, increased with the increase of H_2O_2 stimulation time, whereas cell viability decreased (P<0.01), indicating a time-dependent aggravation of oxidative stress.

The results of qRT–PCR experiments showed that the indicators of intracellular antioxidants in BRL antioxidant enzymes *(Fig. 2-A,B,C)* manifested a significant decline trend with the increase in H_2O_2 stimulation time (P<0.05), reaching a very significant decrease at 6 h. *lncRNA77.2* expression showed the same trend *(Fig. 2-D*, P<0.01). The above data indicated that the antioxidant capacity of BRL cells decreased with the prolongation of H_2O_2 stimulation time.

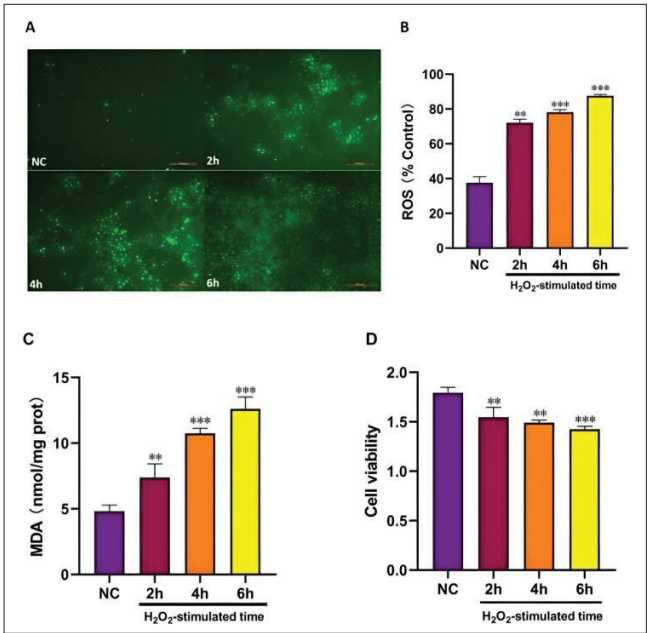

Fig 1. Effects of H_2O_2 action on ROS, MDA and cell proliferation at different time points. **A, B**- ROS analysis of BRL cells at different treatment times for NC, 2 h, 4 h, and 6 h; **C**- MDA levels in BRL; **D**- The effects of hydrogen peroxide action on BRL cell proliferation at different time points. Data are expressed as the ± standard error of the mean (n=3). * Compared with the NC group, * P<0.05, ** P<0.01

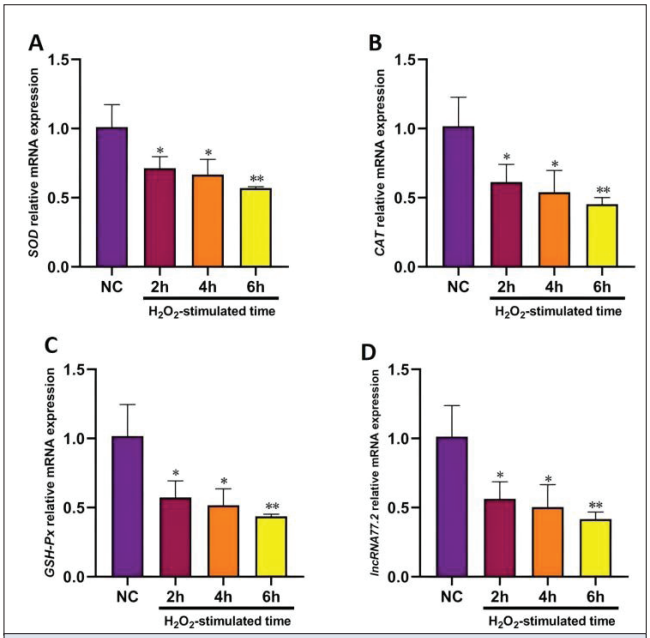

Fig 2. Effects of hydrogen peroxide action at different time points on intracellular antioxidant indexes in BRL. **A**- *SOD / GAPDH;* **B**- *CAT/ GAPDH;* **C**- *GSH-Px*/*GAPDH;* **D**- *lncRNA77.2*. Data are expressed as the ± standard error of the mean (n=3). * Compared with the NC group, * P<0.05, ** P<0.01

Moreover, the expression of Nrf2 and Keap1 in BRL was detected by western blot and found to be significantly decreased with increasing time of H_2O_2 stimulation starting from 2 h *(Fig. 3-A,C*, P<0.05), reaching the lowest value at 6 h (P<0.001), while the apoptosis-related proteins

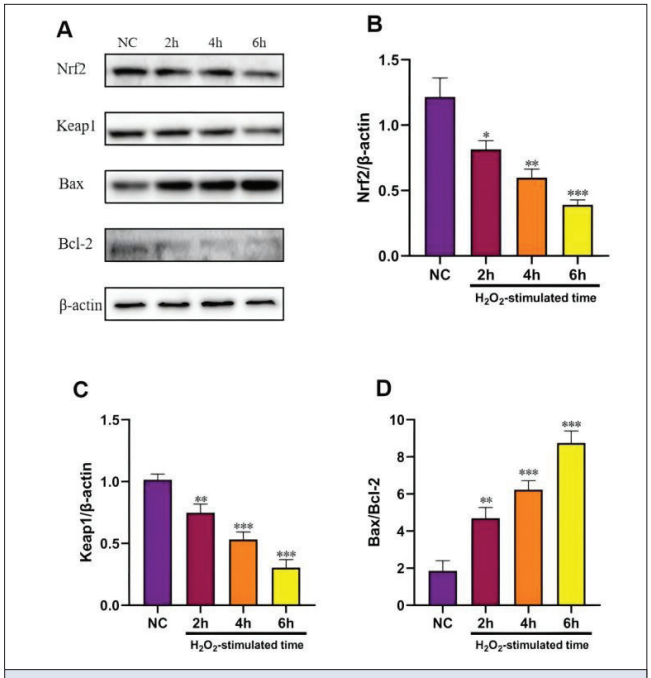

Fig 3. Effects of hydrogen peroxide on oxidation and apoptosis in BRL cells. **A-** Protein expression related to oxidation and apoptosis by Western blot; **B-** Nrf 2 expression analysis; **C-** Keap1 expression analysis; **D-** The analysis of Bax/Bcl-2 expression. Data are expressed as the mean ± standard error (n=3). * Compared with the NC group, * P<0.05, ** P<0.01, *** P<0.001

showed the opposite trend in our study *(Fig. 3-D)*. The above results indicated that oxidative stress decreased the antioxidant capacity and enhanced the apoptotic level in BRL cells. To further explore how *lncRNA77.2* functions as an antioxidant factor during oxidative stress in rat hepatocytes, the time point of 6 h, with the strongest oxidative stress, was selected for subsequent experimental studies.

To explore whether lncRNA77.2 participated in the regulation by H_2O_2-stimulated cytotoxicity, the effect of lncRNA77.2 knockdown was examined in BRL cells. Compared to the H_2O_2+ASO-NC group, the *lncRNA77.2* mRNA level was significantly reduced in the H_2O_2+ASO group, indicating that the *lncRNA 77.2* interference expression was successfully constructed *(Fig. 4-A*, P<0.01). The results of the CCK-8 assay showed that cell viability was significantly reduced in the H_2O_2+ASO group compared to the H_2O_2+ASO-NC group *(Fig. 4-B*, P<0.01). Additionally, the qRT-PCR results showed that the ROS levels in BRL cells were significantly higher in the H_2O_2+ASO group than that in the H_2O_2+ASO-NC group *(Fig. 4-C, D*, P<0.001). Moreover, the level of MDA in BRL cells was significantly increased after *lncRNA77.2* interference *(Fig. 4-E*, P<0.001), while knockdown of *lncRNA77.2* exacerbated H_2O_2-induced oxidative stress.

To verify whether the protective effect of lncRNA77.2 knockdown on H_2O_2-triggered oxidative stress was mediated by antioxidant genes, the expression levels of

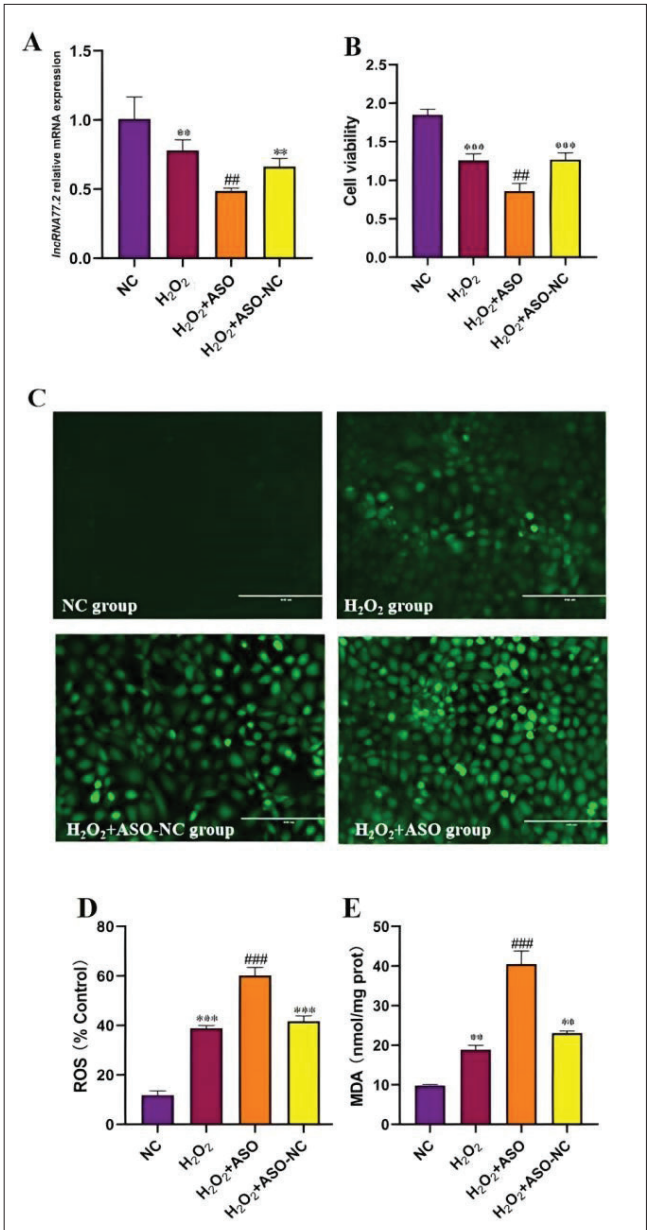

Fig 4. Effects of interfering *lncRNA 77.2* on intracellular cell proliferation and oxidative stress levels in BRL cells. **A-** The expression of *lncRNA77.2* in BRL cells was assessed by qRT-PCR analysis; **B-** Viability of BRL cells was detected using CCK-8 assay; **C-** ROS fluorescence staining, ×200; **D-** Fluorescence intensity of positive ROS staining; **E-** MDA results. Data are expressed as the mean ± standard error (n=3). * Compared with the NC group, # compared to the H_2O_2 + ASO-NC group, ** P<0.01, ##: P<0.01, *** P<0.001, ###: P<0.001

SOD, *CAT*, and *GSH-Px* in BRL cells were analyzed using qRT-PCR after exposure to H_2O_2 for 24 h. As shown in *Fig. 5-A,B,C*, compared to those in the control group, the expression levels of *SOD*, *CAT*, and *GSH-Px* were significantly downregulated in the H_2O_2+ASO group (P<0.01). In summary, *lncRNA77.2* has been shown to play an important role as an antioxidant factor during oxidative stress in BRL cells.

We explored whether *lncRNA77.2* interference affects the oxidative stress and apoptosis of BRL cells. Notably, the

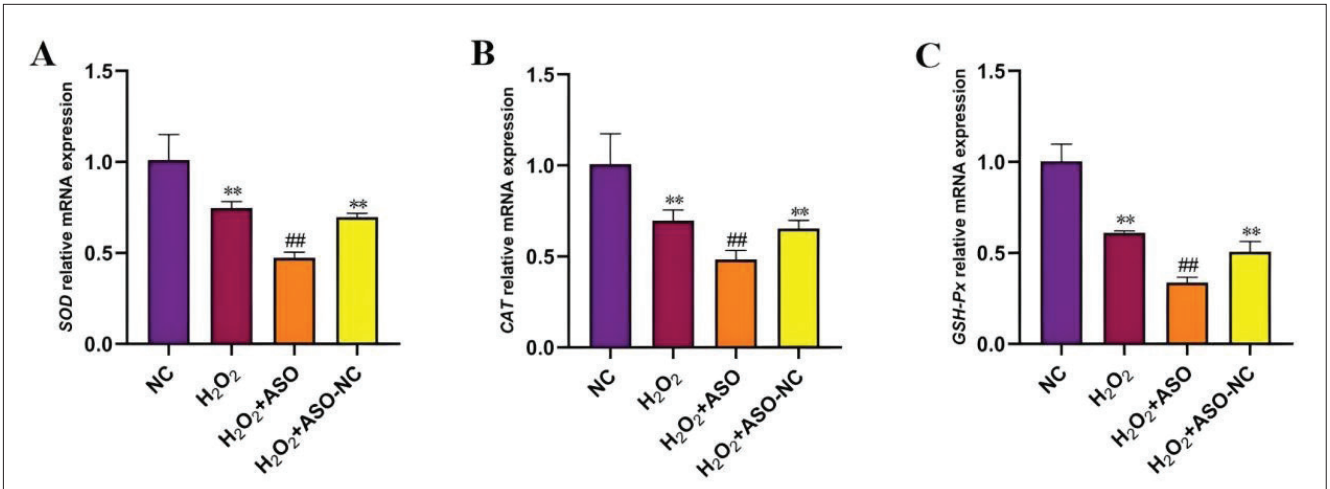

Fig 5. Effects of interfering *lncRNA77.2* on intracellular antioxidant genes in BRL. **A**- *SOD/GAPDH;* **B**- *CAT/GAPDH;* **C**- *GSH-Px/GAPDH.* Data are expressed as the mean ± standard error (n=3). * Compared with NC group, # compared with H_2O_2 + ASO-NC group, ** P<0.01, ##: P<0.01

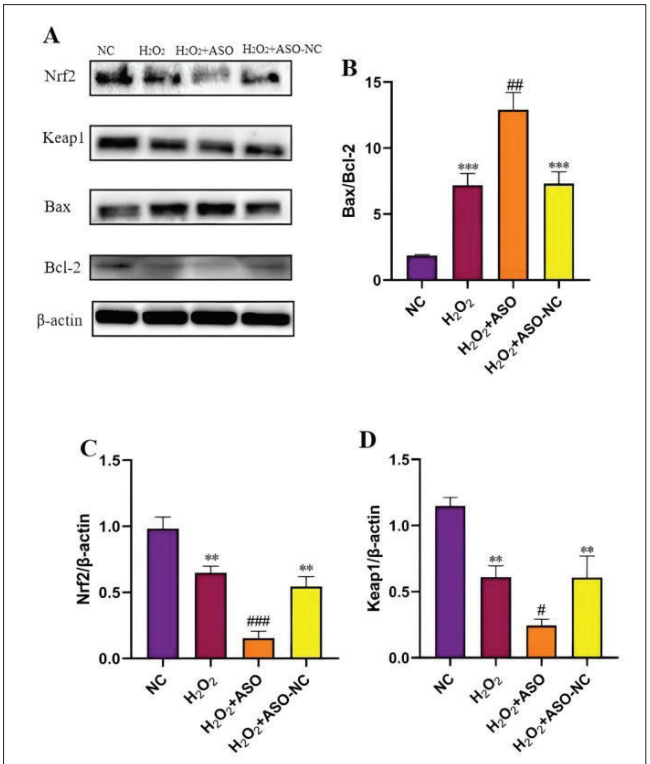

Fig 6. Effects of interfering *lncRNA77.2* on oxidation and apoptosis related proteins in BRL cells. **A**- Western Blot results of Nrf 2, Keap 1, Bax, Bcl-2, β-actin; **B,C,D**- Results of Bax/Bcl-2, Nrf 2/β-actin, Keap1/β-actin. Data are expressed as the mean ± standard error (n=3). * Compared with the NC group, # P<0.05, ** P<0.01, ## P<0.01, *** P<0.001

results of western blotting showed a significant decrease in Nrf2 and Keap1 protein expression and a significant increase in Bax/Bcl-2 protein expression after *lncRNA77.2* interference compared to the H_2O_2+ASO-NC group *(Fig. 6)*. These results suggest that H_2O_2-induced oxidative stress and apoptosis are exacerbated in cells with lncRNA77.2 interference.

The knockdown of *lncRNA77.2* in BRL cells aggravates its oxidative stress, and lncRNA 77.2 can act as an antioxidant factor and play an important role in the regulation of oxidative stress in BRL cells. This study highlights the unprecedented connection of *lncRNA77.2* in oxidative stress, a finding that deepens the understanding of the complex relationship between lncRNA and oxidative stress and provides new ideas and insights into the study of oxidative stress in animals.

Discussion

With the rapid development of science and technology, lncRNA has emerged from what was once regarded as the dark matter of the genome to become a key molecule in regulating gene expression. However, the biological function of lncRNA and stress has been poorly studied. Previous studies by our group have demonstrated that cold stress can induce oxidative stress in the organism. By constructing a cold stress rat model, we screened differentially expressed *lncRNA77.2* in the liver of cold-stress rats by high-throughput sequencing for functional studies. In this study, BRL cells were selected to construct an oxidative stress model via hydrogen peroxide treatment. *lncRNA77.2* interference was performed using antisense oligonucleotide plasmid expression, and the effects of interfering with lncRNA77.2 expression on oxidative stress, proliferation, and apoptosis in rat liver cells after oxidative stress were preliminarily investigated.

Oxidative stress is a hot research topic at present. Under physiological conditions, the oxidation and antioxidant systems of the body are in dynamic balance, and oxidation intermediates such as ROS are produced as a result of an imbalance in the antioxidant system. Excessive ROS can cause oxidative stress and impaired cell metabolic function and directly cause cell death by directly destroying essential proteins, DNA, or lipids [12].

Nrf2 has antioxidant detoxification and cytoprotective effects, and more than 90% of antioxidant genes are

regulated by Nrf2 [13]. Keap1 is a novel multiregional inhibitory protein of the KELCH family, and plays a key role in oxidation-reduction signaling [14]. Nrf2 is found in the cytoplasm with Keap1 and forms a complex after oxidative stress. Following dissociation of the Nrf2 and Keap1 complex, Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE), thus promoting the transcriptional activation of antioxidant enzymes (e.g. CAT GSH-Px and SOD) [15]. Some experiments have found that the Keap1-Nrf2/ARE signaling pathway during oxidative stress can induce the body to produce a series of free radical scavenging enzymes that are resistant to oxidative stress, which is the main mechanism to resist oxidative damage and enhance antioxidant ability [16]. Here, we used the rat hepatocyte oxidative stress model induced by H_2O_2, it was found that with the extension of H_2O_2 stimulation time, the levels of ROS and MDA gradually increased, the mRNA levels of antioxidant enzymes gradually decreased, and the protein expression levels of Nrf2, Keap1, and Bax/Bcl-2 gradually increased, indicating that the prolongation of H_2O_2 stimulation could increase the level of oxidative stress in BRL cells.

It Is generally accepted that there is a close relationship between oxidative stress and lncRNA. Xu et al.[17] found that knockdown of lncRNA KCNQ1OT1 increased miR-137 levels, inhibited the inflammatory response, and alleviated oxidative stress in ox-LDL-treated THP-1 macrophages, suggesting that silencing KCNQ1OT1 suppresses the inflammatory response and oxidative stress induced by ox-LDL through the miR-137/TNFAIP1 pathway in THP-1 macrophages. Furthermore, a study by Shen et al.[18] found that lncRNATUG1 was disrupted, increased cell viability, and downregulated apoptosis and caspase-3 levels. These results suggested that knocking down TUG1 expression may protect LECs from oxidative stress-induced apoptosis. Here, we used ASO to interfere with BRL cell *lncRNA77.2* expression, before treating BRL cells with H_2O_2. The results showed that antioxidant genes SOD, CAT, and GSH-Px relative mRNA expression indicated an obvious downward trend after interfering with *lncRNA77.2*. The protein expression associated with the antioxidant pathways Nrf2, Keap1 also showed the same trend. The above results suggest that different lncRNAs have different regulatory effects on oxidative stress.

In conclusion, our study found that disruption of *lncRNA77.2* accelerates the process of H_2O_2-induced oxidative stress. In conclusion, *lncRNA77.2* was found to be an antioxidant factor important in the regulation of oxidative stress in BRL cells. How signaling pathways are regulated to mitigate liver damage caused by oxidative stress during the antioxidant process requires further investigation. These data provide new insights into the regulatory mechanism of oxidative stress and provide favorable evidence for the functional diversity of lncRNA.

Declarations

Availability of Data and Materials: The data that support the findings ofthis study are available on request fromthe corresponding author (HJ). The data arenot publicly available due to privacy orethical restrictions.

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Conflict of Interest: The authors declared that there is no conflict of interest.

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Author Contributions: HJ designed and supervised the study. YH for mplementation of the experiment and collection of specimens, analysis of data and drafting the paper. LCW, SZY, ZJJ, and YSQ for carrying out the statistical analysis and provided critical comments. HJ checked and improved the manuscript. All authors read and approved the final manuscript.

References

1. Shao ZY, Xue LL, Ji H,Shao BH, Zhan XL, Niu CY, Guo JR, Li SZ, Yang HM: Advances in the study of long-stranded non-coding RNAs and related signaling pathways. *Chinese J Vet Med,* 41 (7): 1438-1443, 2021. DOI: 10.16303/j.cnki.1005-4545.2021.07.32

2. Waterhouse PM, Hellens RP: Plant biology: Coding in non-coding RNAs. *Nature,* 520, 41-42, 2015. DOI: 10.1038/nature14378

3. Z. Durackova: Some current insights into oxidative stress. *Physiol Res,* 59 (4): 459-469, 2010. DOI: 10.33549/physiolres.931844

4. Xiao X, Tan Z, Jia M, Zhou X, Wu K, Ding Y, Li W: Long noncoding RNA SNHG1 knockdown ameliorates apoptosis, oxidative stress and inflammation in models of Parkinson's disease by inhibiting the miR-125b-5p/MAPK1 axis. *Neuropsychiatr Dis Treat,* 17, 1153-1163, 2021. DOI: 10.2147/NDT.S286778

5. Nickerson JA, Momen-Heravi F: Long non-coding RNAs: Roles in cellular stress responses and epigenetic mechanisms regulating chromatin. *Nucleus,* 2024:2350180, 2024. DOI: 10.1080/19491034.2024.2350180

6. Wu GZ, Li XY, Li MY, Zhang Z: Long non-coding RNA MALAT1 promotes the proliferation and migration of Schwann cells by elevating BDNF through sponging miR-129-5p. *Exp Cell Res,* 3 (12):111937, 2020. DOI: 10.1016/j.yexcr.2020.111937

7. Kong X, Liu CX, Wang GD, Yang H, Yao XM, Hua Q, Li XY, Zhang HM, Ma MZ, Su Q, Lv K: LncRNA LEGLTBC Functions as a ceRNA to Antagonize the Effects of miR-34a on the Downregulation of SIRT1 in Glucolipotoxicity-Induced INS-1 Beta Cell Oxidative Stress and Apoptosis. *Oxid Med Cell Longev*, 2019:4010764, 2019. DOI: 10.1155/2019/4010764

8. Zhang P, Sun J, Liang C, Gu B, Xu Y, Lu H, Cao B, Xu H: lncRNA IGHCγ1 acts as a ceRNA to regulate macrophage inflammation via the miR-6891-3p/TLR4 axis in osteoarthritis. *Mediat Inflamm*, 2020:9743037, 2020. DOI: 10.1155/2020/9743037

9. Ray K: Coeliac disease: Lnc13 and coeliac disease: a link to pathogenesis? *Nat Rev Gastro Hepat,* 13 (6): 314-315, 2016. DOI: 10.1038/nrgastro.2016.69

10. Ji H, Niu C, Zhan X, Xu J, Lian S, Xu B, Guo J, Zhen L, Yang H, Li S, Ma L: Identification, functional prediction, and key lncRNA verification of cold stress-related lncRNAs in rats liver. *Sci Rep,* 10:521, 2020. DOI: 10.1038/s41598-020-57451-7

11. Liu CW, Xue LL, Niu CY, Guo JR, Xu B, Zhen L, Ji H: Effect of lncRNA NONRATT021477.2 on the proliferation and apoptosis of BRL cells under oxidative stress conditions. *Chinese J Vet Med,* 43 (3): 539-545, 2023. DOI: 10.16303/j.cnki.1005-4545

12. Ghanim BY, Ahmad MI, Abdallah QM, Qatouseh LA, Qinna NA: Modulation of Nrf 2/ARE pathway - and cell death -related genes during drug-induced liver injury. *Hum Exp Toxicol*, 40 (12): 2223-2236, 2021. DOI: 10.1177/09603271211027947

13. Jagadish N, Parashar D, Gupta N, Agarwal S, Suri V, Kumar R, Suri V, Sadasukhi TC, Gupta A, Ansari AS, Lohiya NK, Suri A: Heat shock protein 70-2 (HSP70-2) is a novel therapeutic target for colorectal cancer and is associated with tumor growth. *BMC Cancer,* 16 (1):561, 2016. DOI: 10.1186/s12885-016-2592-7

14. Yi G, Din JU, Zhao F, Liu X: Effect of soybean peptides against hydrogen peroxide induced oxidative stress in HepG2 cells via Nrf 2 signaling. *Food Funct,* 11 (3): 2725-2737, 2020. DOI: 10.1039/c9fo01466g

15. Sajadimajd S, Khazaei M: Oxidative stress and cancer: The role of Nrf2. *Curr Cancer Drug Targets,* 18 (6): 538-557, 2018. DOI: 10.2174/1568009617666171002144228

16. Kang L, Tian Y, Guo X, Chu X, Xue Y: Long noncoding RNA ANPODRT overexpression protects nucleus pulposus cells from oxidative stress and apoptosis by activating keap1-Nrf2 signaling. *Oxid Med Cell Longev*, 2021:6645005, 2021. DOI: 10.1155/2021/6645005

17. Xu C, Chen L, Wang RJ, Meng J: LncRNA KCNQ1OT1 knockdown inhibits ox-LDL-induced inflammatory response and oxidative stress in THP-1 macrophages through the miR-137/TNFAIP1 axis. *Cytokine,* 155:155912. 2022. 155912. DOI: 10.1016/j.cyto.2022.155912

18. Shen Q, Zhou T: Knockdown of lncRNA TUG1 protects lens epithelial cells from oxidative stress-induced injury by regulating miR-196a-5p expression in age-related cataracts. *Exp Ther Med,* 22:1286, 2021. DOI: 10.3892/etm.2021.10721

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Research Article

Seroprevalence and Risk Factors Associated with *Anaplasma phagocytophilum* Infection in Horses in Egypt

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Abstract

Anaplasma phagocytophilum is a zoonotic obligate intracellular pathogen, that infects horses and is transmitted by *Ixodes* ticks. The seropositivity for this pathogen does not necessarily associate with clinical signs. Limited data about the epidemiology of *A. phagocytophilum* in horses in Egypt are available. The purpose of the study was to determine the seroprevalence of *A. phagocytophilum* in Egyptian horses in three Egyptian governorates and to assess the associated risk factors for infection. A total of 395 serum samples from suspected horses raising in three Egyptian governorates at Northern Egypt were examined using SNAP®4DX® ELISA test. Overall, the seroprevalence of *A. phagocytophilum* was 11.1% (44/395), with highest rate found in Giza governorate 15.7%. The seroprevalence of A. *phagocytophilum* increased significantly in older animals, in thoroughbred horses and during summer. The multivariate logistic regression model revealed that age, thoroughbreds, summer season and presence of ectoparasites were identified as risk factors for *A. phagocytophilum* infection in horses. The results of this study confirmed the presence of antibodies against *A. phagocytophilum* in Egyptian horses. Thus, regular monitoring and genetic identification of pathogens are critical for implementing an effective control program and reducing public health risks.

Keywords: Equine granulocytic anaplasmosis, SNAP test, Risk factors, Horse, Egypt

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Introduction

Anaplasma phagocytophilum is obligate intracellular bacterium, causes granulocytic anaplasmosis, formerly known as granulocytic ehrlichiosis, in a variety of wild and domestic animal species, including horses [1,2]

This zoonotic pathogen is mostly spread throughout the world by *Ixodes* ticks. *A. phagocytophilum* is regarded as an emerging pathogen in humans and it causes a feverish state accompanied by headaches and myalgia [3].

Equine granulocytic anaplasmosis (EGA) varies according to the age of the horse and length of infection, ranging from subclinical to a noticeable condition. The clinical signs of the disease are vague and frequently manifest as ataxia, pyrexia, lethargy, distal limb oedema, and decreased appetite [4,5]. *A. phagocytophilum* may have a detrimental economic impact on the equine sector, because of its ability to inhibit the host defense system and hence cause decreased performance [6].

EGA was initially documented in the United States in 1969 [7] and has since been recorded in Brazil, Israel and Europe including Germany [8-10]. EGA is endemic in some locations but not in others [11]. Seroprevalence investigations in horses have been conducted in numerous European countries such as Italy, Denmark, Sweden, France, Czech Republic and Spain, with seroprevalence rates ranging from 7% in Spain to 73% in Czech Republic [12-18].

The preliminary diagnosis of EGA is based on clinical symptoms or hematological abnormalities such as thrombocytopenia, anaemia and lymphopenia. Moreover, the inclusion bodies (morula) in granulocytes can frequently be identified cytologically to provide a conclusive diagnosis [19,20]. PCR is a sensitive and specific approach for detecting *A. phagocytophilum*-specific DNA, particularly in the early and late phases of the disease where microscopic detection of morulae is challenging [21-25]. EGA can be diagnosed using indirect detection approaches such as immunofluorescence test and ELISA [15]. For a precise diagnosis, four weeks with a four-fold increase in certain antibody titers for EGA is required [26].

In Egypt, *A. phagocytophilum* have been detected using serological and molecular techniques in human and ticks but no available data about the situation of *A. phagocytophilum* in equine.

Therefore, the purpose of this study was to determine the seroprevalence of *A. phagocytophilum* in horses in some localities at Northern Egypt and assess the associated risk factors for the infection.

Material and Methods

Ethical Statement

Benha University's ethical council approved all of the approaches and procedures conducted in this study (ethical number BUFVTM2-03-2023). All approaches were implemented in accordance with the standards and regulations of the Benha university committee. The entire study methodology followed the ARRIVE guidelines.

Study Area

The study was performed during the period between January to December 2023 in three governorates (Giza, Kafr ElSheikh and Qalyubia) located at Northern Egypt, *Fig. 1*.

The climate of Giza has a desert climate according to the Köppen-Geiger classification. During the year, there is virtually no rainfall, and the annual rainfall is 18 mm while the average annual temperature is 22°C. In addition, the climate of Kafr el-Sheikh and Qalyubia governorates has a subtropical desert climate (Classification: BWh), is situated at an elevation of 9 to 51 meters above sea level and average temperature of these areas is 23°C.

Sampling and Sample Size

The sample size was calculated using the following formula based on random sampling strategy with an expected disease prevalence of 50%, an acceptable absolute error of 5%, and a 95% confidence level.

$$N= (1.96)^{2*}P_{exp}\,(1\text{-}P_{exp})/d^2$$

Where n is the calculated sample, P_{exp} is the predicted prevalence rate and d is the absolute precision. A total of 395 serum samples were taken from horses raised in three governorates: Giza (140), Kafr ElSheikh (130), and Qalyubia (125).

All horses had no clinical signs for EGA at time of sampling. Blood (10 mL) was obtained from the jugular vein of presenting horses using a vacutainer tube without anticoagulants. The blood samples were centrifuged at 3000 rpm for 10 min to separate sera which kept at -20°C untill serological analysis.

A questionnaire was used to collect data on sex (male, female), age (1-4, 4-8, >8 years), breed (Arabian, Thoroughbred, mixed), season (summer, spring, winter, and autumn), and presence of ectoparasites (yes or no).

Serological Analysis

In accordance with the manufacturer's instructions, horses were screened using the SNAP® 4Dx test (IDEXX Laboratories, Westbrook, ME, USA), which uses the immunodominant p44 protein of the *A. phagocytophilum*. In brief, serum and conjugate were mixed and then added to the SNAP device's sample well. *A. phagocytophilum* test

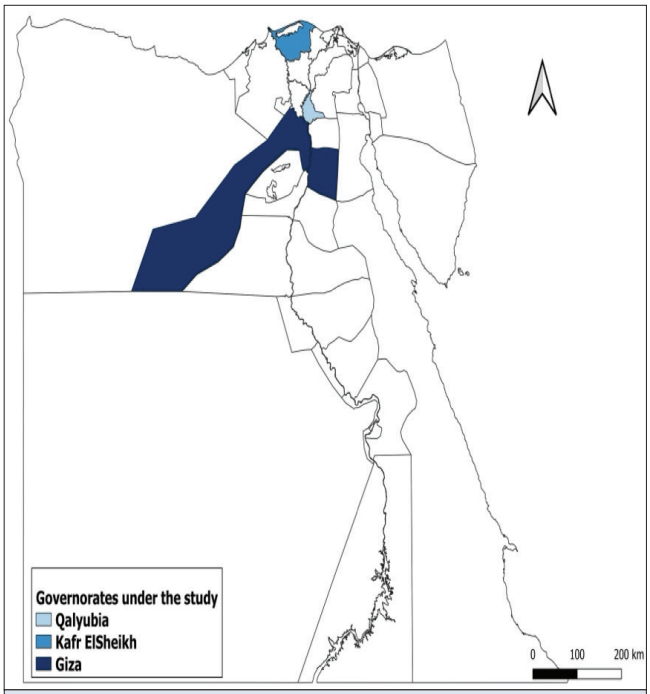

Fig 1. MAP showed location of studied governorates (MAP generated by QGIS software)

results were reported as positive or negative for each horse. The commercial SNAP test has been approved for use with horses despite being created initially for canine testing [27,28].

Statistical Analysis

Microsoft Excel was used to record and code the data from questionnaire surveys before being imported into IBM SPSS version 24 (USA). The relationship between the risk factors (sex, age, breed, season, and presence of ectoparasites) and the *A phagocytophilum* seropositivity was determined using univariate analysis. The significant variables from the univariate analysis were incorporated in a multivariate logistic regression model [29-32]. To determine the degree of association between different parameters and seropositivity, odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.

Results

According to the results of the SNAP test, anti-*A. phagocytophilum* antibodies were found in 11.1% (44/395,

Table 1. *Seroprevalence of Anaplasma phagocytophilum in horses in relation to different factors*

Variable		No of Examined Horses	No of Positive	No of Negative	% of Positive	95% CI	Statistics
Locality	Giza	140	22	118	15.7	10.61-22.64	$\chi2$=4.683 df=2 P=0.096
	Kafr ElSheikh	130	12	118	9.2	5.36-15.44	
	Qalyubia	125	10	115	8.0	4.4-14.1	
Sex	Male	120	10	110	8.3	4.59-14.66	$\chi2$=1.371 df=1 P=0.242
	Female	275	34	241	12.4	8.98-16.78	
Age	1-4years	192	14	178	7.3	4.39-11.86	$\chi2$=10.664 df=2 P=0.005*
	4-8 years	95	9	86	9.5	5.06-17.03	
	>8 years	108	21	87	19.4	13.08-27.9	
Breed	Arabian	145	8	137	5.5	2.82-10.51	$\chi2$=10.523 df=2 P=0.005*
	Thoroughbred	115	21	94	18.3	12.26-26.31	
	Mixed	135	15	120	11.1	6.85-17.52	
Season	Summer	120	21	99	17.5	11.74-25.28	$\chi2$=8.631 df=3 P=0.035*
	Autumn	89	10	79	11.2	6.22-19.47	
	winter	93	8	85	8.6	4.42-16.06	
	Spring	93	5	88	5.4	2.32-11.98	
Presence of ectoparasites	Yes	295	40	255	13.6	10.12-17.94	$\chi2$=6.895 df=1 P=0.009*
	No	100	4	96	4.0	1.57-9.84	
Total		395	44	351	11.1	8.4-14.62	

The result is significant if P<0.05

Table 2. *Multivariate logistic regression analysis for factors associated with Anaplasma phagocytophilum infection in horses*

Variables		B	S.E.	OR	95% CI for OR		P Value
					Lower	Upper	
Age	4-8 years	0.253	0.464	1.3	1.2	3.2	0.029
	>8 years	1.388	0.396	4.0	1.8	8.7	<0.0001
Breed	Thoroughbred	1.449	0.461	4.3	1.7	10.5	0.002
	Mixed	0.789	0.475	2.2	1.4	5.6	0.027
Season	Summer	1.423	0.540	4.2	1.4	12.0	0.008
	Autumn	1.114	0.594	3.0	1.0	9.8	0.061
	Winter	0.419	0.609	1.5	1.2	5.0	0.019
Presence of ectoparasites	Yes	1.374	0.557	4.0	1.3	11.8	0.014

B: Logistic regression coefficient, SE: Standard error, OR: Odds ratio, CI: Confidence interval

95% CI: 8.4-14.62) of the horses that were examined. The highest prevalence was found in the Giza governorate, where it was 15.7% (22/140), while the lowest prevalence was found at qalyubia with 8% (10/125), *(Table 1)*.

The seroprevalence of *A. phagocytophilum* in the horses under examination was not significant associated with both horse sex (P=0.242) and location (P=0.096). The seropositivity of *A. phagocytophilum* increased significantly in thoroughbred horses (18.3%), particularly those Particularly those over eight years old (19.4%). In addition, the highest positive results were highest in summer (21 out of 120; 17.5%), followed by autumn (10 out of 89; 11.2%), and winter (8 out of 93; 8.6%), while the prevalence increased significantly in presence of ectoparasites (40/295; 13.6%) *(Table 1)*.

The multivariate logistic regression model revealed that the likelihood of a positive for *A. phagocytophilum* increased by four times in in older horses over eight years (OR= 4, 95% CI: 1.8-8.7) in thoroughbreds (OR=4.3, 95% CI: 1.7-10.5). Horses were nearly four times more likely to test positive for SNAP in the summer (OR = 4.2) and in the presence of ectoparasites (OR = 4), *(Table 2)*.

Discussion

A. phagocytophilum is an obligate zoonotic intracellular bacterium that affects all mammals, including horses. It has a severe economic impact on the equine industry and reduces performance. To our knowledge, this is the first serological study to investigate presence of antibodies against *A. phagocytophilum* in Egyptian horses.

The study found a seroprevalence of 11.1% using the IDEXX SNAP 4DX Plus Test®, which was similar to the reported prevalences in southwest Virginia (8-11.2%) [33] and Brazil (11.3%) [34].

In addition, high prevalence rates of *A. phagocytophilum* was reported in Scandinavian countries. The prevalence ranged between 17-69% among Swedish horses using PCR [18,35], whereas 22% of horses in Denmark had positive results with ELISA testing [36]. In Europe, the prevalence rate was 5-73% using IFAT and 5% using PCR in the Czech Republic [16,37], 10% based on PCR in Netherlands and 4% using IFAT in Switzerland and ranged between20-26.9% in Germany based on IFAT and SNAP test [26,38],

The status of infection, time of sampling and size, vector distribution, presence of vectors, season, climatic condition, management practices and the tests used to assess infection can all be factors contributing to the fluctuations in global prevalence [39-41].

The current investigation found no significant effect of gender on the prevalence of *A. phagocytophilum*, which is consistent with Hinson et al.[33] and Nogueira et al.[34]. The high incidence was reported in females, which contrasts with the findings of Schäfer et al.[38]. This might be explained by the fact that most of the examined animals in the study were females or by the kind of care the animals received.

Interestingly, the presence of antibodies against *A. phagocytophilum* was significantly associated with the age of horse, with highest prevalence rate in old age. Other studies concluded similar findings [36]. On the contrary, Ribeiro et al.[42] reported no significant effect for age on prevalence of *A. phagocytophilum* in horses and observed high prevalence among young animals. This observation could be explained by the fact that young animals receive more care, while older animals are regularly exposed to illnesses throughout their careers [35,39].

The present finding are broadly in line with observation of Seo et al.[43], where the prevalence rate of *A. phagocytophilum* was higher in thoroughbreds and in presence of ectoparasites in comparison with others.

This could be attributed to thoroughbreds were more exposure for ticks infestation and absence of daily grooming [44]. In addition, the climatic characters of studied areas like relative humidity, temperature are favorable for development of ticks which are the main vectors of pathogen transmission [45].

In the present study, seasonality has a statistically significant effect on seropositivity for *A. phagocytophilum* (P=0.035). This could be due to the highest activity of the vector (*Ixodes* ticks) in late spring/early summer [46-50]. Conversely, Schäfer et al.[38] found that seasonality had no effect on the serological test results for horses which might be due to persist of antibodies titers for at least two years following pathogen exposure.

The results of this study showed that Egyptian horses frequently produce antibodies against *A. phagocytophilum*. The statistical analysis revealed that age, thoroughbreds, summer season and presence of ectoparasites were significantly associated with seroprevalence of *A. phagocytophilum*. Further studies are necessary for identification and genetic characterization of pathogens either in horses or ticks.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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References

1. Nováková M, Víchová B: Granulocytic anaplasmosis-emerging tick-borne disease of humans and animals. *Biologia,* 65, 925-931, 2010. DOI: 10.2478/s11756-010-0119-2

2. Atif FA: *Anaplasma marginale* and *Anaplasma phagocytophilum:* Rickettsiales pathogens of veterinary and public health significance. *Parasitol Res,* 114, 3941-3957, 2015. DOI: 10.1007/s00436-015-4698-2

3. Dumler JS, Choi K-S, Garcia-Garcia JC, Barat NS, Scorpio DG, Garyu JW, Grab DJ, Bakken JS: Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. *Emerg Infect Dis,* 11 (12):1828, 2005. DOI: 10.3201/eid1112.050898

4. Pusterla N, Madigan JE: Equine granulocytic anaplasmosis. *J Equine Vet Sci,* 33 (7): 493-496, 2013. DOI: 10.1016/j.jevs.2013.03.188

5. Saleem S, Ijaz M, Farooqi SH, Ghaffar A, Ali A, Iqbal K, Mehmood K, Zhang H: Equine granulocytic anaplasmosis 28 years later. *Microb Pathog,* 119, 1-8, 2018. DOI: 10.1016/j.micpath.2018.04.001

6. Siska WD, Tuttle RE, Messick JB, Bisby TM, Toth B, Kritchevsky JE: Clinicopathologic characterization of six cases of equine granulocytic anaplasmosis in a nonendemic area (2008-2011). *J Equine Vet Sci,* 33 (8): 653-657, 2013. DOI: 10.1016/j.jevs.2012.10.003

7. Gribble DH: Equine Ehrlichiosis. University of California, Davis, 1970.

8. Von Loewenich F, Stumpf G, Baumgarten B, Röllinghoff M, Dumler J, Bogdan C: A case of equine granulocytic ehrlichiosis provides molecular evidence for the presence of pathogenic *Anaplasma phagocytophilum* (HGE agent) in Germany. *Eur J Clin Microbiol Infect Dis,* 22, 303-305, 2003. DOI: 10.1007/s10096-003-0935-1

9. Levi O, Waner T, Baneth G, Keysary A, Bruchim Y, Silverman J, Harrus S: Seroprevalence of Anaplasma phagocytophilum among healthy dogs and horses in Israel. *J Vet Med Series B,* 53 (2): 78-80, 2006. DOI: 10.1111/j.1439-0450.2006.00911.x

10. Salvagni CA, Dagnone AS, Gomes TS, Mota JS, Andrade GM, Baldani CD, Machado RZ: Serologic evidence of equine granulocytic anaplasmosis in horses from central West Brazil. *Rev Brasil Parasitol Vet,* 19, 135-140, 2010. DOI: 10.1590/s1984-29612010000300002

11. Madigan JE, Hietala S, Chalmers S, DeRock E: Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. *J Am Vet Med Assoc,* 196 (12): 1962-1964, 1990. DOI: 10.2460/javma.1990.196.12.1962

12. Leblond A, Pradier S, Pitel P, Fortier G, Boireau P, Chadoeuf J, Sabatier P: An epidemiological survey of equine anaplasmosis *(Anaplasma phagocytophilum)* in southern France. *Rev Sci Tech,* 24 (3): 899-908, 2005.

13. Passamonti F, Fabrizia V, Katia C, Stefano C, Giacomo C, Luisa MM, Daniela PF, Andrea VS, Mauro C: *Anaplasma phagocytophilum* in horses and ticks: A preliminary survey of Central Italy. *Comp Immunol Microbiol Infect Dis,* 33 (1): 73-83, 2010. DOI: 10.1016/j.cimid.2008.08.002

14. Butler C, Nijhof A, Jongejan F, Van der Kolk J: *Anaplasma phagocytophilum* infection in horses in the Netherlands. *Vet Rec,* 162 (7):216, 2008. DOI: 10.1136/vr.162.7.216

15. Amusategui I, Sainz A, Tesouro MA: Serological evaluation of *Anaplasma phagocytophilum* infection in livestock in northwestern Spain. *Ann N Y Acad Sci,* 1078 (1): 487-490, 2006. DOI: 10.1196/annals.1374.091

16. Praskova I, Bezdekova B, Zeman P, Jahn P: Seroprevalence of *Anaplasma phagocytophilum* in horses in the Czech Republic. *Ticks Tick Borne Dis,* 2 (2): 111-115, 2011. DOI: 10.1016/j.ttbdis.2011.01.002

17. Laus F, Veronesi F, Passamonti F, Paggi E, Cerquetella M, Hyatt D, Tesei B, Fioretti DP: Prevalence of tick borne pathogens in horses from Italy. *J Vet Med Sci,* 75 (6): 715-720, 2013. DOI: 10.1292/jvms.12-0449

18. Egenvall A, Franzén P, Gunnarsson A, Engvall EO, Vågsholm I, Wikström UB, Artursson K: Cross-sectional study of the seroprevalence to *Borrelia burgdorferi* sensu lato and granulocytic *Ehrlichia* spp. and demographic, clinical and tick-exposure factors in Swedish horses. *Prev Vet Med,* 49 (3-4): 191-208, 2001. DOI: 10.1016/S0167-5877(01)00187-8

19. Dzięgiel B, Adaszek Ł, Kalinowski M, Winiarczyk S: Equine granulocytic anaplasmosis. *Res Vet Sci,* 95 (2): 316-320, 2013. DOI: 10.1016/j.rvsc.2013.05.010

20. Madigan J, Gribble D: Equine ehrlichiosis in northern California: 49 cases (1968-1981). *J Am Vet Med Assoc,* 190 (4): 445-448, 1987.

21. Franzén P, Aspan A, Egenvall A, Gunnarsson A, Åberg L, Pringle J: Acute clinical, hematologic, serologic, and polymerase chain reaction findings in horses experimentally infected with a European strain of *Anaplasma phagocytophilum. J Vet Internal Med,* 19 (2): 232-239, 2005. DOI: 10.1892/0891-6640(2005)19<232:achsap>2.0.co;2

22. Pusterla N, Madigan JE: *Anaplasma phagocytophilum* infection. **In,** Equine Infectious Diseases. Chapter 39; pp. 344-347. Elsevier, 2014. DOI: 10.1016/B978-1-4557-0891-8.00039-7

23. Pusterla N, Huder JB, Leutenegger CM, Braun U, Madigan JE, Lutz H: Quantitative real-time PCR for detection of members of the *Ehrlichia phagocytophila* genogroup in host animals and Ixodes ricinus ticks. *J Clin Microbiol,* 37 (5): 1329-1331, 1999. DOI: 10.1128/JCM.37.5.1329-1331.1999

24. Selim A, El-Haig M, Galila ES, Gaede W: Direct detection of *Mycobacterium avium* subsp. *Paratuberculosis* in bovine milk by multiplex real-time PCR. *Anim Sci Pap Rep,* 31 (4): 291-302, 2013.

25. Selim A, Manaa E, Khater H: Molecular characterization and phylogenetic analysis of lumpy skin disease in Egypt. *Comp Immunol Microbiol Infect Dis,* 79:101699, 2021. DOI: 10.1016/j.cimid.2021.101699

26. Gehlen H, Inerle K, Bartel A, Stöckle SD, Ulrich S, Briese B, Straubinger RK: Seroprevalence of *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* infections in German horses. *Animals,* 13 (12):1984, 2023. DOI: 10.3390/ani13121984

27. Chandrashekar R, Daniluk D, Moffitt S, Lorentzen L, Williams J: Serologic diagnosis of equine borreliosis: Evaluation of an in-clinic enzyme-linked immunosorbent assay (SNAP® 4Dx®). *Intern J Appl Res Vet Med*, 6;3, 2008.

28. Thompson D, Thirumalapura NR, Tewari D: Prevalence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* infection in horses from Pennsylvania (2017-2019) using antibody and organism-based detection. *J Am Vet Med Assoc,* 260 (14): 1834-1838, 2022. DOI: 10.2460/javma.22.06.0232

29. Selim A, Khater H, Almohammed HI: A recent update about seroprevalence of ovine neosporosis in Northern Egypt and its associated risk factors. *Sci Rep,* 11 (1): 14043, 2021. DOI: 10.1038/s41598-021-93596-9

30. Selim A, Attia KA, Alsubki RA, Kimiko I, Sayed-Ahmed MZ: Cross-sectional survey on *Mycobacterium avium* Subsp. *paratuberculosis* in Dromedary Camels: Seroprevalence and risk factors. *Acta Trop,* 226:106261, 2022. DOI: 10.1016/j.actatropica.2021.106261

31. Selim A, Manaa EA, Alanazi AD, Alyousif MS: Seroprevalence, risk factors and molecular identification of bovine leukemia virus in Egyptian cattle. *Animals,* 11 (2): 319, 2021. DOI: 10.3390/ani11020319

32. Selim A, Alafari HA, Attia K, AlKahtani MD, Albohairy FM, Elsohaby I: Prevalence and animal level risk factors associated with *Trypanosoma*

evansi infection in dromedary camels. *Sci Rep,* 12 (1):8933, 2022. DOI: 10.1038/s41598-022-12817-x

33. Hinson HL, Lahmers KK, Boes KM, Funk RA: Seroprevalence of *Anaplasma* species in an equine population of Southwest Virginia. *Equine Vet Educ,* 26:3, 2024. DOI: 10.1111/eve.13933

34. Nogueira RdMS, Silva AB, Sato TP, Sá JCd, Santos ACGd, Amorim Filho EF, Vale TLd, Gazêta GS: Molecular and serological detection of *Theileria equi, Babesia caballi* and *Anaplasma phagocytophilum* in horses and ticks in Maranhão, Brazil. *Pesqui Vet Brasil,* 37 (12): 1416-1422, 2017. DOI: 10.1590/S0100-736X2017001200010

35. Janzén T, Petersson M, Hammer M, Aspán A, Dinnétz P: Equine granulocytic anaplasmosis in Southern Sweden: Associations with coniferous forest, water bodies and landscape heterogeneity. *Agric Ecosyst Environ,* 285:106626, 2019. DOI: 10.1016/j.agee.2019.106626

36. Hansen MG, Christoffersen M, Thuesen LR, Petersen MR, Bojesen AM: Seroprevalence of *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in Danish horses. *Acta Vet Scand,* 52:3, 2010. DOI: 10.1186/1751-0147-52-3

37. Hulínská D, Langrová K, Pejčoch M, Pavlásek I: Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction. *Apmis,* 112 (4-5): 239-247, 2004. DOI: 10.1111/j.1600-0463.2004.apm11204-0503.x

38. Schäfer I, Silaghi C, Fischer S, Marsboom C, Hendrickx G, Gehlen H, Müller E: Detection of *Anaplasma phagocytophilum* in horses from Germany by molecular and serological testing (2008-2021). *Vet Parasitol,* 312:109840, 2022. DOI: 10.1016/j.vetpar.2022.109840

39. Selim A, Manaa E, Abdelhady A, Said MB, Sazmand A: Serological and molecular surveys of *Anaplasma* spp. in Egyptian cattle reveal high *A. marginale* infection prevalence. *Iran J Vet Res,* 22 (4):288, 2021. DOI: 10.22099/ijvr.2021.40587.5879

40. Alsubki RA, Albohairy FM, Attia KA, Kimiko I, Selim A, Sayed-Ahmed MZ: Assessment of seroprevalence and associated risk factors for anaplasmosis in *Camelus dromedarius*. *Vet Sci,* 9 (2):57, 2022. DOI: 10.3390/vetsci9020057

41. Selim A, Alanazi AD, Sazmand A, Otranto D: Seroprevalence and associated risk factors for vector-borne pathogens in dogs from Egypt. *Parasite Vectors,* 14, 1-11, 2021. DOI: 10.1186/s13071-021-04670-0

42. Ribeiro AJ, Cardoso L, Maia JM, Coutinho T, Cotovio M: Prevalence of *Theileria equi, Babesia caballi,* and *Anaplasma phagocytophilum* in horses from the north of Portugal. *Parasitol Res,* 112, 2611-2617, 2013. DOI: 10.1007/s00436-013-3429-9

43. Seo MG, Ouh IO, Choi E, Kwon OD, Kwak D: Molecular detection and phylogenetic analysis of *anaplasma phagocytophilum* in horses in Korea. *Korean J Parasitol,* 56 (6):559, 2018. DOI: 10.3347/kjp.2018.56.6.559

44. Hurtado C, Torres R, Pérez-Macchi S, Sagredo K, Uberti B, de Souza Zanatto DC, Machado RZ, André MR, Bittencourt P, Müller A: Serological and molecular detection of *Anaplasma phagocytophilum* in Thoroughbred horses from Chilean racecourses. *Ticks Tick Borne Dis,* 11 (4): 101441, 2020. DOI: 10.1016/j.ttbdis.2020.101441

45. Souza EARd, Araujo AdC, Pires LCSR, Freschi CR, Azevedo SS, Machado RZ, Horta MC: Serological detection and risk factors for equine piroplasmosis in the semiarid region of Pernambuco, Northeastern Brazil. *Rev Brasil Parasitol Vet,* 28, 685-691, 2019. DOI: 10.1590/S1984-29612019088

46. Radwan IT, Baz MM, Khater H, Selim AM: Nanostructured lipid carriers (NLC) for biologically active green tea and fennel natural oils delivery: Larvicidal and adulticidal activities against *Culex pipiens*. *Molecules,* 27 (6):1939, 2022. DOI: 10.3390/molecules27061939

47. Selim A, Abdelhady A, Alahadeb J: Prevalence and first molecular characterization of Ehrlichia canis in Egyptian dogs. *Pak Vet J,* 41 (1): 117-121, 2020. DOI: 10.29261/pakvetj/2020.061

48. Selim A, Khater H: Seroprevalence and risk factors associated with Equine piroplasmosis in North Egypt. *Comp Immunol Microbiol Infect Dis,* 73:101549, 2020. DOI: 10.1016/j.cimid.2020.101549

49. Selim A, Weir W, Khater H: Prevalence and risk factors associated with tropical theileriosis in Egyptian dairy cattle. *Vet World,* 15 (4):919, 2022. DOI: 10.14202/vetworld.2022.919-924

50. Baz MM, Khater HF, Baeshen RS, Selim A, Shaheen ES, El-Sayed YA, Salama SA, Hegazy MM: Novel pesticidal efficacy of Araucaria heterophylla and Commiphora molmol extracts against camel and cattle blood-sucking ectoparasites. *Plants,* 11 (13):1682, 2022. DOI: 10.3390/plants11131682

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Research Article

Assessment of Age-Related Morphological Changes in the Testes of Mali Pig of Tripura, India

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Abstract

This study aimed to investigate the histological, histochemical and electron microscopic features of the testes of Mali pigs of Tripura. The samples were collected from fifteen Mali pigs in five different age groups. Collagen, reticular, elastic, and nerve fibers were observed in the tunica albuginea, seminiferous tubules, germinal epithelium, mediastinum testis and blood vessels across all age groups. Spermatids were found in the seminiferous tubules at three months of age. Histochemical studies revealed glycogen, acidic mucopolysaccharides, keratin and pre-keratin activity in various testicular structures, with staining affinities differing among age groups. Scanning electron microscopy showed the structural morphology of the seminiferous tubules, interstitial tissues, and spermatozoa at different stages of development. The parenchyma of day-old piglets exhibited numerous small round seminiferous or sex cords. Well-defined seminiferous tubules were observed at three months of age and defined spermatogenic cells were present in the lumen at five to six months. The morphological characteristics of the testicular tissues in animals aged five to six months were observed to be almost similar. Sertoli cells, Leydig cells, and spermatozoa were also visualized in the seminiferous tubules under scanning electron microscopy.

Keywords: Histology, Histochemistry, Mali pig, Scanning electron microscopy, Testis

Introduction

The Mali pigs, also known as “local pigs” or “desi pigs,” located in Tripura exhibit very little phenotypic variation among different subgroups. It is believed that domestic pigs in this area share a common origin with the wild pig *Sus scrofa cristatus* [1]. Male pigs weigh an average of 68 kg and reach puberty at around 138.3±6.4 days [2]. The testis is the primary male reproductive organ responsible for sperm production (spermatogenesis). Histological investigation of the testis provides critical insights into the cellular and structural organization that supports male reproductive activity and also helps in understanding the complex structure of the seminiferous tubules, where sperm production occurs and the interstitial cells, which secrete testosterone [3]. This understanding is crucial for comprehending how male fertility and hormone production are controlled. Male germ cell production is the exocrine function, while the production of male sex hormones is its primary endocrine function. Sperm production requires a lower temperature than the average body temperature provided by the scrotum and testicles [4]. Postnatal anatomical investigations of the testes at various ages are necessary to understand anatomical growth and development. The testicular postnatal development of Mali pigs is still unknown and this study is the first to report on the age-related morphological changes in the testes of Mali pigs. The purpose of the study to illustrate the histological, histochemical and scanning electron microscopic features of the postnatal development of the testes. These studies provided the morphological characteristics of the individual regions of the developing testes. While incorporating important literature, it also provides baseline information for future scientific research.

Material and Methods

Ethical Statement

The present study was conducted after approval from the Institutional Animal Ethical Committee (IAEC), Approval reference number. CVSC/CAU/IAEC/22-23/P-13 dated 31st October 2023.

Animals

For this study, 15 male Mali pigs from Tripura were used. The animals were designed for this experiment in five different age groups. Group-I (day-old piglets), group-II (3 months), group-III (4 months), group-IV (5 months) and group-V (6 months) and each of the group was carried three numbers of animals. The samples were collected in November 2023 to May 2024.

Samples

The samples for this study were collected immediately after surgical exposure of the scrotum (castration) with all aseptic precautions. After cleaning, the testicles were recorded for gross and morphometrical characteristics. Subsequently, the biological samples were carried to the department through the ice box for light and electron microscopic studies.

Processing of the Samples for The Light Microscopy

The tissue testicular samples from cranial, middle and caudal regions were cut into 3 to 5 mm thickness and fixed into 10% neutral buffer formalin solutions for the histological and histochemical studies. The tissue processing was carried out by ascending grading of alcohol and paraffined embedded sections were cut into 3 to 5 µm in size [5]. The histological staining was done with Van Gieson's stain (VnGi) for collagen fibers [5], Hartt's stain (Hartt) for elastic fibers [5], Berg's method (Berg) for spermatozoa [5], Bielschowsky's method (Bel) for nerve fibers [5], Gomori's stain (Gomori) for reticular fibers [6] and the histochemical staining was carried out through periodic acid Schiff (PAS) for glycogen, PAS-Alcian blue (PAS-AB) for acidic mucopolysaccharides at pH 2.5 and Ayob- Shklar method for keratin and pre-keratin [5]. The prepared slides were observed under a BX-51 Olympus Advance Trinocular Research Microscope equipped with DP software for computed image analysis.

Processing of the Samples for the Scanning Electron Microscopy

The tissue samples for scanning electron microscopy were cut into 1 to 2 mm sizes and fixed in 2.5% glutaraldehyde solutions in phosphate buffer at pH 7.2 for 4 h at 4°C temperature. Following fixation, the samples were placed in phosphate buffer solutions [7]. The samples were sent to the Sophisticated Analytical Instrumentation Facility (SAIF), North-Eastern Hill University (NEHU), Shillong, Meghalaya, for further processing and imaging under a scanning electron microscope, model no. SEM JEOL JSM 6360, manufactured by Japan Electron Optics Laboratory Company, Limited (Nihon Denshi Kogaku Kenkyujo), Japan.

Results

Histological Observations

The histological study revealed the collagen, reticular, elastic and nerve fibers in all the age groups predominantly. In group-I, the tunica albuginea for day-old piglets were recorded procollagen fibers and the basement membrane of numerous seminiferous or sex cords exhibited elastic and reticular fibers *(Fig. 1-a, Fig. 2-a, Fig. 3-a)*. The lumen of the seminiferous cords was visualized with some gonocytes in the center and undifferentiated Sertoli cells-like structures in the periphery of the cords. No spermatids were present in the seminiferous cords of the day-old piglets *(Fig. 4-a)*. Fine nerve fibers were observed around the seminiferous cords and in the parenchyma of the testes *(Fig. 5-a,b)*.

In group-II, the outermost layer of the testes, the tunica albuginea, was formed by the thick connective tissue and mainly consisted of collagen fibers *(Fig. 1-b)*. The fine elastic fibers and the reticular fibers were observed in the basement membrane of seminiferous tubules along

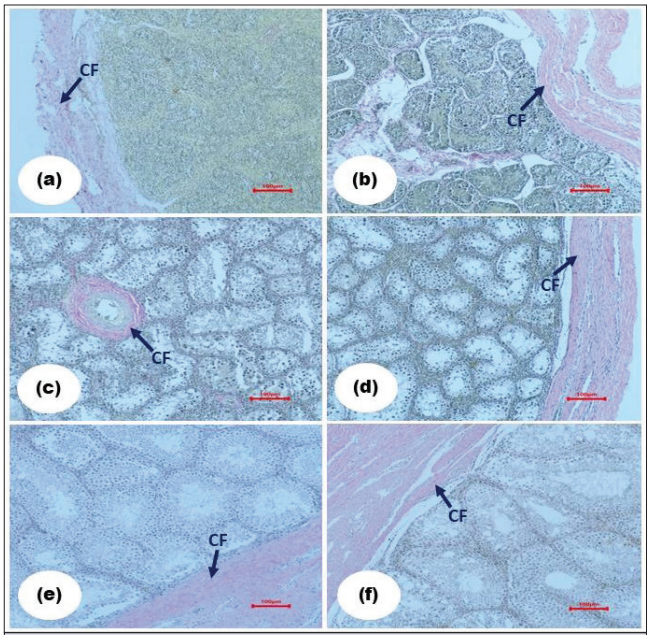

Fig 1. Photomicrographs from group-I showing (a) collagen fibers (CF) in tunica albuginea (VnGi, X100). From group-II showing (b) collagen fibers (CF) in tunica albuginea (VnGi, X100). From group-III showing (c) collagen fibers (CF) in blood vessels (VnGi, X100) and (d) collagen fibers (CF) in tunica albuginea (VnGi, X100). From group-IV showing (e) collagen fibers (CF) in tunica albuginea (VnGi, X100). From group-V showing (f) collagen fibers (CF) in tunica albuginea (VnGi, X100)

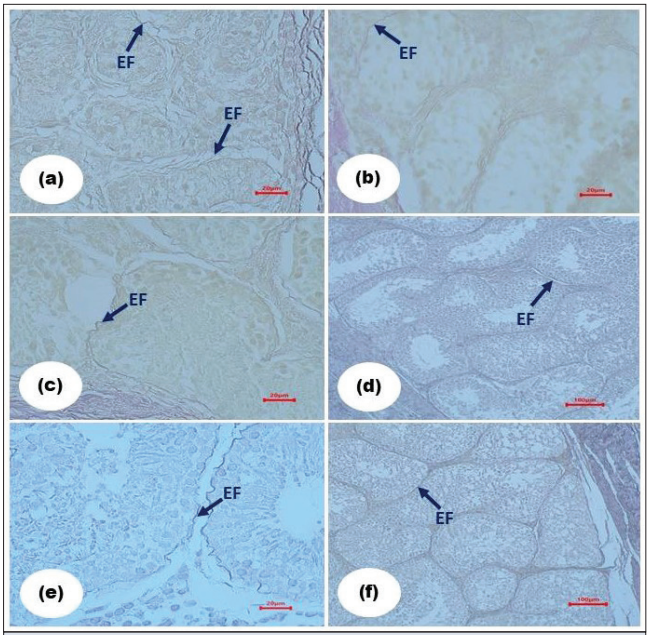

Fig 2. Photomicrographs from group-I showing (a) elastic fibers (EF) in basement membrane of seminiferous or sex cords (Hartt, X400). From group-II showing (b) elastic fibers (EF) in basement membrane of seminiferous tubules (Hartt, X400). From group-III showing (c) elastic fibers (EF) in basement membrane of seminiferous tubules (Hartt, X400). From group-IV showing (d) elastic fibers (EF) in basement membrane of seminiferous tubules (Hartt, X100) and (e) elastic fibers (EF) in basement membrane of seminiferous tubules (Hartt, X400). From group-V showing (f) elastic fibers (EF) in basement membrane of seminiferous tubules (Hartt, X100)

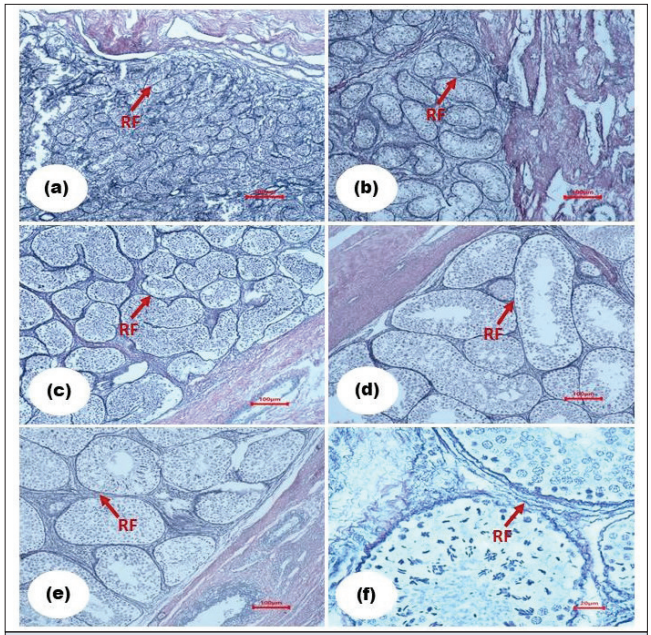

Fig 3. Photomicrographs from group-I showing (a) reticular fibers (RF) in basement membrane of seminiferous cords (Gomori, X100). From group-II showing (b) reticular fibers (RF) in basement membrane of seminiferous tubules and mediastinum testis (Gomori, X100). From group-III showing (c) reticular fibers (RF) in basement membrane of seminiferous tubules (Gomori, X100). From group-IV showing (d) reticular fibers (RF) in basement membrane of seminiferous tubules (Gomori, X100). From group-V showing (e) reticular fibers (RF) in basement membrane of seminiferous tubules (Gomori, X100) and (f) reticular fibers (RF) in basement membrane of seminiferous tubules (Gomori, X400)

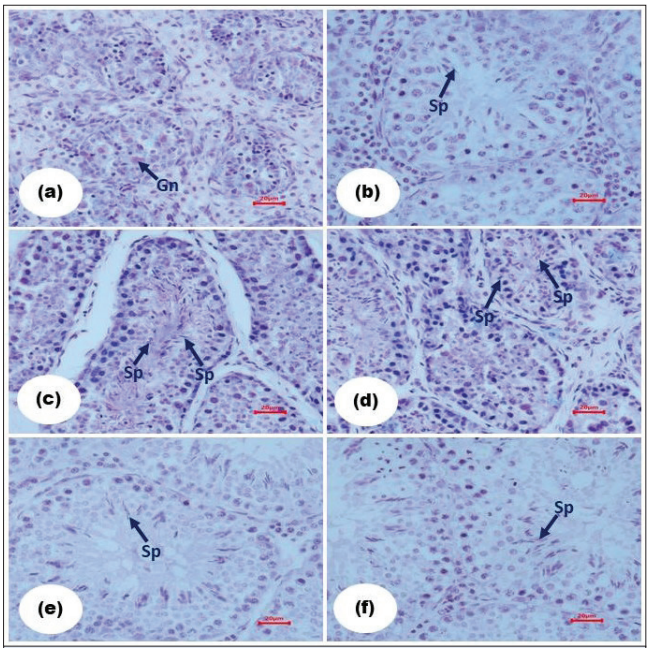

Fig 4. Photomicrographs from group-I showing (a) gonocytes (Gn) in seminiferous cords (Berg, X400). From group-II showing (b) elongated spermatids (Sp) in seminiferous tubules (Berg, X400). From group-III showing (c-d) round to elongated spermatids (Sp) in seminiferous tubules (Berg, X400). From group-IV showing (e) elongated spermatids (Sp) in the seminiferous tubules (Berg, X400). From group-V showing (f) elongated spermatids (Sp) in seminiferous tubules (Berg, X400)

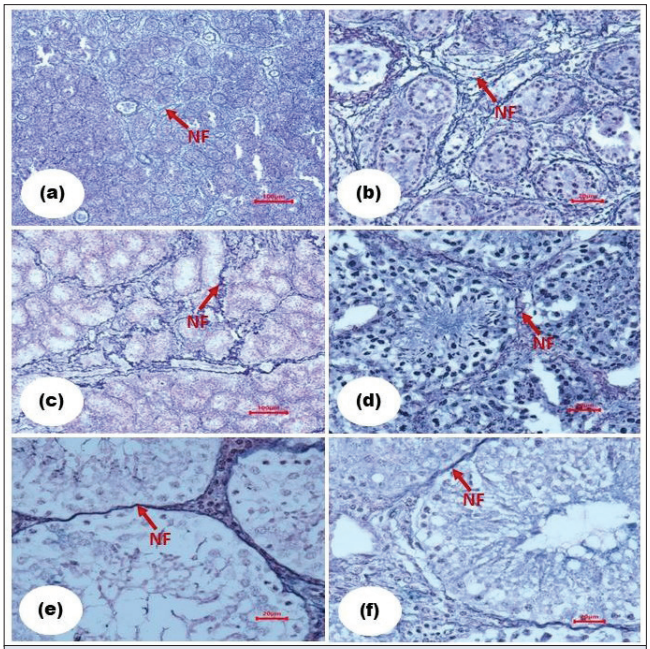

Fig 5. Photomicrographs from group-I showing (a) nerve fibers (NF) around the seminiferous cords (Bel, X100) and (b) nerve fibers (NF) around the seminiferous cords (Bel, X400). From group-II showing (c) nerve fibers (NF) in trabeculae of the testes (Bel, X100). From group-III showing (d) nerve fibers (NF) around the seminiferous tubules (Bel, X400). From group-IV showing (e) nerve fibers (NF) around the seminiferous tubules (Bel, X400). From group-V showing (f) nerve fibers (NF) in between the seminiferous tubules (Bel, X400)

with the lining epithelium of the rete testis *(Fig. 2-b, Fig. 3-b)*. The luminal place of the seminiferous tubules was observed from the 3 months of the age and some of the tubules have recorded few numbers of the elongated spermatids *(Fig. 4-b)*. Nerve fibers were observed in the basement membrane of seminiferous tubules and trabeculae, as well as in the interstitial tissues of the testes *(Fig. 5-c)*.

In group III, the luminal place and the size of seminiferous tubules were observed more in 4 months than in the 3 months of age. The collagen fibers were mainly observed in the tunica fibrosa and trabeculae and around the blood vessels of the testes *(Fig. 1-c,d)*. The elastic and reticular fibers were noticed in the basement membrane of seminiferous tubules and rete testis of the mediastinum *(Fig. 2-c, Fig. 3-c)*. The lumen also contained numerous rounds to elongated spermatids *(Fig. 4-c,d)*. Nerve fibers were found around the seminiferous tubules *(Fig. 5-d)*.

In group-IV, the tunica fibrosa and the tunica vasculosa of the capsule were observed for the collagen fibers *(Fig. 1-e)*. The diameter of the seminiferous tubules was found to be more in 5 months than in 4 months of age. Also, elastic, reticular and nerve fibers were observed in the basement membrane of seminiferous tubules, blood vessels and in

Table 1. *Distribution of histochemical characteristics in the testes of Mali pig of Tripura*

Group	**Testicular Components**	**Histochemical Stains**		
		PAS	PAS-AB	Ayob- Shklar method
Group - I	Tunica albuginea	+	+	-
	Spermatogonia	+	+	-
	Basement membrane of the seminiferous cords	++	+	-
	Interstitial tissue	+	+	+++
	Mediastinum	+	++	-
Group - II	Tunica albuginea	+++	++	-
	Spermatogonia	++	++	-
	Basement membrane of the seminiferous tubules	+++	++	-
	Interstitial tissue	++	+	++
	Mediastinum	++	+	-
Group - III	Tunica albuginea	+++	+	-
	Spermatogonia	++	+	-
	Basement membrane of the seminiferous tubules	+++	++	-
	Interstitial tissue	+++	+	+
	Mediastinum	+++	+	-
Group - IV	Tunica albuginea	+++	++	-
	Spermatogonia	++	+	-
	Basement membrane of the seminiferous tubules	+++	+	-
	Interstitial tissue	+++	+	-
	Mediastinum	+++	+	-
Group - V	Tunica albuginea	+++	+	-
	Spermatogonia	++	+	-
	Basement membrane of the seminiferous tubules	++	+	-
	Interstitial tissue	++	+	-
	Mediastinum	++	+	-

- Absent; + Weak; ++ Moderate; +++ Intense/Strong, (PAS)- Periodic Acid Schiff for glycogen; (PAS-AB)-PAS- Alcian blue for acidic mucopolysaccharides; Ayob- Shklar method for keratin and pre-keratin activity

the rete testis, respectively *(Fig. 2-d,e, Fig. 3-d, Fig. 5-e)*. The widest lumen of the seminiferous were viewed from the 5 months of age and contained numerous spermatids *(Fig. 4-e)*.

In group-V, the histological findings were almost similar for the 5 and 6 months aged animals. The testicular capsule and basement membrane of seminiferous tubules predominantly contained collagen, fine elastic and reticular fibers. In contrast, the nerve fibers were recorded in the periphery of the seminiferous tubules *(Fig. 1-f, Fig. 2-f, Fig. 3-e,f, Fig. 5-f)*. The rounds to elongated shaped spermatids were well observed in the lumen of seminiferous tubules *(Fig. 4-f)*.

Histochemical Studies

In this present investigation, the histochemical distributions for glycogen, acidic mucopolysaccharides and pre-keratin activity were recorded in the cranial, middle and caudal regions of the testes on their developmental basis and observation was recorded in the *Table 1*. The magenta colour was found for the intense reactivity of PAS, which was shown by various locations in the testes. The PAS alcian blue appeared as blue for acidic mucopolysaccharides, whereas the Ayob-Shklar method showed an orange colour for the demonstration of pre-keratin activity in the testicular tissues. No significant differences in histochemical observations were found between the right and left testes.

In group I, weak to moderate activity for the PAS was recorded in the tunica albuginea, basement membrane of the seminiferous cords, mediastinum and the trabeculae of the testes *(Fig. 6-a)*. The mediastinum, tunica albuginea and the interstitial tissue recorded weak reactivity for the acidic mucopolysaccharides and the cytoplasm of some undifferentiated cells in the seminiferous cords showed positive for the pre-keratin activity *(Fig. 7-a, Fig. 8-a)*.

In group II, PAS activity was intense in the tunica albuginea, basement membrane of the seminiferous tubules, trabeculae and moderate PAS affinity was found in the interstitial tissues of the testes *(Fig. 6-b)*. Moderate PAS-AB reactivity was recorded in the tunica albuginea, seminiferous germinal epithelium and the basement membrane of the seminiferous tubules. Weak reactivity for the acidic mucopolysaccharides was found in the interstitial tissues of the testes *(Fig. 7-b)*. Some peritubular and interstitial cells' cytoplasm showed positive pre-keratin activity *(Fig. 8-b,c)*.

In group-III, intense PAS reactivity was recorded in the basement membrane of the seminiferous tubules, trabeculae, blood vessels in the capsules and the interstitial tissues. Spermatids in the seminiferous tubules showed PAS-positive activity from 4 months of age *(Fig. 6-c)*. Weak to moderate acidic mucopolysaccharides were observed in

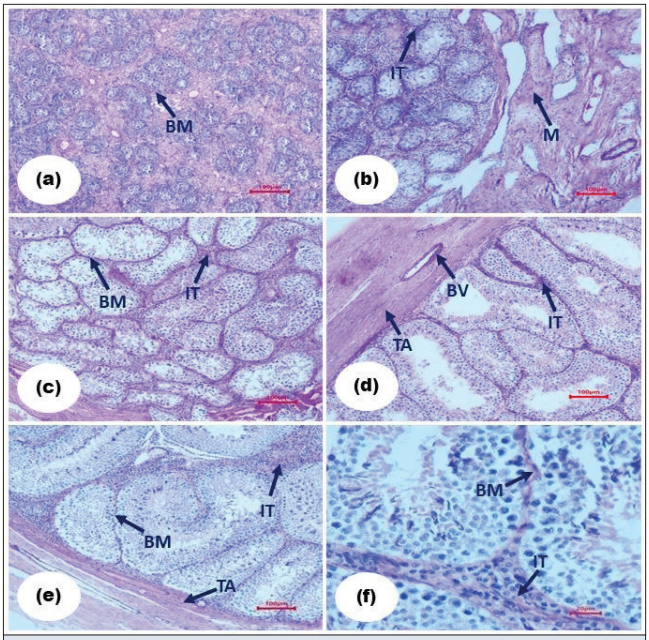

Fig 6. Photomicrographs from group-I showing (a) basement membrane (BM) of seminiferous cords (PAS, X100). From group-II showing (b) interstitial tissue (IT) and mediastinum (M) testis (PAS, X100). From group-III showing (c) basement membrane (BM) of seminiferous tubules and interstitial tissue (IT) in testis (PAS, X100). From group-IV showing (d) tunica albuginea (TA), blood vessels (BV) and interstitial tissue (IT) in testis (PAS, X100). From group-V showing (e) tunica albuginea (TA), blood vessels (BV) and interstitial tissue (IT) in testis (PAS, X100) and (f) basement membrane (BM) of seminiferous tubules and interstitial tissue (IT) in testis (PAS, X400)

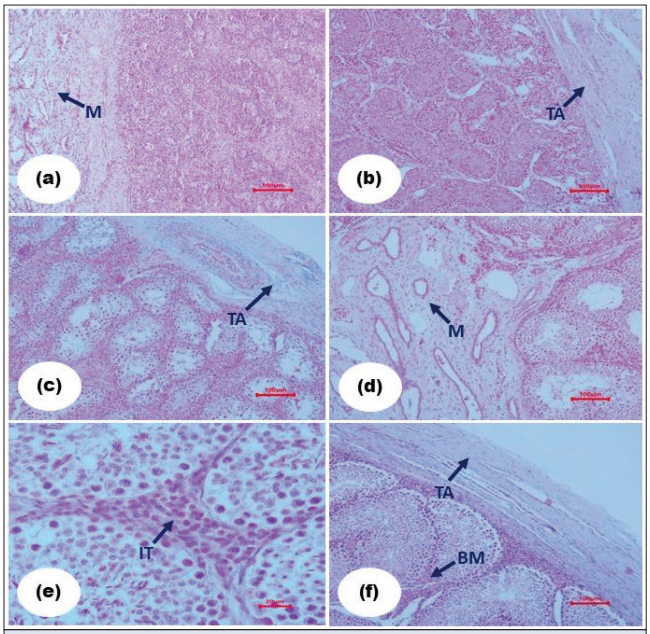

Fig 7. Photomicrographs from group-I showing (a) mediastinum (M) testis (PAS-AB, X100). From group-II showing (b) tunica albuginea (TA) in testis (PAS-AB, X100). From group-III showing (c) tunica albuginea (TA) in testis (PAS-AB, X100). From group-IV showing (d) mediastinum (M) testis (PAS-AB, X100) and (e) interstitial tissue (IT) in testis (PAS-AB, X400). From group-V showing (f) tunica albuginea (TA) and basement membrane (BM) of seminiferous tubules (PAS-AB, X100)

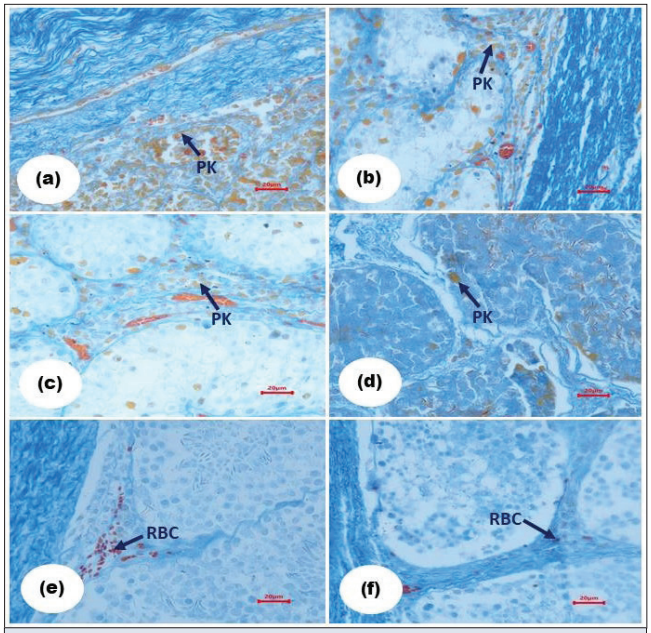

Fig 8. Photomicrographs from group-I showing (a) pre-keratin (Pr) activity in the seminiferous cords (Ayob, X400). From group-II showing (b-c) pre-keratin (Pr) activity in the interstitial cells (Ayob, X400). From group-III showing (d) pre-keratin (Pr) activity in the interstitial cells (Ayob, X400). From group-IV showing (e) erythrocytes (RBC) and negative for pre-keratin (Pr) activity in testis (Ayob, X400). From group-V showing (f) erythrocytes (RBC) and negative for pre-keratin (Pr) activity in testis (Ayob, X400)

the tunica albuginea, blood vessels and interstitial tissues *(Fig. 7-c)*. Few pre-keratin affinities also observed in the interstitial cells of the testes *(Fig. 8-d)*.

In group-IV, intense PAS activity was detected in the tunica albuginea, blood vessels, basement membrane of the seminiferous tubules, mediastinum and interstitial tissues of the testes *(Fig. 6-d)*. Weak PAS-AB reactivity was noticed in the tunica albuginea, seminiferous germinal epithelium, mediastinum and the basement membranes of the seminiferous tubules *(Fig. 7-d)*. No reactivity was noticed for keratin and pre-keratin in 5 months of age *(Fig. 8-e)*.

In group-V, the histochemical studies showed almost the same for the 6 and 5 months of age. A strong affinity for glycogen was observed in the tunica albuginea but was moderate in the basement membrane of the seminiferous tubules and the interstitial tissues of the testes *(Fig. 6-e,f)*. The acidic mucopolysaccharides were found to be weak in the tunica albuginea, blood vessels, interstitial tissues and in the mediastinum of the testes *(Fig. 7-f)*. No reactivity had been shown for the keratin and pre-keratin in the testes *(Fig. 8-f)*.

Scanning Electron Microscopic Studies

In this study, the scanning electron microscopic features were observed in the various locations of the testes during their postnatal development. In group-I, under

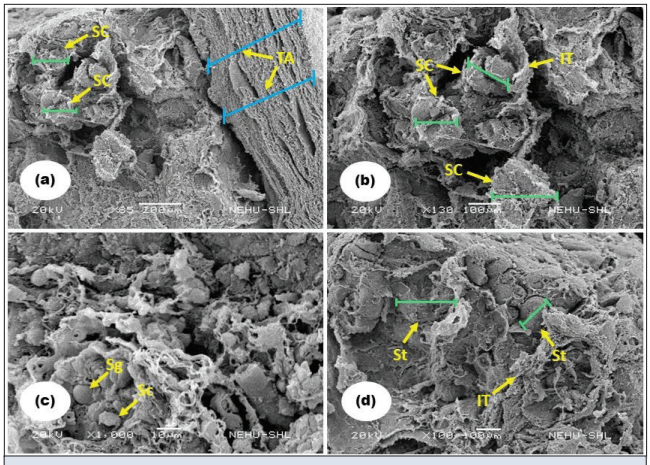

Fig 9. Scanning electron micrographs from group-I showing (a) seminiferous or sex cords (SC) and tunica albuginea (TA) in testis. (b) seminiferous cords (SC) and interstitial tissue (IT) in testis. (c) spermatogenic cell (Sg) and Sertoli cell (Sc) in seminiferous cords. From group-II showing (d) seminiferous tubules (St) and interstitial tissue (IT) in testis

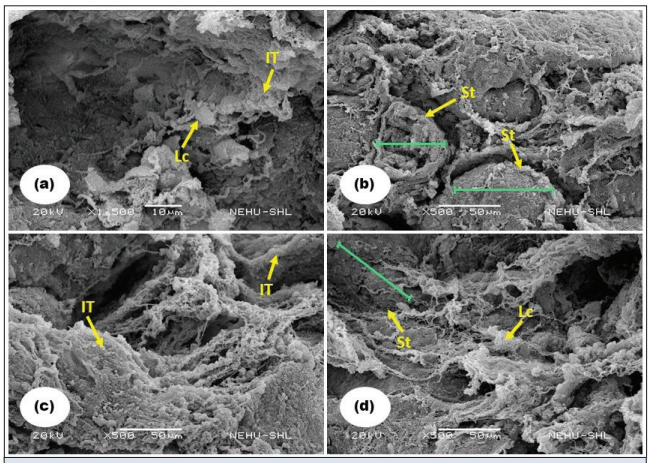

Fig 10. Scanning electron micrographs from group-II showing (a) interstitial tissue (IT) and Leydig cell (Lc) in testis. From group-III showing (b) seminiferous tubules (St) in testes. (c) interstitial tissues (IT) in testes.(d) seminiferous tubules (St) and Leydig cell (Lc) in testis

the scanning electron microscope, the tunica albuginea was viewed as the outermost covering of the testes. The parenchyma of the testes was recorded in numerous small-sized seminiferous or sex cords *(Fig. 9-a,b)*. The seminiferous cords contained round to oval-shaped spermatogonia and spherical-shaped Sertoli cells *(Fig. 9-c)*. The Leydig cells appeared irregularly polygonal in shape for the day-old piglets.

In group-II, well-defined seminiferous tubules were recorded from the 3 months of age *(Fig. 9-d)*. Interstitial tissue was observed between the tubules, which contained spherical to polygonal shaped Leydig cells. Under the scanning electron microscope, it is challenging to differentiate the spermatogonium and spermatogenic cells. Spermatogonium was noticed to be the largest cells in the seminiferous tubules *(Fig. 10-a)*.

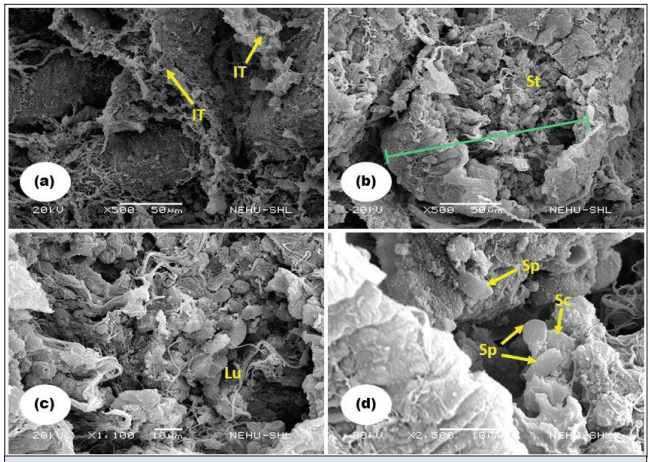

Fig 11. Scanning electron micrographs from group-III showing (a) interstitial tissues (IT) in testes. From group-IV showing (b) seminiferous tubule (St) contained spermatogenic cells in testes. (c) seminiferous lumen (Lu) contained spermatogenic cells in the testes. (d) seminiferous lumen contained spermatozoa (Sp) and Sertoli cells (Sc)

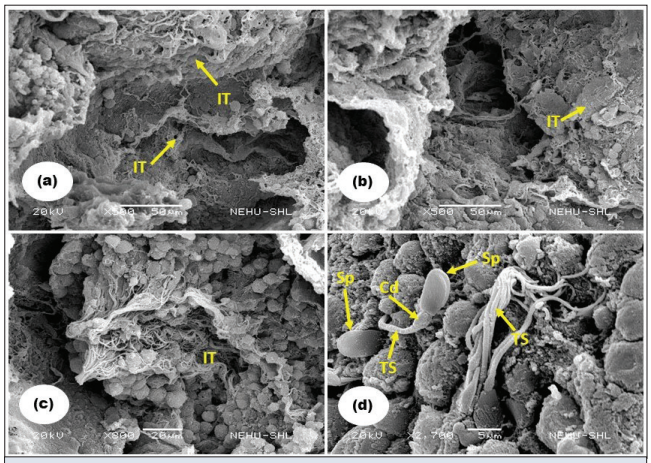

Fig 12. Scanning electron micrographs from group-V showing (a-c) interstitial tissues (IT) contained interstitial cells in testes. (d) the peripheral surface of the seminiferous lumen showing spermatozoa (Sp) and tail (TS) of the spermatozoa contained cytoplasmic droplets (cd) in the testis

In group-III, the scanning electron microscopy revealed numerous spermatogonia and spermatogenic cells in the seminiferous lumen *(Fig. 10-b)*. The round to elongated spermatids and the roughly triangular to elongated-shaped Sertoli cells were noticed in the seminiferous tubules. Additionally, polygonal-shaped Leydig cells were observed in the interstitial tissues *(Fig. 10-c,d, Fig. 11-a)*.

In group-IV, the widest lumen of the seminiferous tubules was viewed from the 5 months of age *(Fig. 11-b)*. The elongated to polygonal-shaped Leydig cells were observed in the in interstitial tissues. Sertoli cell, spermatogonium and spermatogenic cells were found in the luminal place of the seminiferous tubules *(Fig. 11-c)*. The heads of the spermatozoa were observed adjacent to the Sertoli cells in the tubules *(Fig. 11-d)*.

In group-V, the scanning electron microscopic observations were almost similar to the features of 5 and 6 months of age. The seminiferous luminal place exhibited Sertoli cells, spermatogonium and spermatogenic cells *(Fig. 12-a,b,c)*. Leydig cells were also observed in the interstitial tissues. Spermatozoa contained cytoplasmic droplets were seen on the luminal surface of the seminiferous tubules *(Fig. 12-d)*.

Discussion

In this study, the testicular capsule of the day-old piglets (group-I) contained predominantly collagen fibers [8]. The lumen of the seminiferous cords was visualized for some gonocytes and Sertoli cell-like structure in the periphery of the cords [9,10]. The fine elastic and reticular fibers were observed in the basement membrane of numerous seminiferous cords and the nerve fibers were noticed between the seminiferous cords and in the parenchyma of the teste [11]. Furthermore, the tunica albuginea of the testes was recorded for the collagen, elastic and reticular fibers at 3 months (group-II) of age [12,13]. The basement membrane of the seminiferous tubules, rete testis and the mediastinum testis were viewed for fine elastic and reticular fibers [14]. The lumen of the seminiferous tubules contained peripherally located Sertoli cells and a few elongated spermatids [8,15].

In the current investigation, the lumen of the seminiferous tubules observed rounds to elongated shaped spermatids in 4 months (group-III) of age [16]. The basement membrane of the seminiferous tubules and rete testis of the mediastinum testis contained collagen, reticular, and elastic fibers [17,18]. Elastic fibers gradually increased in the periphery of the seminiferous tubules in this study; this might be due to increased tubule elasticity to accommodate more luminal content. The study also observed an increase in the diameter of the seminiferous tubules from age 5 months (group-IV) onward. The basement membrane of seminiferous tubules, blood vessels and the rete testis were noticed for the elastic, nerve and reticular fibers [19]. In the present investigation, the histological findings in 6 months (group-V) were found to be almost similar to those of 5 months of age.

In this present study, PAS reactivity was recorded as weak to moderate in the tunica albuginea, basement membrane of the seminiferous cords, mediastinum and the trabeculae of the testes for group-I animals, which indicates less glycogen contained at their initial stage of life [8]. However, PAS reactivity was strong in the basement membrane of the seminiferous cords for the day-old kids [20,21]. The PAS-AB activity was found to be weak in the mediastinum, tunica albuginea and the interstitial tissues. The cytoplasm of some undifferentiated cells in the seminiferous cords was found positive for the

pre-keratin activity [17]. In the present investigation for group-II, the basement membrane of the seminiferous tubules, trabeculae and tunica albuginea were recorded instances PAS activity. The PAS-AB were moderate in the tunica albuginea, seminiferous germinal epithelium and the basemen membrane but the activity was weak in the interstitial tissues [8]. The pre-keratin activity was observed in the peritubular cells and the interstitial cells for 3 months of age, as reported earlier in pig testis [17].

In this study, the basement membrane of the seminiferous tubules, spermatids trabeculae, blood vessels in the capsules and the interstitial tissues were recorded strong PAS activity for group-III [19]. The peritubular cells and some interstitial cells also showed for pre-keratin affinity. The tunica albuginea, blood vessels, and interstitial tissues were observed to have weak to moderate PAS-AB activity [22]. In the current study, the PAS activity was intense in the tunica albuginea, basement membrane of the seminiferous tubules, mediastinum, interstitial tissues and the blood vessels for group-IV. However, the intense PAS reactivity in the tunica albuginea and basement membrane of the seminiferous tubules of pigs was also recorded earlier [23]. In this study, Sertoli cells were viewed as weak for glycogen activity [17]. The tunica albuginea, seminiferous germinal epithelium, mediastinum and the basement membrane of the seminiferous tubules recorded weak PAS-AB activity, this might provide the proper acidic environment for the spermatogenic cells. No major histochemical differences were observed between the 6 months (group-V) and 5 months aged testes in the present study. Intense PAS activity was noticed in the tunica albuginea, and moderate reactivity was observed in the basement membrane of the seminiferous tubules and interstitial tissues of the testes, which might be the reason for increased spermatozoa production at this stage. However, weak PAS-positive reactivity in the basement membrane of the seminiferous tubules also reported in pig and goat testes [17,21]. The PAS-AB activity was viewed as weak in the tunica albuginea, basemen membranes of the seminiferous tubules, blood vessels, interstitial tissues and the mediastinum of the testes [21]. However, the affinity for the PAS-AB activity was recorded as intense in the basement membranes of the seminiferous tubules and tunica albuginea of pig testes [8]. No reactivity was recorded for the keratin and pre-keratin in the testes of 6 months of age.

In the present investigation, the tunica albuginea was viewed under the scanning electron microscope as the most covering of the testes for group-I animals. Numerous small sized seminiferous or sex cords were viewed in the parenchyma of the testes. The interstitial tissues were viewed for irregular polygonal-shaped Leydig cells in the day-old piglets. However, Leydig cells were also viewed in the electron and light microscopic studies for piglets and microminipigs, respectively [8,24]. Under scanning electron microscopy, well-formed seminiferous tubules were observed from the testes of 3 months (group-II) of aged. The spherical to polygonal-shaped Leydig cells were noticed in the interstitial tissue and spermatogonium was noticed the largest cells in the seminiferous tubules [25]. In the current study, the seminiferous lumen was recorded in numerous spermatogonium and spermatogenic cells for the testes of group-III animals. The interstitial tissues were recorded and elongated to round-shaped Leydig cells [26]. In this study, the widest lumen of the seminiferous tubules was recorded from the 5 months (group-IV) of the aged. The polygonal-shaped Leydig cells were observed in the interstitial tissues [27]. The seminiferous luminal place was noticed for the Sertoli cells and the spermatogonium and spermatogenic cells [11]. In this study, the scanning electron microscopic features were observed to be almost similar for the 6 (group-V) and 5 months of the aged animals. The Sertoli cells and the spermatogonium and spermatogenic cells were viewed in the seminiferous tubules, whereas the Leydig cells were recorded in the interstitial tissues [7]. The data presented in this study can be the foundation for future research on the reproductive systems of domestic and wild animals in the era of artificial intelligence [28]. The present study revealed the histological, histochemical, and scanning electron microscopic characteristics of the testes based on the post-natal development of Mali pigs in Tripura. The research provides morphological insights into the testes and contributes valuable baseline information for future scientific studies on the male genital system of domestic animals.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author (O.P. Choudhary) upon reasonable request

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Declaration of Generating Artificial Intelligence: This article and/or tables and figures were not written/created by Artificial Intelligence (AI) and AI-assisted technologies.

Ethical Statement: The present study was conducted after approval from the Institutional Animal Ethical Committee. (Approval reference number. CVSC/CAU/IAEC/22-23/P-13 dated 31st October 2023).

Author's Contributions: Writing the original draft and design, R.S and P.C.K; methodology, R.S and A.K; planned and investigation, R.S, P.C.K and A.K; software, R.S, A.K, and P.J.D; formal analysis, R.S, A.K, P.J.D and O.P.C; writing-review, editing and visualization, R.S, P.J.D and O.P.C. All authors have read and agreed to the published version of the manuscript.

References

1. Dandapat A, Choudhury KD, Debbarma C, Das MK: Phenotypic characterization of Mali pig in Tripura, India. *Chest Livest Res Rural Dev,* 22 (4): 0-7, 2010.

2. Debbarma A, Koloi S, Sarkar D, Tripura S, Debbarma A, Debbarma K: Livestock and fodder production scenario of Tripura. *J Pharma Innov,* 10 (5): 18-20, 2021.

3. Rajathi S, Ramesh G, Kannan TA, Raja K: Postnatal morphological development of male reproductive organs in guinea pigs. *Haryana Vet,* 60 (2): 285-288. 2021.

4. Konig HE, Liebich HG: Veterinary anatomy of domestic animals. 6th ed., 413-419, Manson Publishing Ltd, London, UK, 2014.

5. Luna LG: Manual of histologic staining methods, Armed Forces Institute of Pathology. 3rd ed., 76-163, McGraw Hill Book Co London, New York, 1968.

6. Bancroft JD, Gamble M: Theory and Practice of Histological Techniques. 6th ed., Churchill Livingstone, Elsevier, China, 2008.

7. Penchev G: Postnatal development of interstitial endocrine (Leydig) cells in pig's testes-electron microscopy study. *Bulg J Vet Med,* 14 (3): 131-136, 2011.

8. Babu LS: Gross anatomical, histomorphological and ultrastructural studies of testis during postnatal development in pig *(Sus domesticus). PhD Thesis*. Maharashtra Animal and Fishery Sciences University, Bombay Veterinary College, Nagpur, India, 2012.

9. Kim S, Shin T: Immunohistochemical study of osteopontin in boar testis. *J Vet Sci,* 8 (2): 107-110, 2007. DOI: 10.4142/jvs.2007.8.2.107

10. Contreras-Ortiz AJ, Vigueras RM, Mendoza-Elvira SE, Martínez-Castañeda FE, Gutiérrez-Pérez O, Trujillo-Ortega ME: Postnatal testicular development in Vietnamese pot-bellied pigs. *Acta Histochem,* 123 (5): 151741, 2021. DOI: 10.1016/j.acthis.2021.151741

11. Sarma K, Devi J: Changes in the seminiferous epithelium of the testes during postnatal development in Assam goat. *Anat Res Int,* 2012 (1):620924, 2012. DOI: 10.1155/2012/620924

12. Bhagyalakshmi J, Balasundaram K, Selvaraj P, Kathirvel S, Balachandran P, Chandana GSS: Gross, histology and histochemical analysis of the testis in dog *(Canis lupus familiaris). J Entomol Zool Stud,* 8 (4): 69-73, 2020.

13. Abhin-Raj KP, Sreeranjini AR, Maya S, Vasudevan VN, Sunilkumar NS: Histomorphological studies on the testicular capsule of crossbred Large White Yorkshire boars *(Sus scrofa domesticus). J Vet Anim Sci,* 53 (2): 154-157, 2022. DOI: 10.51966/jvas.2022.53.2.154-157

14. Singh TS, Kalita PC, Choudhary OP, Doley PJ, Kalita A: Gross morphological, histological and histochemical studies on the epididymis of local pig (Zovawk) of Mizoram. *J Anim Res,* 9 (6): 855-861, 2019. DOI: 10.30954/2277-940X.06.2019.9

15. Khan SA, Kalhoro IB, Gandahi JA, Vistro WA, Kalwer Q, Ali I, Khokhar TA, Mangi MH: Puberty associated micro anatomical modification in testes and epididymis of Teddy goat *(Capra hircus). Pure Appl Biol,* 8 (2): 1281-1286, 2019. DOI: 10.19045/bspab.2019.80070

16. Neves ES, Chiarini-Garcia H, França LR: Ultrastructural observation of the mule testis indicates normal function of somatic cells. *Anim Reprod,* 2 (4): 263-271, 2018.

17. Sikarwar S: Gross and histological studies on the testes of large white Yorkshire pig (*Sus scrofa*). *PhD Thesis*. Rajasthan University of Veterinary and Animal Sciences, College of Veterinary and Animal Science, Bikaner, Rajasthan, India, 2018.

18. Sikarwar S, Mathur R, Dangi A: Histological studies of seminiferous tubules in the testes of large white Yorkshire pig *(Sus scrofa). J Pharm Innov,* 11 (7): 505-508, 2022.

19. Gofur MR, Khan MZI, Karim MR, Islam MN: Histomorphology and histochemistry of testis of indigenous bull *(Bos indicus)* of Bangladesh. *Bangladesh J Vet Med*, 6 (1): 67-74, 2008. DOI: 10.3329/bjvm.v6i1.1341

20. Sarma K, Kalita SN, Devi J: Postnatal age related histochemical studies on the testes in Assam goat *(Capra hircus). Vet Pract,* 18 (1): 74-76, 2017.

21. Sarma K, Kalita SN, Devi J: Histochemical studies on the testicular stroma, seminiferous tubules and the Leydig cells in Assam goats. *Explor Anim Med Res,* 8 (2): 168-172, 2018.

22. Singh A: Gross and histological studies on the testes of Marwari sheep *(Ovis aries). MVSc Thesis*. Rajasthan University of Veterinary and Animal Sciences, College of Veterinary and Animal Science, Bikaner, Rajasthan, India, 2013.

23. Singh TS, Kalita PC, Choudhary OP, Kalita A, Doley PJ: Histological, micrometrical and histochemical studies on the testes of large white Yorkshire pig *(Sus scrofa domesticus). Indian J Anim Res,* 54 (12): 1595-1598, 2020. DOI: 10.18805/ijar.B-3914

24. Kangawa A, Otake M, Enya S, Yoshida T, Shibata M: Histological changes of the testicular interstitium during postnatal development in microminipigs. *Toxicol Pathol*, 47 (4): 469-482, 2019. DOI: 10.1177/0192623319827477

25. Sarma K: Studies on post natal development of testis and epididymis in relation to thyroid activity in Assam goats *(Capra hircus). PhD Thesis*. Assam Agricultural University, College of Veterinary Science, Assam, India, 2009.

26. Singh TS: Comparative gross morphological, light and electron microscopic studies on the testes and epididymis of Zovawk and Large White Yorkshire pig. *MVSc Thesis*. Central Agricultural University, College of Veterinary Sciences and Animal Husbandry, Imphal, Manipur, India, 2018.

27. Yazama F, Nishida T, Kurohmaru M, Hayashi, Y: A scanning electron microscopy of the interstitial tissue of the boar testis. *Exp Anim,* 39 (1): 117-120, 1990. DOI: 10.1538/expanim1978.39.1_117

28. Choudhary OP, Saini J, Challana A: ChatGPT for veterinary anatomy education: An overview of the prospects and drawbacks. *Int J Morphol,* 41(4):1198-1202, 2023. DOI: 10.4067/S0717-95022023000401198

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Research Article

Evaluating *Linum usitatissimum* Seeds Extract as Potential Alternative Biochemical and Therapeutic Agent Against Induced Coccidiosis in Broiler Chicken

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Abstract

Coccidiosis is a significant disease of poultry and is usually treated using various synthetic anticoccidial drugs. However, the effectiveness of this approach has been compromised due to drug resistance. Medicinal plants are being considered as potential alternatives to these drugs. As part of ongoing research, an investigation was conducted to assess the anticoccidial potential of *Linum usitatissimum* seed extract (LUE) and its impact on hematological parameters in broiler chickens with experimental coccidiosis. A total of 108 broiler chicks were divided into six groups. The chicks in Groups I, II, and III were given plant extract at three different concentrations: 4%, 5%, and 6%, respectively, at one week of age. Group IV was the positive control - these chickens were treated with toltrazuril. Group V served as the negative control, meaning they were infected but not given any medication. Group VI was the normal control group. All groups, except Group VI, were orally infected with 60,000 sporulated oocysts when they were 18 days old. After 7 days of giving orally infection, six birds from each group were euthanized so that we could examine their feed conversion ratio (FCR), oocyst score, lesion score, fecal score, serum chemistry and hematology. The results showed that the *L. usitatissimum* extract exhibited anticoccidial activity. It improved the FCR and reduced lesion, oocyst, fecal scores and blood and serum chemistry.

Keywords: Anticoccidial, Chicken, Coccidiosis, *Linum usitatissimum*

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Introduction

Protozoa of the genus *Eimeria*, single-cell parasites from the phylum Apicomplexa, with complicated and several life cycle phases, cause avian coccidiosis [1]. *Eimeria* species mainly affect avian intestines, causing symptoms such as bloody diarrhea, poor feed conversion ratio (FCR), and even death. This results in considerable financial losses for chicken farming. *E. tenella* is one of the most harmful *Eimeria* species, causing cecal coccidiosis in hens. Each year, the poultry sector suffers significant financial losses due to coccidiosis, which starts with the intake of sporulated oocysts [2]. Due to quick sporulation process, naturally occurring coccidian oocysts abound and generate millions of oocysts [3]. Although Sulfanilamide was first anticoccidial utilized as a treatment against coccidiosis in poultry, a range of anticoccidial feed additives and antibiotics have also been developed and used. Synthetic chemicals and anti-coccidials are usually employed to control coccidiosis. However, reasonable use of anticoccidial medications resistance has evolved [4]. Furthermore, time is needed to locate some alternative tools for efficient coccidiosis control. Based on their Therapeutic and immunomodulatory activity,

various plants were documented as anticoccidial and immunomodulatory over the past ten years [5-7]. Using antioxidant-rich and biological active plant extracts has become especially important in view of resistance to synthetic antibiotics, phenols, flavonoids, tannins and saponins are being used as an alternate approach to treat coccidiosis [8,9]. Like other plant with anthelmintic and therapeutic activity, *Linum usitatissimum* often referred as linseed and locally known Alsi contains numerous pharmacological properties against different parasitic and bacterial infections due to its diverse antioxidant chemicals [10]. Therefore, the present study was designed to evaluate the anticoccidial capacity of *L. usitatissimum* seed against induced infection in chickens which was caused by *Eimeria* based on existing literature including antioxidant properties.

Material and Methods

Ethical Approval

The research is conducted by the approval from ethical committee of University of Agriculture Faisalabad under the PSF, Project No. 185 and PARB, Project No. 358 (No. 628/6-08-2013)

Plants Material

After procuring *L. usitatissimum* (Alsi) seeds from the local market and they were crushed using a grinder machine. Afterwards, we prepared an aqueous methanolic extract of *L. usitatissimum* using Soxhlet's apparatus (Velp Italy) following the method described by Abbas et al.[11]. The suspension was evaporated in a rotary evaporator (Heidolph Germany) at a temperature not exceeding 50°C. The prepared *L. usitatissimum* extract was then stored in a refrigerator at 4°C after freeze drying until further use.

Parasite

The parasite material was collected from outbreak cases in Faisalabad as well as the intestines of naturally infected chickens with *Eimeria*. To induce sporulation, the material was immersed in a potassium dichromate solution (2.5%) at 25-29°C and 60-80% humidity. The sample was next inspected under a microscope [12].

The number of sporulated oocysts was calculated using the Modified McMaster procedure. The material was put into the chambers of the McMaster slide and left alone for 2-3 min to let the sporulated oocysts to float and become visible. The slide was next examined under a microscope at low (10x) and high (40x) magnification

Experimental Design

A total of 108 broiler chicks were obtained and divided into six groups, with 18 birds in each group. Groups I, II, and III were given plant extract at three different concentrations (4%, 5%, and 6%, respectively) at one week of age. Group IV was kept as the positive control (infected plus toltrazuril® from A&K Pharmaceuticals, Faisalabad, provided at a rate of 1 mL/L of water). Group V served as the negative control (infected and non-medicated), and group VI as the normal control (non-infected and non-medicated). By the time the chickens reached eighteen days, all groups except group VI had been orally infected with 60.000 sporulated oocysts. Six birds from each group were sacrificed seven days after the inoculation to collect data on FCR, oocyst score, lesion score, fecal score, serum chemistry, and hematology.

Evaluation of Anticoccidial Activity

The potential of (LUE) as an anticoccidial treatment was investigated using metrics such as (FCR), lesion score [13] and oocyst score [14]. The fecal scores of birds in each group were monitored to assess the severity of illness at day 3 to 7. To evaluate the fecal score in chickens, a standard method is used. The optimal time to assess the emergence of illness is between the third and seventh days after inoculation. The fecal score chart ranges from 1 to 5, representing increasing degrees of disease progression [15].

Hematological Parameters

The collected blood samples were tested for packed cell volume (PCV) by following the microhematocrit method with slight modification. Hemoglobin level (Hb) was determined using Sahli's device. Erythrocyte and leukocyte counts were performed using a hemocytometer using Natt and Herrick solution under compound microscope at 10x.

Serum Chemistry

The plant extract was evaluated for toxic possession, cellular injury, and serum samples using various imported assays (Merck, Germany) to determine the levels of serum enzymes (LAT, LDH, Creatinine) [16].

Statistical Analysis

Statistical analysis was performed using the ANOVA approach and SAS statistical analysis software version 9 [17]. The data was considered statistically significant with a P value <0.05.

Results

All the groups offered with *L. usitatissimum* extract (LUE) revealed improved FCR at classified doses in *Table 1*. However, the admirable result showed by the group which was administered with higher dose and the results were similar (P>0.05) to standard medicine (Toltrazuril®)

A lower lesion score at graded doses was observed in (LUE) treated group in *Table 2*. However, the admirable result showed by the group which was administered with

Table 1. *Feed conversion ratio (FCR) of plant extract treated groups*

Groups	Feed Consumed (g)	Ending Weight (g)	Feed Conversion Ratio (g/g)*
L. usitatissimum 4%	920.1	418.18	2.20
L. usitatissimum 5%	990.2	405.45	2.44
L. usitatissimum 6%	941.5	408.90	2.30
Positive control	910.16	409.86	2.22
Negative control	964.4	368.40	2.61
Normal Control	916.6	422.6	2.16

**Due to feeding in group statistical analysis was not achievable because FCR is simple ratio*

Table 2. *Lesion score of different plant extract treated groups*

Groups	0	+1	+2	+3	+4	Mean
L. usitatissimum 4%	0	1	2	2	1	1.83 ± 0.54^{b}
L. usitatissimum 5%	0	2	2	2	0	1.50 ± 0.51^{c}
L. usitatissimum 6%	0	3	3	0	0	1.66 ± 0.83^{bc}
Positive control	1	3	2	0	0	1.33 ± 0.40^{d}
Negative control	0	0	0	3	3	3.33 ± 0.51^{a}
Normal Control	0	0	0	0	0	0

Means with different superscripts are significantly different (P<0.05) from each other
+1: No lesions, +2: Very few, +3: Large amount, +4: Blood and Death

Table 3. *Oocyst score of treated groups with plant extract*

Groups	0	+1	+2	+3	+4	+5	Mean
L. usitatissimum 4%	0	1	3	1	1	0	2.00 ± 0.75^{b}
L. usitatissimum 5%	0	2	2	2	0	0	1.50 ± 0.51^{c}
L. usitatissimum 6%	0	3	3	0	0	0	1.66 ± 0.54^{bc}
Positive control	0	2	3	1	0	0	1.66 ± 0.75^{bc}
Negative control	0	0	0	3	2	1	3.83 ± 0.40^{a}
Normal Control	–	–	–	–	–	–	–

Means with different superscripts are significantly different (P<0.05) from each other
0: No oocyst, +1: 1-10 oocyst/Field, +2: 11-20 oocyst/Field, +3: 21-50 oocyst/Field, +4: 51-100 oocyst/Field, +5: More than 100 oocyst/Field

Table 4. *Fecal score of groups treated with Plant Extract*

Groups	Day 3rd	Day 4th	Day 5th	Day 6th	Day 7th
L. usitatissimum 4%	–	2.04 ± 0.75^{ab}	2.15 ± 0.75^{b}	1.12 ± 0.63^{b}	–
L. usitatissimum 5%	–	1.34 ± 0.51^{b}	2.01 ± 0.63^{b}	1.52 ± 0.54^{b}	–
L. usitatissimum 6%	–	1.52 ± 0.54^{ab}	1.68 ± 0.51^{b}	1.51 ± 0.54^{b}	–
Positive control	–	1.51 ± 0.83^{ab}	1.68 ± 0.51^{b}	1.34 ± 0.51^{b}	–
Negative control	–	2.68 ± 1.40^{a}	2.99 ± 0.42^{a}	2.71 ± 0.79^{a}	–
Normal Control	–	–	–	–	–

Means with different superscripts are significantly different (P<0.05) from each other

Table 5. *Serum enzymes values of groups treated Plant Extract (Mean±SD)*

Groups	ALT	ASAT	LDH	Urea	Creatinine
L. usitatissimum 4%	9.46±0.91^{b}	180.72±10.08^{b}	476.01±16.91^{b}	5.40±0.81^{b}	0.21±0.02^{b}
L. usitatissimum 5%	9.57±0.97^{b}	182.60±12.14^{b}	483.95±21.52^{b}	5.55±0.97^{b}	0.18±0.02^{b}
L. usitatissimum 6%	9.74±1.38^{b}	177.55±14.72^{b}	477.24±22.21^{b}	5.60±1.02^{b}	0.19±0.03^{b}
Positive control	9.69±1.15^{b}	181.85±10.15^{b}	477.42±21.15^{b}	5.20±0.48^{b}	0.19±0.02^{b}
Negative control	24.62±2.31^{a}	288.87±36.21^{a}	891.96±22.16^{a}	20.60±1.12^{a}	0.70±0.03^{a}
Normal Control	8.98±1.78^{b}	195.03±13.46^{b}	471.45±15.78^{b}	5.45±0.59^{b}	0.16±0.02^{b}

Means with different superscripts are significantly different (P<0.05) from each other

Table 6. *Hematological values o of groups treated Plant Extract (Mean±SD)*

Groups	PCV %	Hb g/dL	RBC 10^{6}/µL	WBC 10^{3}/µL
L. usitatissimum 4%	29.14±1.67^{a}	10.22±1.06^{a}	3.99±0.76^{a}	21.67±2.93^{b}
L. usitatissimum 5%	28.20±1.61^{a}	11.43±1.35^{a}	3.64±0.86^{a}	22.74±2.70^{b}
L. usitatissimum 6%	27.01±1.66^{a}	11.81±1.28^{a}	3.41±0.71^{a}	21.66±1.64^{b}
Positive control	24.17±2.14^{a}	11.20±0.64^{a}	3.27±0.71^{b}	21.41±2.78^{b}
Negative control	20.15±1.15^{b}	8.91±0.82^{b}	1.88±0.12^{c}	34.01±5.04^{a}
Normal Control	25.34±1.03^{a}	11.0±1.34^{a}	3.15±0.58^{ab}	22.51±3.26^{b}

Means with different superscripts are significantly different (P<0.05) from each other

a higher dose and the results were similar (P>0.05) to standard medicine (Toltrazuril®)

All groups treated with *L. usitatissimum* extract (LUE) showed minimal oocyst scores at graded doses in *Table 3*. However, the admirable result showed by the group which was administered with higher dose and the results were similar (P<0.05) to standard medicine (Toltrazuril®)

L. usitatissimum extract (LUE) administered groups showed minimal fecal score at graded doses in *Table 4*. However, the admirable result showed by the group which was administered with higher dose and the results were similar (P<0.05) to standard medicine (Toltrazuril®)

Minimal serum enzyme values in all (LUE) administered groups were observed in *Table 5*. However, the admirable result showed by the group which was administered with higher dose and the results were similar (P>0.05) to standard medicine (Toltrazuril®)

Maximum hematological (PCV, Hb, RBCs and WBCs) values were observed in *Table 6*. However, the admirable result showed by the group which was administered with higherdose and the results were similar (P>0.05) to standard medicine (Toltrazuril®)

Discussion

Recent reports have explored alternative methods for treating coccidiosis by using plant antioxidant composites such as phenols, flavonoids, tannins, and saponins [1,4,11,18]. This investigation found that *L. usitatissimum* shows anticoccidial activity and has positive effects on serum chemistry, hematological values, oocyst score, lesion score, and fecal score. The results were identical to those of accomplished with the traditional medication (Toltrazuril®). Specifically, *L. usitatissimum*, when administered at doses of 4%, 5%, and 6%, confirmed a dose-dependent potential to combat mixed *Eimeria* infections. The maximum concentration confirmed results corresponding to the Toltrazuril® dealt with group (P>0.05). Previous studies inspecting the capacity of various herbal extracts to combat coccidiosis have also mentioned similar findings, with the effectiveness of the extracts varying depending on the dosage administered [19-21].

In a study, the weight gains in broiler chicks infected with *Eimeria* was significantly reduced by an ethanolic extract from Carica papaya leaves [22]. Studies showed that orally administered *Ageratum conyzoides* extract to 28-day-old chicks increased the number of red blood cells (RBCs), white blood cells (WBCs), and PCV in the treated birds. This eventually resulted in a decrease in infection levels and excretion of oocysts. The antioxidant chemicals found in *A. conyzoides*, including flavonoids, phenols, conyzorium, methexnebilitin, and quercetin, may reduce the oxidative stress caused by coccidiosis. Likewise, *A. conyzoides* has been found to have anticoccidial properties. This means that it can diminish *Eimeria* infection by inquisitive with lipid peroxidation [8,23]. The study found that the groups given the plant extract had lower levels of certain serum enzymes similar to the control groups, indicating that the

extract did not have any harmful effects. Hepatotoxicity can be determined by looking at the levels of AST and ALT, while nephrotoxicity can be determined by studying the levels of serum creatinine and urea.

A recent study showed that pulp of olive fruit when combine with vitamin C gives positive results on hatchability, weight gain, and improved growth rate [24]. Recent study of catechin *Uncaria gambir* extract which is planned on broiler birds to see meat quality, growth rate, serum /Plasma values and also antioxidant activity gives favorable results [25].

There are some other studies which showed the antioxidant, biomedical, immunomodulatory, antibacterial, resistant free effects of plant material, zinc oxide, Sodium Alginate and nanoparticles on animals [26].

The findings mentioned above indicate that plant-derived extracts could effectively help in dealing with chicken coccidiosis and its associated toxicity. These results also highlight the potential of *L. usitatissimum* as a safe and herbal anticoccidial agent, which warrants further research into the plant's active substances and modes of action. By incorporating *L. usitatissimum* into chicken health management, the risk of drug resistance may be reduced, and overall chicken health may be improved. This could present a sustainable and successful alternative to synthetic anticoccidial medicines

Declarations

Availability of Data and Materials: Research data will be provided by the author (K. Hussain) on request.

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Ethical Approval: The research is conducted by the approval from ethical committee of University of Agriculture Faisalabad under the PSF, Project No. 185 and PARB, Project No. 358 (No. 628/6-08-2013)

Competing Interest: There is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): This whole article is free from any AI tool.

Author Contributions: KH apprehended and planned the study; AA, AR, MUW, BA, MASM did work on methodology and RZA, MAZ, JAK, MAR and investigated the data and help in the writeup of the manuscript

References

1. Abbas A, Alkheraije KA: Immunomodulatory effects of *Carica papaya* extract against experimentally induced coccidiosis in broiler chickens. *Pak Vet J,* 43 (3): 628-632, 2023. DOI: 10.29261/pakvetj/2023.089

2. Chapman HD: Milestones in avian coccidiosis research: A review. *Poult Sci,* 93, 501-511, 2014. DOI: 10.3382/ps.2013-03634

3. Fall AD, Gbati OB, Diatta W, Lapo RA, Diatta-Badji K, Dieng M, Ibra S, Dieng SIM, Bassene E, Pangui LJ: Anticoccidian activity of ethanol roots extract of *Cassia sieberiana* DC in chickens. *European J Med Plants,* 11, 1-7, 2016. DOI: 10.9734/EJMP/2016/21343

4. Hussain K, Abbas A, Alanazi HAH, Alharbi AMA, Alaiiri AA, Rehman A, Waqas MU, Raza MA, Yasin R, Ahmad B, Bano N, Khera HURA: Immunomodulatory effects of *Artemisia brevifolia* extract against experimentally induced coccidiosis in broiler chicken. *Pak Vet J,* 43 (2): 333-338, 2023. DOI: 10.29261/pakvetj/2023.026

5. Khalifa WH, Sallam MG, Kamel NN, Samy A, Yassein SA, El-Mallah GM, Abusinaa GE: Using *in-ovo* injection of olive pulp extract and vitamin C to improve hatchability, post hatch growth performance, carcass traits and some biochemical blood analysis in broiler chickens. *Int J Vet Sci,* 12 (3): 353-359, 2023. DOI: 10.47278/journal.ijvs/2022.200

6. Liaqat I, Noor S, Qureshi AS, Ali S, Liaqat I, Al-Arifa N, Alam S, Ajmal A, Zia T, Munawar M: Biosynthesis and evaluation of *Cinnamomum zeylanicum* nanomaterials for the treatment of polycystic ovary syndrome in mice. *Pak Vet J,* 43 (1): 118-124, 2023. DOI: 10.29261/pakvetj/2023.004

7. Farieha, Jahan N, Rehman KU, Ali S: Effect of herbal mixture as angiotensin-converting enzyme inhibitor in angiotensin-II dependent hypertension. *Pak Vet J,* 39 (1): 25-30, 2019. DOI: 10.29261/pakvetj/2018.103

8. Nweze NE, Obiwulu IS: Anticoccidial effects of *Ageratum conyzoides*. *J Ethnopharmacol,* 122 (1): 6-9, 2009. DOI: 10.1016/j.jep.2008.11.014

9. Velázquez-Antunez J, Olivares-Perez J, Olmedo-Juárez A, Rojas-Hernandez S, Villa-Mancera A, Romero-Rosales T, Zamilpa A, Gonzalez-Cortazar M: Biological activity of the secondary compounds of *Guazuma ulmifolia* leaves to inhibit the hatching of eggs of *Haemonchus contortus*. *Pak Vet J,* 43 (1): 55-60, 2023. DOI: 10.29261/pakvetj/2022.075

10. Al-Saeed FA, Bamarni SSI, Iqbal KJ, Rehman TU, Faruk AZ, Mahmood S, Şahin T, Ölmez M, Riaz R: *In vitro* anthelmintic efficacy of *Haloxylon salicornicum* leaves extract using adult *Heamonchus contortus* worms. *Pak Vet J,* 43 (1): 91-96, 2023. DOI: 10.29261/pakvetj/2022.091

11. Abbas A, Iqbal Z, Abbas RZ, Khan MK, Khan JA, Sindhu ZD, Mahmood MS, Saleemi MK: *In vivo* anticoccidial effects of *Beta vulgaris* (sugar beet) in broiler chickens. *Microb Pathog,* 111, 139-144, 2017. DOI: 10.1016/j.micpath.2017.07.052

12. Ryley JF, Meade R, Burst JH, Robinson TE: Methods in coccidiosis research separation of oocysts from faeces. *J Parasitol,* 73, 311-326, 1976. DOI: 10.1017/S0031182000046990

13. Johnson J, Reid WM: Anticoccidial drugs lesion scoring techniques in battery and floor pen experiments with chickens. *Exp Parasitol,* 28, 30-36, 1970. DOI: 10.1016/0014-4894(70)90063-9

14. Hilbrich P: Krankheiten des geflugelsunterbesonderedberucksichitigung der haltung und futterung. Hermann kuhn kg. Schwenningen am neckar Germany, 1978.

15. Habibi H, Firouzi S, Nili H, Razavi M, Asadi SL, Daneshi S: Anticoccidial effects of herbal extracts on *Eimeria tenella* infection in broiler chickens: *In vitro* and *in vivo* study. *J Parasit Dis,* 2014. DOI: 10.1007/s12639-014-0517-4

16. Abbas RZ, Iqbal Z, Khan MN, Zafar MA, Zia MA: Anticoccidial activity of *Curcuma longa* L. in broiler chickens. *Braz Arch Biol Technol,* 53, 63-67, 2010. DOI: 10.1590/S1516-89132010000100008

17. SAS: SAS Statistical Software Version 9.1. SAS Institute Inc. Cary, NC, USA, 2004.

18. Molan AL, Liu Z, Shampa DE: Effect of pine bark *(Pinus radiata)* extracts on sporulation of coccidian oocysts. *Fol Parasitol,* 56, 1-5, 2009. DOI: 10.14411/fp.2009.001

19. Dkhil MA, Abdel-Baki AS, Wunderlich F, Sies H, Al-Quraishy S: Anticoccidial and anti-inflammatory activity of garlic in murine *Eimeria papillata* infections. *Vet Parasitol,* 175, 66-72, 2011. DOI: 10.1016/j.vetpar.2010.09.009

20. Yang WC, Tien YJ, Chung CY, Chen C, Chiou WH, Hsu SY, Liu HY, Liang CL, Chang CLT: Effect of *Bidenspilosa* on infection and drug

resistance of Eimeriain chickens. *Res Vet Sci,* 98, 74-81, 2015. DOI: 10.1016/j.rvsc.2014.11.002

21. Wang D, Zhou L, Li W, Zhou H, Hou G: Anticoccidial effect of *Piper sarmentosum* extracts in experimental coccidiosis in broiler chickens. *Trop Anim Health Prod,* 5, 1071-1078, 2016. DOI: 10.1007/s11250-016-1034-5

22. Nghonjuyi NW, Tiambo CK, Kimbi HK, Manka'a N, Juliano, F. Lisita RS: Efficacy of ethanolic extract of *Carica papaya* leaves as a substitute of sulphanomide for the control of coccidiosis in KABIR chickens in cameroon. *J Anim Health Prod,* 3 (1): 21-27, 2015. DOI: 10.14737/journal.jahp/2015/3.1.21.27

23. Hussain K, Alsayeqh AF, Abbas A, Abbas RZ, Rehman A, Zaib W, Rehman TU, Mahmood MS: Potential of *Glycyrrhiza glabra* (Licorice) extract an alternative biochemical and therapeutic agent against coccidiosis in broiler chickens. *Kafkas Univ Vet Fak Derg,* 28 (5): 585-591, 2022. DOI: 10.9775/kvfd.2022.27620

24. Ramaiyulis, Mairizal, Salvia, Fati N, Malvin T: Effects of dietary catechin *Uncaria gambir* extract on growth performance, carcass characteristics, plasma lipids, antioxidant activity, and nutrient digestibility in broiler chickens. *Int J Vet Sci,* 12 (2): 169-174, 2023. DOI: 10.47278/journal.ijvs/2022.177

25. Elbehary M, Dowidar YA, Ashour AM, Abd El Fattah EM, Monir A: Effect of *in vitro* maturation medium supplementation with chitosan nanoparticles on the river buffalo cumulus-oocyte complexes. *Int J Vet Sci,* 12 (3): 341-346, 2023. DOI: 10.47278/journal.ijvs/2022.188

26. El-Hamaky AMA, Hassan AA, Wahba AKA, El Mosalamy MMEA: Influence of copper and zinc nanoparticles on genotyping characterizations of multi-drug resistance genes for some calf pathogens. *Int J Vet Sci,* 12 (3): 309-317, 2023. DOI: 10.47278/journal.ijvs/2022.195

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Research Article

Molecular and Pathological Detection of Jaagsiekte Sheep Retrovirus in Lung Tissues of Sheep

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Abstract

Ovine pulmonary adenocarcinoma (OPA) is a disease of sheep that is caused by a *Betaretrovirus* named exogenous Jaagsiekte sheep retrovirus (exJSRV). This virus causes oncogenic transformation in lungs, and symptoms develop related to the growing tumors. Disease develops slowly with a long incubation time ranging 2-4 years. Currently there is no serological test to evaluate the presence in the flock and also disease can mostly be diagnosed post mortem. The aim of this study is to determine characterization and the molecular presence exJSRV types in circulation. In this study lung tissues of 25 suspected cases were investigated. Initial diagnosis is made by histopathological (HP) and immunohistochemical (IHC) methods. Hematoxylin & Eosin (H&E) staining was used for examining histopathological changes. Anti JSRV capsid antibody was used with Streptavidin-Biotin peroxidase method. Slides were examined under light microscope and photographs were taken. All 25 cases were diagnosed as OPA with these methods. Lung tissues embedded in paraffin were used as material for nucleic acid extraction. Envelope gene of JSRV nucleic acid was chosen for investigating with reverse transcription polymerase chain reaction (RT-PCR). Since paraffinized tissue blocks were used, sensitivity was not high and only 10/25 tissues were deemed positive. Positive amplicons were sent to sequencing. A phylogenetic tree was constructed after analyzing the sequences. Also predicted amino acid sequences were analyzed. In conclusion we found both type 1 and type 2 exJSRV have been circulating in the region and changes in amino acids were detected which could lead to possible differentiation in pathogenesis.

Keywords: Histopathology, Immunohistochemistry, Jaagsiekte sheep retrovirus, Phylogenetic analysis, Polymerase chain reaction, Sheep

Introduction

Ovine pulmonary adenocarcinoma (OPA) or Jaagsiekte sheep retrovirus (JSRV) is a neoplastic disease characterized by tumor lesions in the lung. This condition is caused by the exogenous form of JSRV (exJSRV) [1,2]. JSRV is the only known virus for naturally causing lung cancer in animals [3]. Sheep is the natural host of the virus and there are studies that report the disease in goats. Causative agent is classified in *Retroviridae* under genus *Betaretrovirus* and species *Betaretrovirus ovijaa* which has single stranded, positive sense RNA genome consisting of approximately 7400 bases. JSRV also has an endogenous form (enJSRV) which is integrated in DNA of many sheep but not associated with the disease [1,4-6]. JRSV genome codes four viral proteins namely *env*, *gag*, *pol*, *pro.* Among these proteins *env* is responsible for inducing oncogenic transformation [6,7]. exJSRV is suggested to have two types. Type 1 is based on South African, Kenyan origin and type 2 is United Kingdom or American origin [1,5]. However, these two types are not reported to have any differences in pathogenicity. Enzootic nasal tumor virus (ENTV) is also another *Betaretrovirus* that is closely related to JSRV and cause a disease with similar course [3,7]. Other *Lentiviruses* (such as Visna-Maedi disease) may also cause coinfection in some cases which may mask diagnosis or worsen the outcome of the disease [8].

World Organisation of Animal Health (WOAH) has reported [8] OPA is widespread around the world where there is sheep livestock, causing economic loss. Affected animals have symptoms related to lung lesions; dyspnea,

cough, nasal discharge is most common in the early stages of the disease [6]. As the disease progresses, symptoms become more apparent, animals have difficulty moving and excess (mucous) fluid is discharged from nares when hind legs are elevated. This is called wheelbarrow test and it is the main clinical symptom leading to diagnosis. Clinical symptoms are worsened by secondary infections and death is commonly caused the secondary infectious agents. Bacterial or verminous bronchopneumonia is common with OPA cases and can be seen solely or in combination in necropsy [6-8].

Disease has a long incubation period, ranging from months to years. The time disease is most prevalent is between ages 2 to 4, although it can rarely be seen younger animals [8,9]. Transmission of the virus occurs mostly with aerosols and lamb can also be infected with the virus by milk/colostrum. Transplacental crossing of the virus does not have a significant role in transmission. Virus is present in lung fluid, tumors, regional lymphoid tissues and sometimes in peripheral blood [6-8].

Although virus genome has been extensively studied molecularly and many strains are sequenced, no permissive cell culture has been found to propagate the virus. Only some tumor cells from young lambs can be used to maintain the virus for a short period [8]. No circulating antibodies develop after OPA infection [5]. Viral proteins can be found only in tumor cells but not anywhere else [6,10]. There are some related problems caused by aforementioned information; firstly, no vaccine is available, secondly no routine early diagnostic test detection is present and thirdly no seroprevalence test can be applied to monitor the disease in a flock. Isolation or compulsory slaughter of the infected animals can be considered the only method of prevention [6,8].

The aim of this study is to investigate the presence of exJRSV from paraffin embedded tissue blocks and determine the molecular characterization of circulating isolates. Data such as exJSRV types that is in circulation cannot be identified without molecular analyses, for this reason sequencing and phylogenetical analyses were mandatory.

Material and Methods

Ethical Approval

The study was approved by Kafkas University Animal Experiments Local Ethics Committee (Decision Number: KAÜ-HADYEK/2021-180.)

Material

Material of the study consists of 25 sheep lung tissues embedded in paraffin which belong to Pathology Department's tissue archive. Lung tissues were collected between years 2010-2018. These tissues belong to animals suspected of the disease.

Histopathological (HP) Examinations

Lung tissues were fixed in 10% formalin and routine procedures were followed for paraffin embedding. Sections were taken into slides and Hematoxylin & Eosin (H&E) staining was used for examining histopathological changes. Detailed examinations and photographing were done under light microscope.

Immunohistochemical (IHC) Examinations

Streptavidin-Biotin peroxidase method was used for immunostaining according to manufacturer instructions (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL). Anti JSRV capsid (CA) antibody (Supplied by Professor Massimo Palmarini, used with dilution 1/1500 and incubated overnight at 4ºC) was applied after antigen retrieval and blocking of non-specific proteins. Reactions were generated using aminoethyl carbazole (AEC) as chromogen (Thermo Scientific, TA-125-HA). Contrast staining was done with hematoxylin. Slides were mounted with AEC specific mounting media. Phosphate Buffer Saline (PBS) was used instead of primary antibody for control slides.

Slides were examined under light microscope (Olympus Bx53) and photographs were taken with Cell^P software (Olympus Soft Imaging Solutions GmbH, 3,4). Detailed analyzes were done with Image J software (1.51j8).

Nucleic Acid Extraction

Five to ten sections of 10 microns were taken from paraffin embedded tissue blocks and put to 1.5 mL tubes for each case. The extraction was carried out according to Pikor et al.[11]. The method described yields in total DNA and RNA if no RNase is added. For this reason, no RNase was added to yield RNA as much as possible. This step is the only modification of the aforementioned method.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Since exJSRV is RNA form it was necessary to perform reverse transcription before submitting samples to polymerase chain reaction (PCR). This was achieved using onestep RT-PCR kit by Hibrigen (Türkiye). The primer pair (targeted for envelope KT279065.1 gene), was used as reported by Mansour et al.[9]. The conditions were modified, for reverse transcription 40 min at 55ºC, 95ºC5 min, was used for initial denaturation, 35 cycles of 95ºC denaturation 1 min, 58ºC 1 min of annealing and 72ºC 1 min for extension followed by final extension at 72ºC for 10 min. Expected amplicon size was 398 base pairs.

Phylogenetic Analysis

Positive amplicons (10 in total) were sent to a commercial company (BM Laboratuvar Sistemleri, Ankara) for Sanger sequencing. Sequence assembly and editing were performed using Bioedit (Version 7.0.5.3) and Clustal W [12], before comparing with the GenBank nucleotide sequence database for sequence similarities using the Basic Length Alignment Search Tool (BLAST) software of the National Center for Biotechnology Information (NCBI) [13]. Phylogenetic analysis was performed using MEGA7 software's neighbour-joining method [14] and the evolutionary distances between different sequences were calculated by the Kimura two-parameter model. The confidence level of the NJ tree was assessed by bootstrapping using 1000 replicates. A table of amino acid comparison was also prepared according to the sequence data to examine the differences.

Results

Histopathological Results

Several widespread tumor foci of varying dimensions were observed in lung tissues. These foci consisted mostly of papillary extensions or acinar structures originating from bronchiolar or alveolar cells. Tumoral cells were in the shape of cuboidal or columnar. Mitotic figures were very few. Stroma layers of tumors were thickened because of the increased count of mononuclear cells and extension of the connective tissue. Alveolar macrophages and mononuclear cells were present around the neoplastic foci. In some cases, parasitic agents were also identified in addition to OPA findings and there was neutrophil leukocyte infiltration in some cases, indicating secondary bacterial infection *(Fig. 1-a,b)*.

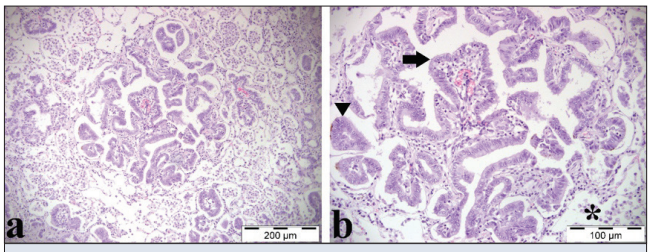

Fig 1. a: Lung, Tumoral foci, **b:** Papillary *(arrow)* and acinar *(arrowhead)* proliferations that extend to the lumen, alveolar macrophages *(asterisk)* in the tumoral vicinity

Immunohistochemical Results

JSRV positive reactions were mostly observed in cytoplasms of cuboidal or columnar cells that form papillary or acinar structures. There was also positivity in cytoplasms of alveolar macrophages in some areas surrounding tumoral region *(Fig. 2-a,b)*.

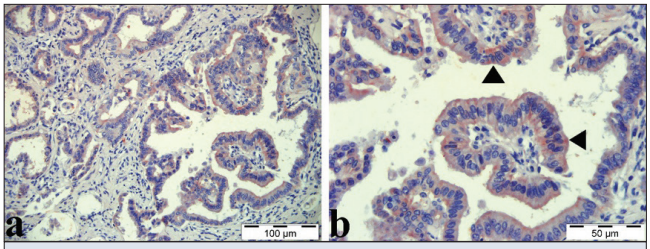

Fig 2. a-b: JSRV positive reactions *(arrowheads)* in cytoplasm of the tumoral cells in papillary and acinar tumoral regions

Molecular Results

Ten of the 25 samples of paraffin embedded tissues were positive with the RT-PCR procedure and had correct size amplicons visualized at 398 bp. 7 amplicons were sequenced and deposited to GenBank with accession numbers OM953765, OM953766, OM953767, OM953768, OM953769, OM953770, OM953771. Other 3 amplicons could not be sequenced due to low nucleic acid concentration.

A phylogenetic tree was constructed with the reference sequences obtained from GenBank. Endogenous forms of JSRV and exogenous JSRV reference sequences of type 1 and type 2 were included. ENTV references were added to indicate the close distance relation and other retroviruses were included for outgrouping. OM953768 and OM953770 were clustered with type 2 exJSRV references and our other remaining 5 isolates were clustered with type 1 exJSRVs *(Fig. 3)*.

Histopathological results belonging to all 25 cases indicated characteristic OPA findings. These results were further

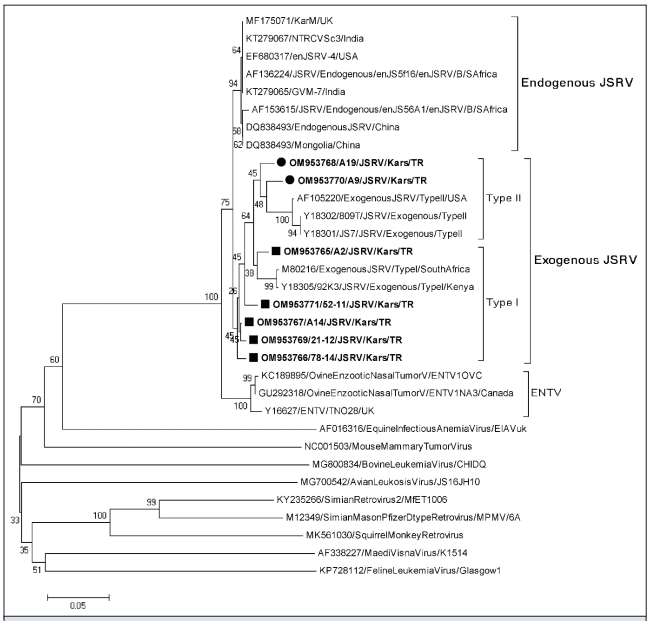

Fig 3. Phylogenetic tree constructed using *env* gene reference strains and our isolates. Black circles are type 2 isolates of this study and black squares are type 1 isolates of this study

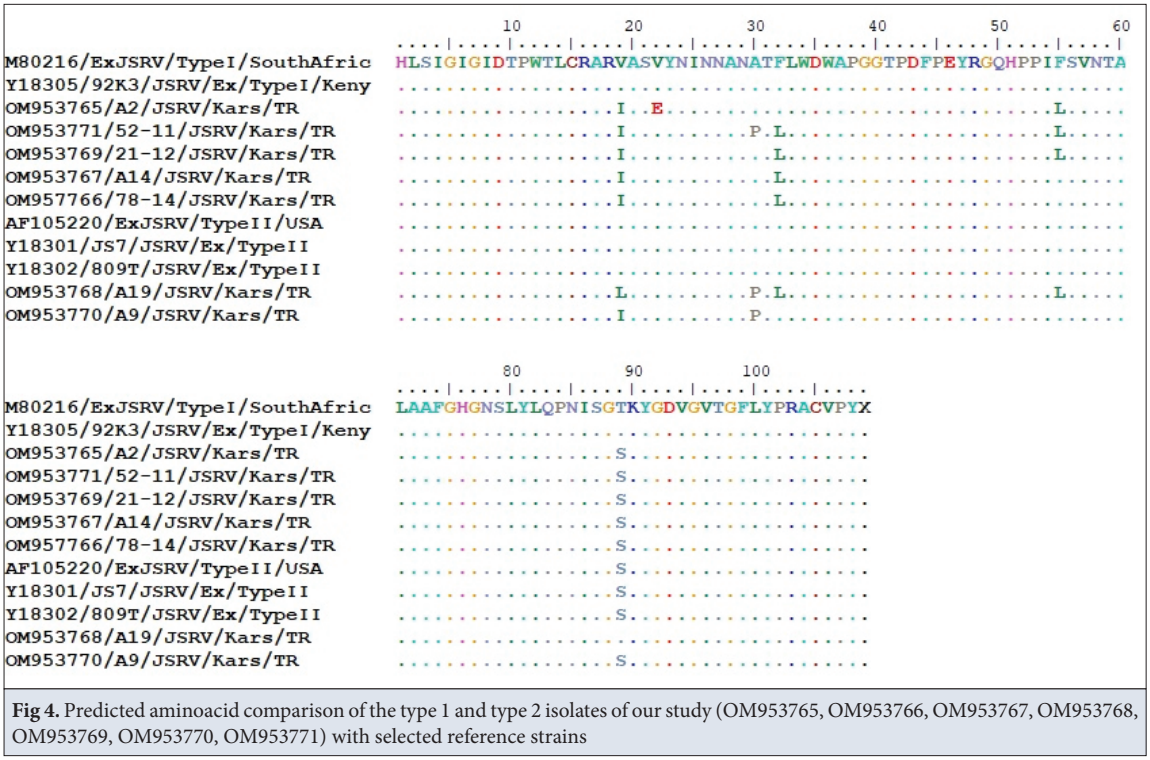

Fig 4. Predicted aminoacid comparison of the type 1 and type 2 isolates of our study (OM953765, OM953766, OM953767, OM953768, OM953769, OM953770, OM953771) with selected reference strains

confirmed with immunohistochemical results which were stained with spesific antibody to JSRV envelope. According to these two tests, all tissues were diagnosed as OPA. However, it was not possible to detect exJSRV nucleic acid in all tissues with onestep RT-PCR. RNA of the virus was found in 10 of the 25 tissues. This is not an indication the remaning 15 cases did not contain nucleic acid, only disadvantage of using paraffin embedded tissue blocks. These 15 tissues were regarded as false negatives as histochemical and immunohistochemical findings confirmed presence of OPA.

Predicted amino acid content comparison based on sequence is shown in *Fig. 4*. There are certain changes that is distinctive in our isolates for both type 1 and type 2. There is valine to isoleucine change in 19th position. While all references on this position have valine, our isolates have isoleucine, except one type 2 isolate (OM953768) which has leucine. This change is the most consistent among our isolates although there are some other changes as shown in the *Fig. 4*.

Discussion

OPA can either be misdiagnosed because of secondary infections or undiagnosed because of early stage. Animals are not admitted to veterinary clinics because prognosis is bad when clinical manifestations are seen. Most studies obtain samples by going to the abattoir to specifically look for OPA [7]. We selected paraffin embedded tissue blocks as material because prevalence of the disease is not known in the region. Before performing RT-PCR, tissue slides were first examined histopathologically. Using only histopathology can lead to false positivity because of the similar lesions [7,10]. This can be prevented using confirming with immunohistochemistry. Considering this fact, all tissues were initially diagnosed as OPA histopathologically and confirmed with immunohistochemistry. This was done because there was no positive control for RT-PCR; hence histopathological and immunohistological results were regarded as positives when performing RT-PCR. While all of the 25 tissues were positive with H&E and IHC, only 10 of them were deemed positive after visualizing amplicons after RT-PCR. This is possibly the result of RNA degrading mainly by the processes of making paraffin blocks, storage time and with the application/incubation time of the extraction procedure and expected [11]. As a result, other 15 tissues can be regarded as false negatives when these conditions are considered. This does not present a problem for the study as we did not investigate prevalence. But this is a general problem in diagnosing OPA prevalence as the most suitable material for early-stage detection is peripheral blood and it is known to give false negative results [7,10].

Our location is situated in the Northeastern region of Türkiye and animal husbandry is the main sources of its income. There are 500.000-600.000 small ruminant bred as livestock [15,16]. Mean altitude is 2000 meters, region has large meadows which is used for extensive breeding of small ruminants. Climate of the region has short summer time and harsh winter conditions. Small ruminant breeding is mostly done pastoral/extensively, but harsh

winter conditions limit this time [15]. Animals are reared in closed spaces wintertime, for this reason diseases which spread by aerosols and contact have higher incidences, including OPA [7]. Unfortunately, there is not enough data to determine the prevalence of the disease. But the disease has been previously reported from our country and also our province. Initial reports of the disease focused on histopathological means [17,18]. In the following years, reports on the presence of the disease were published from the Eastern region of the country [19-23]. Currently, disease has been studied in our province [24-28] and neighbouring province [29].

When predicted amino acid sequence table *(Fig. 4)* is examined, there are changes among our regional isolates and reference strains. We can also see some changes in reference strains like in 89th position as some has threonine and others serine. These changes can be of importance as *env* is the gene that is responsible for oncogenic transformation [6,7]. Further research needs to be performed in this regard.

We were able to find both type 1 and type 2 exJSRV from the material we used. This proves both types are in circulation in our country. ExJSRV type 1 is suggested to be of South African origin and type 2 is United Kingdom or American origin [1,5]. Considering this fact, both types were possibly come into our country from an animal movement. Earlier reports of OPA, while some used molecular techniques [21,22,26], do not contain information about types. Because of this we cannot make any assumptions about previous existence of type 1 and type 2. Our findings suggest the need to design further studies for investigating possible differences in pathogenesis of both types as evidence of circulating types are revealed.

OPA can be considered endemic where clinical manifestations are seen, as virus would spread to the flock before symptoms appear. There is currently no vaccine or routine diagnostic test for early-stage disease. Due to lack of early detection diagnostic tests; serosurveys or eradication programmes cannot be planned/executed. Development of a reliable diagnostic test which can be applied ante mortem easily, to crowded sheep flocks and populations is still needed to establish preventive measures.

In conclusion two different types of the exJSRV were discovered with molecular characterization using paraffin embedded lung tissues. We believe our results will contribute to the literature and draw further investigations on OPA.

Declarations

Financial Support: The authors declare that this study has received no financial support.

Ethical Statement: The study was approved by Kafkas University Animal Experiments Local Ethics Committee (Decision Number: KAÜ-HADYEK/2021-180)

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available within the manuscript.

Conflict of Interest: The authors declared that there is no conflict of interest.

Generative Artificial Intelligence: No kind of artificial intelligence tool was used in preparing this manuscript.

Author Contributions: NC, EK: Idea and study design. EB, NH: Sample collection; NC, EB HN: Laboratory analyses; VY, MOT, NC: Analysis of data; NC, EK: Manuscript preparation. All authors have read and approved the final manuscript.

References

1. Bai J, Zhu RY, Stedman K, Cousens C, Carlson J, Sharp JM, DeMartini JC: Unique long terminal repeat U3 sequences distinguish exogenous Jaagsiekte sheep retroviruses associated with ovine pulmonary carcinoma from endogenous loci in the sheep genome. *J Virol,* 70 (5): 3159-3168, 1996. DOI: 10.1128/JVI.70.5.3159-3168.1996

2. Palmarini M, Cousens C, Dalziel RG, Bai J, Stedman K, DeMartini JC, Sharp JM: The exogenous form of Jaagsiekte retrovirus is specifically associated with a contagious lung cancer of sheep. *J Virol,* 70 (3): 1618-1623, 1996. DOI: 10.1128/JVI.70.3.1618-1623.1996

3. Palmarini M, Mura M, Spencer, TE: Endogenous betaretroviruses of sheep: teaching new lessons in retroviral interference and adaptation. *J Gen Virol,* 85 (1): 1-13, 2004. DOI: 10.1099/vir.0.19547-0

4. Palmarini M, Sharp JM, de las Heras M, Fan H: Jaagsiekte sheep retrovirus is necessary and sufficient to induce a contagious lung cancer in sheep. *J Virol,* 73 (8): 6964-6972, 1999. DOI: 10.1128/JVI.73.8.6964-6972.1999

5. DeMartini JC, Carlson JO, Leroux C, Spencer T, Palmarini M: Endogenous retroviruses related to Jaagsiekte sheep retrovirus. **In,** Fan HY, Fan H (Eds): Jaagsiekte Sheep Retrovirus and Lung Cancer. 117-137, Springer, Berlin, 2003.

6. Quintas H, Pires I, Garcês A, Prada J, Silva F, Alegria N: The diagnostic challenges of ovine pulmonary adenocarcinoma. *Ruminants,* 1 (1): 58-71, 2021. DOI: 10.3390/ruminants1010005

7. Toma C, Bâlteanu VA, Tripon S, Trifa A, Rema A, Amorim I, Pop RM, Popa R, Catoi C, Taulescu M: Exogenous Jaagsiekte sheep retrovirus type 2 (exJSRV2) related to ovine pulmonary adenocarcinoma (OPA) in Romania: Prevalence, anatomical forms, pathological description, immunophenotyping and virus identification. *BMC Vet Res,* 16 (1): 296, 2020. DOI: 10.1186/s12917-020-02521-1

8. WOAH: World Organisation of Animal Health in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.8.8 Ovine Pulmonary Adenocarcinoma. 2021. https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.08.08_OPA.pdf; *Accessed:* 10.10.2023.

9. Mansour KA, Al-Husseiny SH, Kshash QH, Jassim A: Clinical-histopathological and molecular study of ovine pulmonary adenocarcinoma in Awassi sheep in Al-Qadisiyah Province, Iraq. *Vet World,* 12 (3): 454-458, 2019. DOI: 10.14202/vetworld.2019.454-458

10. De Las Heras M, Ortín A, Salvatori D, Pérez de Villareal M, Cousens C, Miguel Ferrer L, Miguel Cebrián L, García de Jalón JA, Gonzalez L, Michael Sharp J: A PCR technique for the detection of Jaagsiekte sheep retrovirus in the blood suitable for the screening of ovine pulmonary adenocarcinoma in field conditions. *Res Vet Sci,* 79 (3): 259-264, 2005. DOI: 10.1016/j.rvsc.2005.02.003

11. Pikor LA, Enfield KS, Cameron H, Lam WL: DNA extraction from paraffin embedded material for genetic and epigenetic analyses. *J Vis Exp,* (49):e2763, 2011. DOI: 10.3791/2763

12. Hall TA: BioEdit: A user-friendly biological sequence align-ment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser,* 41, 95-98, 1999.

13. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res,* 25 (17): 3389-3402, 1997. DOI: 10.1093/nar/25.17.3389

14. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol,* 28 (10): 2731-2739, 2011. DOI: 10.1093/molbev/msr121

15. Demir M: Kars ilinde büyük ve küçükbaş hayvancılık; Livestock breeding in Kars province. *Dogu Cogr Derg,* 21 (35): 39-62, 2016. DOI: 10.17295/dcd.33180

16. TUIK: Turkish Institute of Statistics in Yearly Report. 2016. http://www.turkiyekoyunkeci.org/tr/HaberIcerikDetay/kucukbas-hayvan-sayimiz-44-milyon-34-bin-basa-yukseldi/36760; *Accessed:* 18.01.2023.

17. Kıran MM: Pathological investigations on sheep pulmonary adenomatosis. *Eurasian J Vet Sci,* 9 (1): 41-45, 1993.

18. Kıran MM, Çiftçi MK, Erer H, Ortatatlı M, Hatipoğlu F, Tuzcu M: Koyun pulmoner adenomatozisinin eksfoliyatif sitoloji ve bronkoalveolar lavaj yöntemleri ile tanısı ve sonuçların histopatolojik bulgularla karşılaştırılması. *Eurasian J Vet Sci,* 16 (1): 59-68, 2000.

19. Beytut E, Sözmen M, Erginsoy S: Immunohistochemical detection of pulmonary surfactant proteins and retroviral antigens in the lungs of sheep with pulmonary adenomatosis. *J Comp Pathol,* 140 (1): 43-53, 2009. DOI: 10.1016/j.jcpa.2008.10.003

20. Sozmen M, Beytut E: An investigation of growth factors and lactoferrin in naturally occurring ovine pulmonary adenomatosis. *J Comp Pathol,* 147 (4): 441-451, 2012. DOI: 10.1016/j.jcpa.2012.04.004

21. Can-Sahna K, Eroksuz Y, Berber E, Sozdutmaz I: Detection of exogenous Jaagsiekte sheep retrovirus in Turkey. *Indian J Anim Res,* 49 (4): 498-502, 2015. DOI: 10.5958/0976-0555.2015.00053.9

22. İlhan F, Vural SA, Yıldırım S, Sözdutmaz İ, Alcigir ME: Expression of p53 protein, Jaagsiekte sheep retrovirus matrix protein, and surfactant protein in the lungs of sheep with pulmonary adenomatosis. *J Vet Diagn Invest,* 28 (3): 249-256, 2016. DOI: 10.1177/1040638716636939

23. Özkan C, Yıldırım S, Huyut Z, Özbek M: Selected tumour biomarker levels in sheep with pulmonary adenomatosis. *J Vet Res,* 64 (1): 39-44, 2020. DOI: 10.2478/jvetres-2020-0017

24. Karakurt E, Beytut E, Dağ S, Nuhoğlu H, Yıldız A, Kurtbaş, E: Assessment of MDA and 8-OHdG expressions in ovine pulmonary adenocarcinomas by immunohistochemical and immunofluorescence methods. *Acta Vet Brno,* 91 (3): 235-241, 2022. DOI: 10.2754/avb202291030235

25. Karakurt E, Beytut E, Dağ S, Nuhoğlu H, Yıldız A, Kurtbaş E: The relationship between metastasis and MMP-9 in sheep with pulmonary adenocarcinomas. *Kafkas Univ Vet Fak Derg,* 28 (2): 211-216, 2022. DOI: 10.9775/kvfd.2021.26678

26. Karakurt E, Coşkun N, Beytut E, Keleş ÖF, Dağ S, Yılmaz V, Nuhoğlu H, Yıldız A, Kurtbaş E: Evaluation of the relationship between inflammatory reaction and interleukins in ovine pulmonary adenocarcinomas. *Vet Res Forum,* 14 (1): 1-6, 2023. DOI: 10.30466/vrf.2022.542311.3279

27. Karakurt E, Beytut E, Dağ S, Nuhoğlu H, Yıldız A, Kurtbaş E: Immunohistochemical detection of pro-inflammatory and anti-inflammatory interleukins in the lungs of sheep with Jaagsiekte. *TJVR,* 6 (1): 9-14, 2022. DOI: 10.47748/tjvr.1037041

28. Karakurt E, Beytut E, Dağ S, Nuhoğlu H, Yıldız A, Kurtbaş E: Immunohistochemical detection of TNF-α and IFN-γ expressions in the lungs of sheep with pulmonary adenocarcinomas. *Acta Vet Eurasia,* 48 (3): 161-166, 2022. DOI: 10.5152/actavet.2022.21124

29. Bolat İ, Yildirim S, Sağlam YS, Çomakli S, Kiliçlioğlu M, Dereli E: Investigation of the effects of pulmonary adenomatosis on oxidative DNA damage, inflammation, apoptosis, and autophagy in lung tissues in sheep. *Small Ruminant Res,* 23010717:1, 2024. DOI: 10.1016/j.smallrumres.2023.107171

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Short Communication

Association of Two ISSR Markers with the Growth Traits of Saburai Does *(Capra hircus)*

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Abstract

Saburai goat *(Capra hircus)* developed by the farmers at Tanggamus Regency, Indonesia and originated from the crossbreeding between Boer buck and Ettawa grade doe for meat production purpose. This study aimed to evaluate Saburai goat based on two ISSR markers of $(AG)_9C$ and $(GA)_9C$. This study uses 28 adult Saburai does. Results showed that a total of 13 DNA fragments were observed according to two ISSR markers with a total of 11 polymorphic loci. However, the PIC value in $(AG)_9C$ ISSR marker was higher than $(GA)_9C$ ISSR marker. According to both ISSR markers, Saburai goat can be characterized into two clusters. In conclusion, the genetic diversity in Saburai goats is high and potential to improve with molecular selection.

Keywords: Genetic diversity, Indonesia, ISSR marker, Polymorphic, Saburai goat

Introduction

Saburai *(Capra hircus)* is an Indonesian crossbred goat developed through a grading up program by farmers in Tanggamus Regency, Lampung (one of Indonesia›s provinces). The breed is a mix of 75% Boer buck and 25% Ettawa-grade doe. Since 2015, Saburai has been recognized as an official Indonesian native goat breed by the Indonesian Ministry of Agriculture through decision No. 359/Kpts/PK.040/6/2015. Presently, Saburai goats were bred for meat production with 35.93±7.16 kg (bucks) and 31.77±7.58 kg (does) of yearling weight and 1.64±0.56 of litter size. As the native Indonesian crossbred goat, assessing the genetic diversity of the Saburai goat is important for genetic conservation and evaluation of population structure. Inter simple sequence repeat (ISSR) is a molecular marker technique that uses polymerase chain reaction (PCR) to amplify DNA with a single primer made up of a microsatellite sequence. The ISSR method is known for being quick, easy to use, highly reproducible, and polymorphic. Despite the need for genetic characterization, ISSR markers can select sheep with a high breeding value for body weight.

Recently, the two ISSR markers of $(AG)_9C$ and $(GA)_9C$ have been used to study the genetic diversity in ruminant livestock animals such as cattle [1], goats and sheep [2]. Unfortunately, there are limited studies for assessing both ISSR markers in goats. Previously, researching with other

ISSR markers to observe the genetic diversity in the goat population without an association study [3]. Therefore, the current study aimed to determine the association of $(AG)_9C$ and $(GA)_9C$ ISSR markers with the body weight of Saburai does. Furthermore, this study was conducted to observe the genetic diversity in Saburai does based on both ISSR markers. These findings are important for evaluating the genetic potential of Saburai does concerning growth traits.

Material and Methods

Ethic Approval Statement

This study was performed under the animal ethics protocols of the Animal Science Faculty, Universitas Lampung, Indonesia (Certificate No: 6410/UN26.14/2023)

Animals and Research Site

Twenty-eight (28) unrelated Saburai does were selected for the experiment. The does were collected from Tanggamus Regency, Lampung Province of Indonesia. This research site located at latitude 5°05'-5°56'S and longitude 104°18'-105°12'E and placed at 2.115 m asl with 27.3-29.6°C of air temperature, 72.1-81.8% of relative humidity and 2.066.5 mm of rainfall per year.

Management of Animal and Data Records

The management of Saburai goat husbandry implemented by the livestock farming group in Gisting Atas Subdistrict aims to maintain animal health, increase production, and ensure business sustainability. The primary feed for Saburai goats in this group consists of green fodder like gamal, kaliandra, and odot grass, which are known for their high nutritional content. Additionally, farmers use plant residues as supplemental feed. The feeding schedule is three times a day: morning, noon, and afternoon. The Gemar Menanam Hijauan (GMH) program encourages members to plant their own animal feed, which helps ensure a year-round supply of high-quality feed.

The goats' housing is managed using a well-structured system, typically elevated pens to maintain cleanliness and animal health. These pens provide ample space for movement and are equipped with efficient waste disposal systems, essential to prevent disease transmission.

The breeding management in this group has focused on Saburai goat reproduction since 2014. They use natural mating methods while keeping several bucks to avoid inbreeding, ensuring good genetic diversity in their herd.

DNA Extraction

The blood samples were taken from each animal using venoject and vacutainer tubes containing EDTA, then stored at -20°C for subsequent DNA extraction. The DNA extraction was conducted using the Genomic DNA Extraction Kit (Geneaid, Taiwan) following the manufacturer's instructions. Nonetheless, individual body weight data at birth, weaning (4 months old), and yearling (12 months old) were collected from each animal for the association study.

DNA Amplification

The DNA amplification was performed in a total volume of 10 µL containing 3 µL of DNA template, 5 µL of PCR Master Mix (DreamTag™, USA), 0.5 µL of $(AG)_9C$/$(GA)_9C$ ISSR primer and 1.5 µL of free-nuclease water. The amplification of each primer consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min. The electrophoresis for DNA visualisation was performed at 100 V for 35 min with 2% agarose gel stained with safe DNA gel stain (SYBR™, USA). The DNA fragments'visualisation was captured by G-Box Documentation System (Syngene, UK).

Data Analysis

Five genetic diversity parameters were calculated in this study belonging to the total of DNA fragments, number of polymorphic loci, number of effective allelles (n_e), Shannon's diversity index (I) and polymorphic informative content (PIC). While, the n_e, I and PIC values were calculated referring as follows [4]:

$$n_e = 1/\sum P_{ij}^2 \quad (1)$$

$$I = -\sum P_i \ln(P_i) \quad (2)$$

$$PIC = 1-\sum P_i^2 \quad (3)$$

where, n_e is the number of effective alleles; I is the Shannon's diversity index, PIC is the polymorphic informative content and P_i is the frequency of the j^{th} pattern in the i^{th} band. The dendrogram of animals was computed with a Heatmapper computer program. The association study of ISSR markers was performed by comparing the growth traits of animals at different clusters using General Linear Model (GLM) methods and analyzed with the SPSS 16.0 computer program.

Results

Sixteen (16) DNA loci were obtained in Saburai does based on two ISSR markers belonging to eleven (11) DNA patterns for $(AG)_9C$ and five (5) DNA patterns for $(GA)_9C$ *(Table 1)*. Three DNA loci of S1.2 (5 animals), S1.4 (5 animals) and S1.5 (4 animals) were shown as the three common DNA loci in $(AG)_9C$ ISSR marker for experimental animals. While S2.3 (12 animals) and S2.4 (10 animals) were shown as the two common DNA loci

Table 1. *Polymorphisms of two ISSR markers in Saburai does*

ISSR Marker	Locus Name	Number of Fragment	Length (bp)	N	Frequency
$(AG)_9C$	S1.1	6	350, 500, 650, 780, 1500, >3000	1	0.04
	S1.2	5	500, 650, 780, 1500, >3000	5	0.19
	S1.3	5	350, 500, 650, 780, >3000	2	0.07
	S1.4	5	500, 550, 650, 780, >3000	5	0.19
	S1.5	4	500, 650, 780, >3000	4	0.15
	S1.6	4	500, 550, 650, 780	1	0.04
	S1.7	3	500, 650, >3000	3	0.11
	S1.8	2	500, 650	3	0.11
	S1.9	2	500, 550	1	0.04
	S1.10	1	550	1	0.04
	S1.11	1	500	1	0.04
$(GA)_9C$	S2.1	4	500, 600, 650, >3000	2	0.07
	S2.2	4	300, 490, 500, >3000	1	0.03
	S2.3	3	300, 500, >3000	12	0.43
	S2.4	2	300, 500	10	0.36
	S2.5	1	500	3	0.11

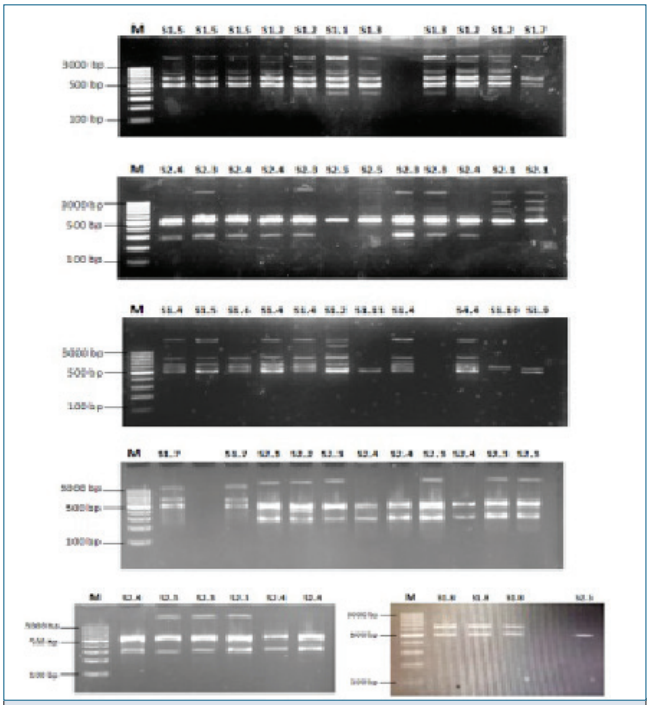

Fig 1. Pattern of DNA fragments in Saburai does based on PCR-ISSR analysis with $(AG)_9C$ (S1.- code) and $(GA)_9C$ (S2.- code) ISSR Markers on 2% agarose gel. M: DNA ladder 100 bp

Table 2. *Genetic diversity in two ISSR markers of Saburai does*

ISSR Marker	Total Number of Fragment	Number of Polymorphic Locus	Frequency of Polymorphic Locus	Number of Effective Allele (n_e)	Shannon's Diversity Index (I)	PIC
$(AG)_9C$	7	6	0.86	7.59	2.50	0.87
$(GA)_9C$	6	5	0.83	3.01	1.26	0.67

PIC = polymorphic informative content

in the $(GA)_9C$ ISSR marker for experimental animals. In addition, the number of DNA fragments in both ISSR markers ranged from 1 to 6 bands for $(AG)_9C$ and 1 to 4 bands for $(GA)_9C$ *(Fig. 1)*. Therefore, the PIC and I values in the $(AG)_9C$ were higher than $(GA)_9C$ *(Table 2)*. In general, two clusters of Saburai does were determined according to two ISSR markers such as cluster 1 with 15 animals) and cluster 2 with 13 animals *(Fig. 2)*. Therefore,

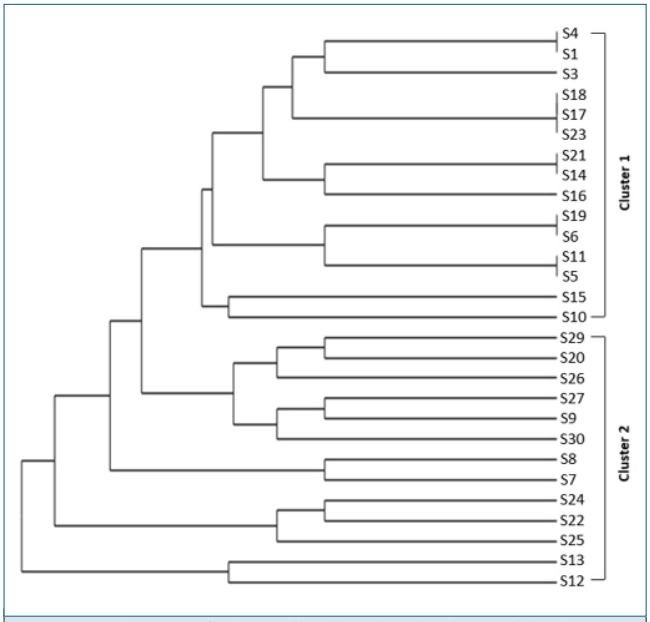

Fig 2. Dendrogram of Saburai does based on $(AG)_9C$ and $(GA)_9C$ ISSR markers. S- code is the sample ID

Table 3 . *Comparation between the growth traits of Saburai does from different clusters*

Parameter	Cluster 1 (N=15)	Cluster 2 (N=13)	P-Value
Birth weight (kg)	3.27±0.81	2.97±0.72	0.634
Weaning weight (kg)	17.08±3.24	15.90±2.90	0.634
Yearling weight (kg)	38.25±9.71	34.67±8.69	0.634
Preweaned daily gain (kg/day)	0.11±0.02	0.10±0.01	0.631
Postweaned daily gain (kg/day)	0.09±0.03	0.08±0.02	0.544

N = number of animal

the association analysis revealed that the growth traits of Saburai does at cluster 1 were higher than in cluster 2 but it's not significantly different *(Table 3)*.

Discussion

In Saburai does, the PIC value of ISSR markers in $(AG)_9C$ was higher than $(GA)_9C$. A high PIC value also indicated high genetic diversity in the observed animals. Thus, the PIC value is classified in low (<0.30), moderate (0.31-0.50) and high (>0.50) categories. In Tuvinian short-tailed sheep, the PIC value of $(AG)_9C$ ISSR markers was about 0.20-0.0.40 [5] and lower than in animals under study. Contrastly, there are 21 DNA loci of $(AG)_9C$ ISSR markers were observed in Tuvinian short-tailed sheep and higher than in Saburai does. In Kermani sheep, a total of 28 DNA loci of $(AG)_9C$ and 36 DNA loci of $(GA)_9C$ were observed and higher than in Saburai does [6]. In Mehraban sheep, total 28 and 36 of DNA loci were observed with $(AG)_9C$ and $(GA)_9C$ ISSR markers, respectively [7]. In goats, the PIC value ranged from 0.09-0.19 for $(AG)_9C$ and 0.14-0.21 for $(GA)_9C$ [2].

The variation of DNA loci can be affected by the type of species and breed. As the crossbred goat, Saburai had higher genetic diversity and its supporting these findings. According to the quantitative genetic aspect, the heritability (h^2) value of growth traits in Saburai goat from low (<0.10) to moderate (0.11-0.30) categories such as 0.24±0.08 for body weight, 0.29±0.17 for weaning weight and 0.10±0.08 for yearling weight [8]. Hence, the quantitative genetic aspects explained that Saburai goat had high genetic diversity and possible to improve their productivity traits with conventional and molecular selection methods. Hence, the ISSR analysis revealed that Saburai does at cluster 1 had higher growth traits in comparison to cluster 2 but it is not different significantly.

In the present study, the genetic diversity of Saburai does was able to be observed based on two ISSR markers. A study of microsatellite markers was reported able to identify the genetic diversity in Indonesian native goat breeds [9]. Therefore, a molecular selection involving functional genes may be used to obtain the genetic markers for growth traits. The growth hormone (GH) gene is one of candidate genes that had a potency for molecular

selection of growth traits in Saburai goats [10] and Merino cross sheep [11] in Indonesia. Subsequently, Myostatin (MSTN) gene was polymorphic in Saburai goat and can be used for molecular selection of growth traits [12]. Furthermore, assessing the genome-wide association study (GWAS) can detect many potential candidate genes across the autosomal chromosome regions of goats accurately [13,14]. Despite this, a whole genome sequencing (WGS) technique can obtain the genetic markers in the livestock accurately [15].

In conclusion, two ISSR markers of $(AG)_9C$ and $(GA)_9C$ were polymorphic in Saburai does. However, the ISSR marker of $(AG)_9C$ had a higher PIC value rather than $(GA)_9C$. In this study, Saburai does can be characterized into two clusters based on both ISSR markers. Commonly, the average growth traits in the does on cluster 1 were higher than those in cluster 2. In the future, further research involving large samples and ISSR markers is important to select Saburai goats based on specific genetic markers accurately.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available to the corresponding author (W.P.B. Putra).

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References

1. Maherovska OM: Selection and assessment of ISSR-markers for Analysis of separate cattle populations. *Anim Breed Genet,* 61, 137-145, 2021. DOI: 10.31073/abg.61.15

2. Askari N, Abadi MM, Baghizadeh A: ISSR markers for assessing DNA polymorphism and genetic characterization of cattle, goat and sheep populations. *Iranian J Biotechnol,* 9 (3): 222-229, 2011.

3. Kostova M, Bojinov B: Application of ISSR markers for detection of genetic variation in two bulgarian autochthonous goat breeds. *Bulg J Agric Sci,* 24 (6): 1109-1113, 2018.

4. Kayis SA, Hakki EE, Pinarkara E: Comparison of effectiveness of ISSR and RAPD markers in genetic characterization of seized marijuana *(Cannabis sativa* L.) in Turkey. *Afr J Agric Res,* 5, 2925-2933, 2010.

5. Stolpovsky YA, Kol NV, Evsyukov AN, Ruzina MN, Shimiit LV, Sulimova GE: Analysis of the genetic structure of Tuvinian short-fat-tailed sheep populations with the use of the ISSR-PCR method. *Russ J Genet,* 46 (12): 1660-1669, 2010. DOI: 10.1134/S1022795410120094

6. Mohammadabadi M: Inter-simple sequence repeat loci associations with predicted breeding values of body weight in Kermani sheep. *Genet 3rd Millenn,* 14 (4): 4383-4390, 2016.

7. Zamani P, Akhondi M, Mohammadabadi MR, Saki AA, Ershadi A, Banabazi MH, Abdolmohammadi AR: Genetic variation of mehraban sheep using two intersimple sequence repeat (ISSR) markers. *Afr J Biotechnol,* 10 (10): 1812-1817, 2011.

8. Sulastri S, Siswanto S, Adhianto K: Genetic parameter for growth performance of Saburai goat in Tanggamus district, Lampung province, Indonesia. *Adv Anim Vet Sci,* 6 (11): 486-491, 2018. DOI: 10.17582/journal.aavs/2018/6.11.486.491

9. Pakpahan S, Widayanti R, Artama WT, Budisatria IGS, Raadsma HW, Margawati ET: Genetic diversity analysis of Indonesian indigenous goat breeds based on microsatellite markers. *Small Ruminant Res,* 225:107011, 2023. DOI: 10.1016/j.smallrumres.2023.107011

10. Prastowo S, Herowati N, Widyas N, Pambuko G and Vanessa R: Allele frequency changes of growth hormone gene in Boer goat crossbred population and its association to body measurements. *JIT,* 21 (1): 18-26, 2021. DOI: 10.24198/jit.v21i1.33425

11. Putra WPB, Margawati ET, Raadsma HW, Tyasi TL: Polymorphism of growth hormone (GH/HaeIII) gene and its association with the performance of Merino cross rams. *World Vet J,* 14 (2): 151-157, 2024. DOI: 10.54203/scil.2024.wvj19

12. Dakhlan A, Putra WPB, Santosa PE, Kurniawati D: Novel polymorphisms in caprine *Myostatin* gene and its relationship with growth traits in Saburai does *(Capra hircus). Pak J Biol Sci,* 26 (7): 380-385, 2023. DOI: 10.3923/pjbs.2023.380.385

13. Zhang L, Wang F, Gao G, Yan X, Liu H, Liu Z, Wang Z, He L, Lv Q, Wang Z, Wang R, Zhang Y, Li J, Su R: Genome-wide association study of body weight traits in inner Mongolia cashmere goats. *Front Vet Sci,* 8:752746, 2021. DOI: 10.3389/fvets.2021.752746

14. Sun X, Jiang J, Wang G, Zhou P, Li J, Chen C, Liu L, Li N, Xia Y, Ren H: Genome-wide association analysis of nine reproduction and morphological traits in three goat breeds from Southern China. *Anim Biosci,* 36, 191-199, 2023. DOI: 10.5713/ab.21.0577

15. Zhong J, Ma Z, Chai Z, Wang H, Zhang C, Ji Q, Xin J: Whole genome sequencing of the Dzo: Genetic implications for high altitude adaptation, sterility, and milk and meat production. *Kafkas Univ Vet Fak Derg,* 24 (6): 835-844, 2018. DOI: 10.9775/kvfd.2018.20022

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Case Report

Topical Xenogeneic Exosome Therapy in a Dog with Toxic Epidermal Necrolysis

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Abstract

This report describes a positive outcome of topical exosome therapy for toxic epidermal necrolysis (TEN) in a dog. Enrofloxacin was administered subcutaneously one day before and for 7 days after the surgery for urolithiasis. Fourteen days after discontinuing the treatment, the dog was presented to the clinic for disseminated superficial tissue loss in the dorsal region which is associated with adverse drug reaction based on the scores for assessment of drug causality for epidermal necrolysis. Bovine-derived cord blood exosome was applied in the dose of 1 million/kg, twice a day, with intradermal and spraying routes to multiple points around the wound. The dog was monitored weekly, and complete recovery was observed 58 days after treatment. This report shows that topical xenogeneic exosome may be an alternative treatment approach for wound healing in dogs.

Keywords: Dogs, Enrofloxacin, Exosome, Toxic epidermal necrolysis

Introduction

Toxic epidermal necrolysis (TEN) is a life-threatening immune-mediated skin reaction most often triggered by particular medications. Stevens-Johnson syndrome (SJS) and TEN are on the same spectrum and are clinically distinguished by the percentage of body surface area detached. In humans and dogs, SJS, SJS/TEN, or TEN is characterized by more than 10%, 10-30%, and 30% skin detachment of the total body surface area, respectively. Although there is no definitive information on TEN pathophysiology, the activation of T cells in response to a drug or infection and the subsequent development of epidermal necrosis is emphasized [1]. ALDEN score, the algorithm of drug causality for epidermal necrolysis, describes SJS/TEN in humans [2] and dogs [3].

Enrofloxacin is a fluoroquinolone antibiotic used widely in veterinary medicine due to its potent Gram-positive and Gram-negative activity; however, it is more toxic to eukaryotic cells than many other groups of antibiotics [4]. Enrofloxacin administration may inhibit cell proliferation, decrease metabolic activity, negatively affect the bone marrow, and result in tissue necrosis [5]. Enrofloxacin can also induce an inflammatory response at the injection site and affect the cell count and protein levels of the immune system [6].

Exosomes are nano-sized bio-vesicles released into surrounding body fluids upon the fusion of multivesicular bodies and the plasma membrane. They were shown to carry cell-specific cargos of proteins, lipids, and genetic materials, and can be selectively taken up by neighboring or distant cells far from their release, reprogramming the recipient cells upon their bioactive compounds. The native role of extracellular vesicles in mediating the transfer of biomolecules between cells has raised the possibility of

using them as therapeutic vehicles. Exosomes reflect the biophysical features of mesenchymal stem cells (MSCs) and are considered more effective than MSCs themselves. Exosomes have many beneficial effects on skin care as they contain various biological molecules that can help promote skin repair and regeneration [7,8].

Case reports and controlled studies are needed to assess whether exosome application could be an alternative treatment approach in dogs and cats. Thus, herein, a successful result of topical exosome treatment in a dog with TEN following subcutaneous enrofloxacin administration was presented.

Case History

Written informed consent was obtained from the owner for the participation of the animal in this study. An 8-year-old, male, 12 kg body weight, sterile Cavalier King Charles Spaniel was brought to a private clinic on 23.09.2023 with the complaint of inability to urinate. Physical examination did not reveal specific signs except pain in response to abdominal palpation *(Table 1)*. Complete blood count (CBC) (Mindray 2800Vet) and serum biochemistry profile (FujiFilm Dri-Chem 4000i) were within their references (data not shown). Subsequently, 14 stones obstructing the urethra were detected by the abdominal radiography. The patient was considered to be suitable for inhalation anesthesia and abdominal surgery based on the pre-operative evaluation for anesthesia. Stones in the urethra were successfully removed with the surgery (24.09.2023).

The patient was administered 1 mL enrofloxacin (Baytril-K 5%, 50 ml, Flacon, Bayern, Istanbul/ Türkiye) subcutaneously for 7 days and 1.5 mL meloxicam (Bavet Meloxicam, 20 mL, Flacon, Bavet, Istanbul/ Türkiye) for 3 days. The dog was discharged after 6 days (30.09.2023)

Table 1. *Some physical and hematological test results before and 58 days after the topical exosome treatment in the dog*

Parameter	Pre-exosome Treatment	Post-exosome Treatment	Reference Range [12]
Temperature °C	39.2	39.0	37.2-39.3
Heart rate bpm	136	132	100-140
Respiration rpm	22	20	<20
Capillary re-filling time sec	1	1	<1
WBC x10^9/L	18.1	9.0	6.0-15.0
Lymphocyte x10^9/L	2.2	1.9	1.0-4.8
Monocyte x10^9/L	0.5	0.3	0.2-1.4
Eosinophils x10^9/L	0.70	0.33	0.1-1.2
Granulocyte x10^9/L	15.4	6.8	3.0-11.5
Erythrocyte x10^{12}/L	6.7	5.93	5.5-8.5
Hemoglobin g/dL	13.1	10.9	12.0-18.0
Hematocrit %	43.3	37.7	37.0-55.0
MCHC g/dL	31.2	28.9	30.0-38.0
MCV fL	65.6	63.6	60.0-77.0
MCH pg	19.9	18.3	19.5-24.5
RDW %	13.3	15.0	12.0-14.9
Platelets x10^9/L	191	151	200-500
MPV fL	9.4	9.6	8.0-14.1
PDW %	18.1	17.4	12.0-17.5
PCT %	0.11	0.144	0.14-0.46

Table 2. *Some biochemical parameters before and 58 days after the topical exosome treatment in the dog*

Parameters	Pre-exosome Treatment	Post-exosome Treatment	Reference Range [12]
ALP IU/L	496	120	20-60
ALT IU/L	180	41	15-60
BUN mg/dL	10.9	13.7	9-29
Creatinine mg/dL	0.63	0.71	0.4-1.4
Glucose mg/dL	133	110	75-128
TP g/dL	6.8	6.8	5.8-7.3

ALP: Alkalen phosphatase, ALT: Alaline aminotransferase, BUN: Blood ure nitrogen, TP: Total protein

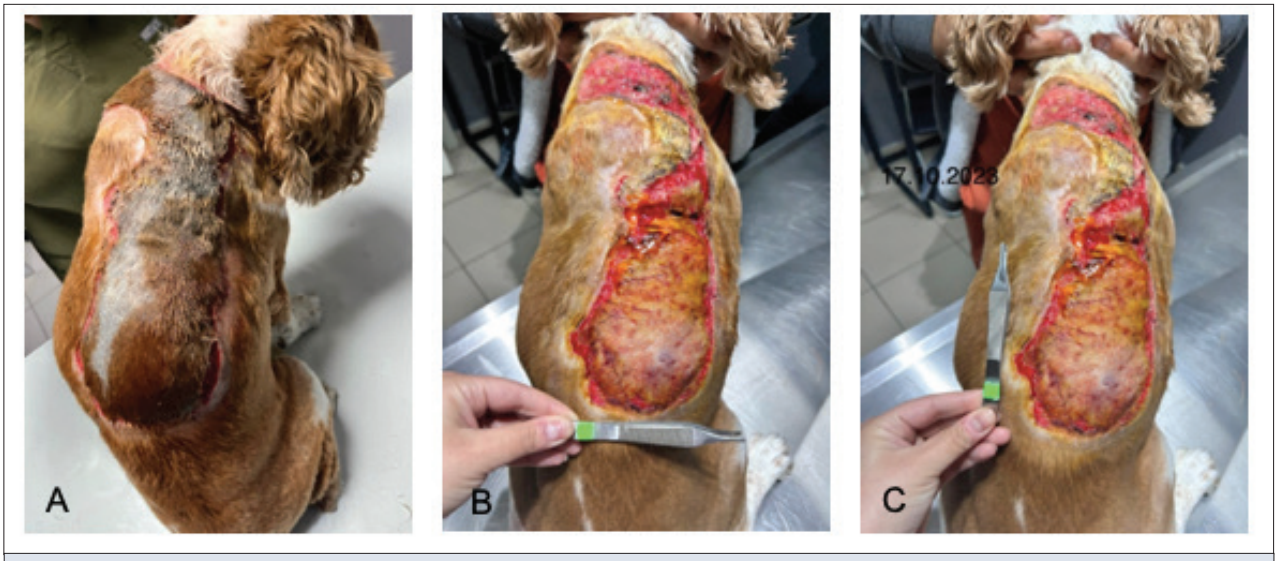

Fig 1. Following the enrofloxacin injection on day 5, the dog was presented to the clinic with the complaint of rigid and crusted swelling on his back (A). The dog was sedated for cleaning necrotized areas, showing a wound with extensive tissue loss (B and C). TEN was diagnosed based on the epidermal necrolysis of the skin surface covering more than 30% of the body

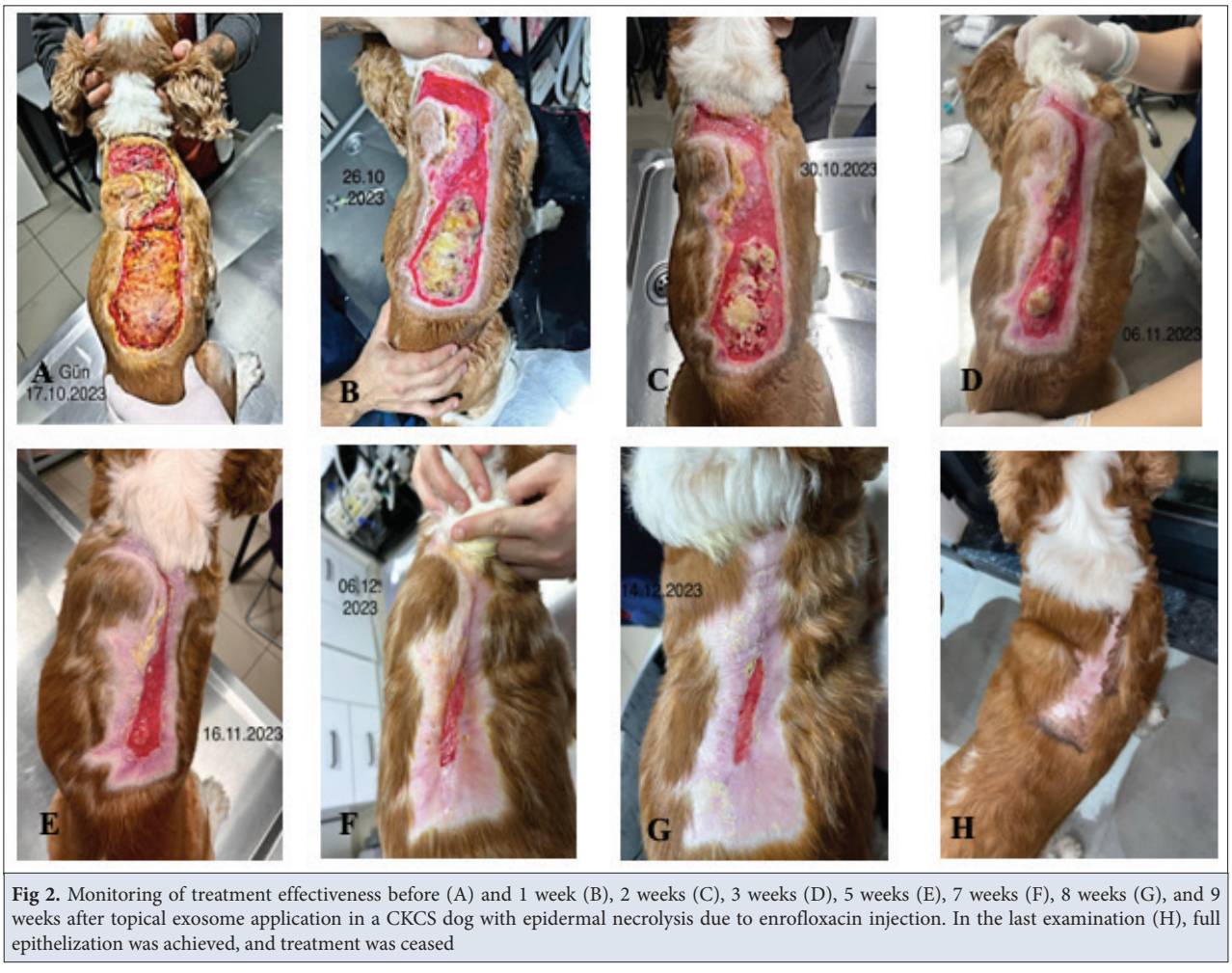

Fig 2. Monitoring of treatment effectiveness before (A) and 1 week (B), 2 weeks (C), 3 weeks (D), 5 weeks (E), 7 weeks (F), 8 weeks (G), and 9 weeks after topical exosome application in a CKCS dog with epidermal necrolysis due to enrofloxacin injection. In the last examination (H), full epithelization was achieved, and treatment was ceased

with the urethral catheter removed. On 14.10.2023, the patient was brought back to the clinic with the complaint of swelling and pruritis on his back where the area was rigid and crusted on palpation *(Fig. 1-A)*. The CBC and serum biochemistry panel showed slightly elevated leukocytes, eosinophils, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and glucose *(Table 1, Table 2)*, compared to their references [9].

The patient was hospitalized and topical standard treatments were applied for 5 days, using an antiseptic solution (Chlorhexidine, Crystaline^{R} solution, Animal Health, Türkiye) to prevent the dog from secondary bacterial infections, NSAID (Meloxicam, Bavet Meloxicam 20 Flacon, Türkiye) for pain management, and antihistaminic to relieve allergy symptoms (Cetirizine, Zyrtec 10 mg tablet, UCB Pharma, Türkiye) as suggested [1], but it was not beneficial because of too much necrotized tissue. The necrotized areas were cleaned under sedation with butorphanol (Butomidor Flacon, 0.2 mg/kg, im; InterHas, Türkiye) (17.10.2023), and a wound with extensive tissue loss was observed *(Fig. 1-B,C)*. Microbiologic examination of the skin swap did not reveal the presence of any bacteria or fungus. Considering the withdrawal time and duration of the drugs, epidermal necrolysis was considered to be associated with the enrofloxacin administration rather than meloxicam [4]. ALDEN score (+3) was found to be most likely drug-induced TEN [2,3-7,10].

To increase epithelization and wound healing faster, xenogeneic exosome obtained from the umbilical cord of calves was purchased (5 billion exosomes/mL, 5 ml Flacon; Morphiya, Mage Group Biotechnology Company, London, UK). The exosome was isolated according to the recommended procedure [11]. The dose (1 million/kg) used in the study was adapted from the previous study [12]. It was applied intradermally at 0.05 mL with an intradermal needle to multiple points (peripherally with 1 cm intervals) around the wound. In the following days, the wound area was first cleaned with crystalline, and then 1 mL exosome was diluted with 5 mL normal saline and applied twice daily to the area by spray for the first 10 days. In the following days, 0.5 mL exosome was diluted with 2 mL normal saline, and the same procedure was followed thereafter. During this period, the wound area was protected from external factors only by covering it with sterile gauze, and no other treatment was applied. The area of the skin wound was minimalized from day to day, and the healing process was monitored by the weekly photos *(Fig. 2)*. After 51 days, the patient was discharged to home since the skin wound was minimalized. In the last examination (58th day of the treatment), full recovery *(Table 1, Table 2)* or full epithelization was achieved, and thus treatment was ceased *(Fig. 2-H)*.

Discussion

Exosomes are being investigated for their biomarkers and therapeutic potential in humans and experimental animals, especially in hepato-renal diseases. Topical exosome therapy is also used for cosmetics, skin care, tissue regeneration, and dermatological disease in humans. However, no study has evaluated its effectiveness in improving acute skin wound healing in dogs. Therefore, we presented here a successful outcome of xenogeneic topical exosome therapy in a dog with a large-sized skin wound.

In this dog, TEN was suspected since huge epidermal necrolysis of the skin surface (more than 30% of the whole-body surface area) occurred after enrofloxacin injection *(Fig. 1)*. Other possible etiologies such as traumatic burns, thermal injury, erythema multiforme, vasculitis with ischemic necrosis, pemphigoid, and bullous immune diseases, systemic lupus erythematosus [1], and endocrinopathies such as hypothyroidism and hyperadrenocorticism were excluded based on the history, clinical and hemato-biochemistry findings *(Table 1, Table 2)*, as well as appearance of the skin lesion *(Fig. 1)*. Observed increases in serum ALP and ALT, two weeks after enrofloxacin treatment in this dog were found to be associated with TEN [13]. Similar to this concept, hepatitis and cholestatic liver disease were reported as the most common complications seen in patients with TEN [13]. The exact mechanism between TEN and liver diseases has not been explained yet [13]. In this case, enrofloxacin administration may be a possible reason for observed increases in serum hepatic injury marker and eosinophil count in peripheral blood [13]. Since the enrofloxacin use causes an increase in pro-inflammatory factors (IL-1β, TNF, etc.) and induces an inflammatory response at the injection site [4], it may have a role in developing large-sized epidermal necrolysis in the presented case.

Following the diagnostic work-up, treatment options were considered, and it was included using conventional techniques; antiseptic solution to prevent the dog from secondary bacterial infections, NSAID such as meloxicam for pain management, and antihistaminic such as cetirizine, as suggested [1]. Since the dog did not give a positive response to these medications and had a huge skin lesion with tissue loss, we decided to use topical xenogeneic (bovine-derived cord blood) exosome as an alternative treatment approach. Exosomes are beneficial for skin care since they are filled with proteins, lipids, and other molecules that can help to promote healing, hydration, and the protection of the skin. These molecules can help to boost collagen production, reduce inflammation, and protect the skin from environmental stressors. Additionally, exosomes can help increase the efficacy of other active ingredients, such as hyaluronic acid, peptides, and antioxidants [8]. Although many studies have been conducted on exosome therapy in human and animal models in recent years, no clear results have been obtained and also its routine clinical use has not been realized yet. Therefore, the subject needs further studies with more cases in human and veterinary medicine.

Studies show the differences in the sources and applications of exosomes. Since MSC-derived exosomes are most commonly used in these studies, their usage was preferred

in the presented case. Exosomes have been used topically for wound healing or parenterally for various diseases, such as renal failure, liver damage, and tumors, in humans and experimental animals. In a recently published study, prepared exosomes were injected into the peripheral and central areas of the wound immediately after injury and then for two weeks (daily for the first week and every other day for the second week) [14]. In that study [14], the wound closure rate was higher in the exosome group compared to the placebo control group on day 14. In the presented case, successful re-epithelialization and wound healing were observed from the first week of exosome treatment, and the wound size decreased significantly from week to week. Another study showed that ulcer surface area decreased 12 weeks after direct and intramuscular exosome administration in diabetic patients [15]. Perianal fistule was reported to have 56% closure at 24 weeks following intralesional exosome administration in humans [16]. The time to heal TEN-related wounds was reported to be maximally 65 days after the comprehensive treatment including immunosuppressive drugs and immunoglobulins in humans [17]. In the presented case, full recovery was observed at 58 days without additional local and parenteral drug applications *(Fig. 2)*.

This case report has some limitations. Firstly, since enrofloxacin and meloxicam are injected simultaneously, it was not easy to decide which medication led to epidermal necrolysis in the dog. It should be kept in mind that quinolones and oxicam NSAIDs are definitely on the list of drugs found to be associated with TEN [10]. Considering the withdrawal time and duration of meloxicam usage, epidermal necrolysis could have resulted from enrofloxacin administration rather than meloxicam. In addition, when the ALDEN scores were calculated for both drugs, enrofloxacin was found to be a more potent factor in causing skin lesions in this case. Secondly, it would be better if the skin biopsy and histopathologic examination could have been performed. The owner did not permit skin biopsy due to the necessity for anesthesia in this procedure. Despite this limitation, ALDEN scores and others such as the Naranjo Algorithm Adverse Drug Reaction Probability Scale with or without histopathologic evaluations can be used to describe the presence of TEN in humans [2] and dogs [1].

As a result, this case report provides us with an alternative treatment approach for topical xenogeneic exosome application in large-sized skin wounds such as TEN in dogs. Bovine-derived exosomes could be used safely without complication to shorten the improvement period of skin lesions in clinical practice.

Declarations

Availability of Data and Materials: In this case, the original images obtained during the study are available from the corresponding author (ZY) on request.

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Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): We declared that the article, tables, and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: ZY, PLK, and TV performed diagnostic and therapeutic procedures; exosomes were obtained from ŞU and SC. TV wrote the article's draft, and ZY and SU revised it before submission. All approved the final version of the paper.

References

1. Lecru LA, Combarros D, Castilla-Castaño E, Delverdier M, Cadiergues MC, Pressanti C: Case report: Positive outcome of a suspected drug-associated (ımmune mediated) reaction in a 4-year-old male French bulldog. *Front Vet Sci,* 8:728901, 2021. DOI: 10.3389/fvets.2021.728901

2. Moral L, Toral T, Gilabert A, Sánchez A, Silvestre JF, Marco FM: Drug reexposure in children with severe mucocutaneous reactions. *Allergol Immunopathol (Madr),* 50 (1): 104-107, 2022. DOI: 10.15586/aei.v50i1.382

3. Banovic F, Olivry T, Bazzle L, Tobias JL, Atlee B, Zabel S, Hensel N, Linder KE: Clinical and microscopic characteristics of canine toxic epidermal necrolysis. *Vet Pathol,* 52 (2): 321-330, 2015. DOI: 10.1177/0300985814537530

4. Grabowski Ł, Gaffke L, Pierzynowska K, Cyske Z, Choszcz M, Wegrzyn G, Wegrzyn A: Enrofloxacin - The ruthless killer of eukaryotic cells or the last hope in the fight against bacterial ınfections? *Int J Mol Sci,* 23 (7):3648, 2022. DOI: 10.3390/ijms23073648

5. Han Y, Hong-Gu J: Determination of the toxicity of enrofloxacin on mouse bone marrow cells. *J Prev Vet,* 43, 141-145, 2019. DOI: 10.13041/jpvm.2019.43.4.141

6. Gorla N, García Ovando H, Larripa I: Chromosomal aberrations in human lymphocytes exposed in vitro to enrofloxacin and ciprofloxacin. *Toxicol Lett,* 104 (1-2): 43-48, 1999. DOI: 10.1016/s0378-4274(98)00230-6

7. Sanz-Ros J, Mas-Bargues C, Romero-García N, Huete-Acevedo J, Dromant M, Borrás C: Extracellular vesicles as therapeutic resources in the clinical environment. *Int J Mol Sci,* 24 (3):2344, 2023. DOI: 10.3390/ijms24032344

8. Shi H, Wang M, Sun Y, Yang D, Xu W, Qian H: Exosomes: Emerging cell-free based therapeutics in dermatologic diseases. *Front Cell Dev Biol,* 9:736022, 2021. DOI: 10.3389/fcell.2021.736022

9. Villiers E, Ristic J: BSAVA Manual of Canine and Feline Clinical Pathology. 1-614, Cambrian Printer, Aberystwyth, UK, 2016.

10. Sassolas B, Haddad C, Mockenhaupt M, Dunat A, Liss Y, Bork K, Haustein UF, Vileuf D, Roujeau JC, Le Louet H: ALDEN, an algorithm for assessment of drug causality in Stevens-Johnson Syndrome and toxic epidermal necrolysis: Comparison with case-control analysis. *Clin Pharmacol Ther,* 88 (1): 60-68, 2010. DOI: 10.1038/clpt.2009.252

11. Koçak P, Unsal N, Canikyan S, Kul Y, Cohen SR, Tiryaki K, Duncan D, Schlaudraff KU, Ascher B, Tiryaki TE: The effect of hybrosome (umbilical cord blood exosome-liposome hybrid vesicles) on human dermal cells *in vitro*. *Aesthet Surg J Open Forum,* 5:ojad039, 2023. DOI: 10.1093/asjof/ojad039

12. Prasai A, Jay JW, Jupiter D, Wolf SE, El Ayadi A: Role of exosomes in dermal wound healing: A systematic review. *J Invest Dermatol,* 142 (3): 662-678.e8, 2022. DOI: 10.1016/j.jid.2021.07.167

13. Jha AK, Suchismita A, Jha RK, Raj VK: Spectrum of gastrointestinal involvement in Stevens-Johnson syndrome. *World J Gastrointest Endosc,* 11 (2): 115-123, 2019. DOI: 10.4253/wjge.v11.i2.115

14. Rasti M, Parniaei AH, Dehghani L, Nasr Esfahani S, Mirhendi H, Yazdani V, Azimian Zavareh V: Enhancing the wound healing process through local injection of exosomes derived from blood serum: An *in vitro* and *in vivo* assessment. *Regen Ther,* 19 (26): 281-289, 2024. DOI: 10.1016/j.reth.2024.06.004

15. Dash NR, Dash SN, Routray P, Mohapatra S, Mohapatra PC: Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. *Rejuvenation Res,* 12 (5): 359-366, 2009. DOI: 10.1089/rej.2009.0872

16. de la Portilla F, Alba F, García-Olmo D, Herrerías JM, González FX, Galindo A: Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: Results from a multicenter phase I/IIa clinical trial. *Int J Colorectal Dis,* 28 (3): 313-323, 2013. DOI: 10.1007/s00384-012-1581-9

17. Widgerow AD: Toxic epidermal necrolysis - management issues and treatment options. *Int J Burns Trauma,* 1 (1): 42-50, 2011.

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LETTER TO THE EDITOR

New Information About Godlewsky's Contributions to Turkish Veterinary Medicine History Extracted from a Document

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Dear Editor

An archive document representing an important turning point in the history of Turkish veterinary medicine reveals that Tophane-i Amire was not only important as the industrial center of the Ottoman Empire, but also in terms of military veterinary services. This document *(Fig. 1)* dated December 2, 1844[1*] shows that the First-Class Military Veterinarian Godlewsky, who came from Prussia to the Ottoman lands to provide veterinary education, was concerned not only with the treatment of the horses of the Imperial Army, but also with the health of the animals in Tophane-i Amire. This document sheds light on the beginning of scientific veterinary education in the Ottoman Empire and adds a new dimension to the history of veterinary medicine. Tophane deserves to be remembered not only as a military industrial center, but also as a place that contributed to the development of veterinary medicine with the health services it provided.

Godlewsky, a First-Class Military Veterinarian, was tasked with the establishment of a veterinary school, marking the commencement of scientific veterinary education in the Ottoman Empire. His principal duty involved training soldiers from the Imperial Guard in the identification and treatment of illnesses affecting military horses. In addition to this, he was assigned the responsibility of caring for the horses of two artillery regiments and offering his services to four cavalry regiments stationed in the vicinity

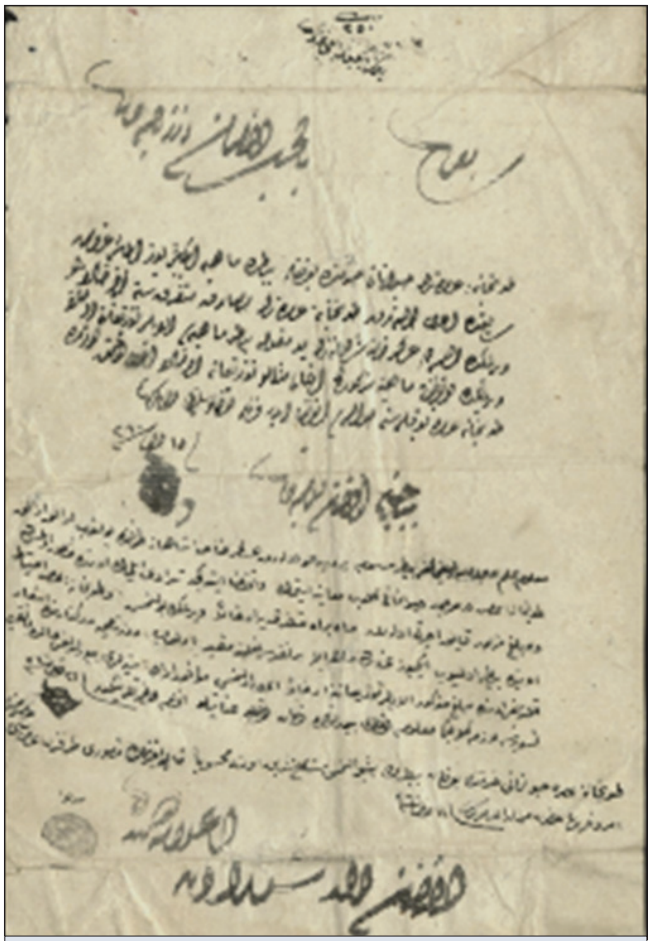

Fig 1. Vet. Med. Godlewsky's letter of assignment

[1] * CAS. 100/4523

of Istanbul during critical periods. In exchange for his services, Godlewsky was granted a monthly salary of 1.750 kuruş, in addition to provisions such as bread, rice, and meat, and an allowance of 150 kuruş for the upkeep of his two horses [1,2]. While these responsibilities and compensations were previously documented as part of Godlewsky's contributions to the Ottoman Empire, an archival record indicates that he also provided veterinary care for the animals of the Tophane-i Amire, receiving an extra monthly payment of 250 kuruş (It corresponds to approximately 17.75 grams of gold. [3,4]) for this service. The expenses incurred were covered by the minor expenditure budget of the Tophane-i Amire. Furthermore, the document discloses that Godlewsky, who oversaw the horses of the Imperial Guard, occasionally conducted inspections and administered treatment to the horses in Tophane as well. Prior to Godlewsky's appointment, the primary caretaker of the horses was a military chief farrier who received a monthly wage of 200 kuruş[2*].

The recently discovered document demonstrates the breadth of Godlewsky's responsibilities to the Empire and positions him as a significant figure in Turkish and world veterinary history. His contributions warrant lasting acknowledgment and admiration.

Declarations

Availability of Data and Materials: The data in this lettering are available from the corresponding authors (S.V.G.) in case of a request.

Competing Interest: The authors declare that they have no conflict of interest.

Author Contributions: The contributions of the authors to the letter to the editor are equal.

References

1. Bekman M: Veteriner Tarihi. 113-227, Ankara Basım ve Cildevi, Ankara, 1940.

2. Godlewsky, Sommer: Türkiye'de Veteriner Hekimlik (19'uncu Yüzyıl Ortalarında). Çev. Prof. Dr. Nihal Erk, Ankara Üniversitesi Veteriner Fakültesi Yayınları, Ankara, 1972.

3. Şen H: Bir parasal istikrarsızlık örneği: Osmanlı Devleti'nde paranın tağşişi. *Active Bankacılık ve Finans Dergisi*, 33, 50-60, 2003.

4. Tekeli İ, İlkin S: Para ve Kredi Sisteminin Oluşumunda Bir Aşama: Türkiye Cumhuriyet Merkez Bankası. 2th ed., 46, T.C. Merkez Bankası, Ankara, 1997.

2 * CAS. 100/4523

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• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

OPEN ACCESS STATEMENT

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 30, Issue: 6, Year: 2024

ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• Preliminary Evaluation Process

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• Peer-review Process

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• Publication Process of an Article

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

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ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (E-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

2- The official language of our journal is English.

3- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and other files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

4- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

5- Authors should know and take into account the "Generative Artificial Intelligence (AI)" and other matters listed in the **"Ethical Principles and Publication Policy"** section regarding scientific research and authors.

6- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

7- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

8- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university

9- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

10- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

11- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

12- The editorship may request the language editing of the manuscript submitted to the journal. If the article is accepted, it will not be published without language editing. Before publication, a declaration and/or certificate stating that proofreading is done by a registered company will be requested from the corresponding author.

13- No fee is charged at any stage in Kafkas Üniversitesi Veteriner Fakültesi Dergisi (No APC/APF)

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover Letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title Page

• Title, Running Title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

- Declarations

• Availability of Data and Materials

• Acknowledgements

• Funding Support

• Competing Interests

• Generative Artificial Intelligence (AI)

• Authors' Contributions

Further Considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided