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RESEARCH ARTICLE

MOLECULAR DETECTION OF *TRYPANOSOMA EVANSI* USING ROTAT 1.2 (VSG) GENES IN CATTLES FROM BORNO AND GOMBE STATES, NIGERIA

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ABSTRACT

Trypanosoma evansi (*T. evansi*) causes surra, an important haemoprotozoan disease of economic importance that affects a wide range of hosts. The research was designed to detect *T. evansi* using a molecular method in cattle from Gombe and Borno States, Nigeria. Blood samples from 455 apparently healthy cattle were collected from the month of May to October, 2018 in different Local Government areas. Microscopy detection revealed an overall prevalence rate of 11.8% in Gombe State and 9.0% in Borno State during the study period. Out of the 455 blood samples collected, 50 randomly selected samples, with 25 each from Borno and Gombe states, were subjected to molecular analysis for identification of *T. evansi*. Twenty-one positive samples of *T. evansi* were detected in 50 cattle using PCR, targeting the RoTat 1.2 variable surface glycoproteins (VSG) gene. PCR-positive samples were furtherly phylogenetically analysed. Sequence analysis of the Rotat 1.2 VSG gene showed high variability between the isolates from this study and the isolates earlier deposited in the GenBank. Microscopic examination exhibited a very low sensitivity, whereas PCR using RoTat 1.2 VSG gene showed more sensitivity in the diagnosis of *T. evansi* from cattle. Conclusively, the phylogenetic results obtained from the Neighbor joining technique suggest that the isolate of *T. evansi* in this study is not related to the isolates from Egypt, Kenya, Sudan and India.

Keyword: Cattle, Nigeria, RoTat 1.2 Gene, *Trypanosoma evansi*

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INTRODUCTION

Trypanosoma evansi (*T. evansi*) is the most common pathogenic trypanosome worldwide and causes the sickness known as "surra" (Luckins, 1988; Cadioli et al., 2006). It is pathogenic to numerous species of both domestic and wild animals and has a broad host range (Franke et al., 1994a; Herrera et al., 2002). Biting flies, belonging to species such as *Tabanus*, *Stomoxys*, *Haematopota*, *Lyperosia*, and *Chrysops spp*. are the main mechanical method of transmission (Fraser, 1909; Nieschulz, 1926; 1927a; Nieschulz, 1927b; Luckins, 1999) of the disease. The most significant host in Africa is camels (Dia et al., 1997), however, cattle have also been identified as the second most highly vulnerable animals (Mahmoud and Gray, 1980). Experimental research conducted in Nigeria has confirmed that *T. evansi* can infect donkeys, cattle, sheep, and goats; however, the disease has a long course in these animals (Ilemobade, 1971; Audu et al., 1999; Shehu et al., 2006). There are considerable differences in the severity of syndromes caused by *T. evansi* infection in different geographical areas of its occurrence, depending on the virulence of the strain, the susceptibility of the host (Sawitri et al., 2022) and the presence of other concurrent diseases. Although *T. evansi* is primarily a parasite of camels, there is a likelihood that animals herded together with camels may become infected with *T. evansi* and serve as a reservoir (Aregawi et al., 2019). As such, there is the need to have a better understanding of disease susceptibility of various animal species with respect to *T. evansi* infection.

Light microscopy of various preparations (wet mount, thin and thick blood films, and buffy coat technique) is the most widely used and accessible diagnostic test for trypanosomosis. *T. evansi* in stained blood smears under the light microscope is described as having a large size body (25–35 μm), small and subterminal kinetoplast, thin posterior extremity, large undulating membrane, central nucleus, and free flagellum, according to Desquesnes et al. (2013). However, it is time-consuming, unsuitable for use in field conditions, and has a low sensitivity and specificity (Eisler et al., 2004; Kumar et al., 2012). There has been a number of advancements in trypanosomosis diagnosis recently, leading to a better understanding of the disease's location and mode of transmission- a must for any effective treatment (Magez et al., 2021). These developments have made it possible to accurately identify and detect several Trypanosoma species (Traub et al., 2005). Although enzyme-based immunosorbent assay (ELISA) was thought to have improved pathogen determination sensitivity, antigen detection with monoclonal antibody (mAb)-based ELISA is highly unreliable because immune active agents in the blood can't tell the difference between active infections and past infections (Geysen et al., 2003). More sensitivity and reliability were available than with earlier approaches with the introduction of polymerase chain reaction (PCR), a revolutionary technique for direct parasite detection and identification. PCR analysis (Masiga et al., 1992; Ngomtcho et al., 2017; Weber et al., 2019) and Trypanosoma species-specific DNA probes (Habeeb et al., 2021) have greatly improved the identification and comprehension of trypanosome diversity, particularly in light of the high prevalence of mixed trypanosome infections that are found in the field (Solano et al., 1995; Woolhouse et al., 1996). There is a paucity of information on the disease pattern in different livestock species in Africa, including Nigeria. In Nigeria, extensive work has been done on animal trypanosomosis (Shehu et al., 2006; Karshima et al., 2016; Dabo and Maigari 2018; Umeakuana et al., 2019; Weber et al., 2019), but little has been done on infection due to *T. evansi,* especially in cattle within the study area. Since there is a considerable difference in the severity of syndromes caused by *T. evansi* infection in different geographical areas of its occurrence, it is desirable to evaluate the prevalence of cattle infected with this strain of *T. evansi* in cattle in the study area.

MATERIALS AND METHODS

Ethical approval

All applicable national and institutional guidelines for the care and use of animals were followed. All procedures performed in the studied animals were following the ethical standards of the Faculty of Veterinary Medicine Committee on Animal Use and Care, where the study was conducted.

Study areas and sampling points

This study was carried out in Borno and Gombe States of Northeastern Nigeria (Figure 1). Borno state lies between longitude 120° 8E and latitude 10° 23N and the climate, ecology and vegetation is Sahelian, semi-arid and savannah with flooded pastures towards Lake Chad and mountainous areas in the Southeast. The State shares international borders with Niger, Cameroun and Chad Republics, which have similar relative humidity during the dry season and rainy season (Ishaku and and Majid, 2010).

Sampling was conducted in three urban and rural areas—Konduga, Jere, and Maiduguri—selected from among the 27 Local Government Areas (LGAs) of Borno State. Similarly, Dukku, Gombe, and Yamaltu Deba were sampled from 11 LGAs of Gombe State, Nigeria.

Study design

This study was carried out in two phases, via: Phase I: Microscopic examination of thin blood smears for identification of *Trypanosoma* parasite, Phase II: Molecular detection and characterization of Rotat 1.2 (VSG) genes of *T. evansi* by using polymerase chain reaction (PCR).

Study animals and sample collection

This study was conducted from May to October, 2018. A non-probability convenience sampling method was used with emphasis on areas having large populations of cattle. A total number of 455 randomly selected apparently heathy cattle from selected livestock markets and farms were examined for *T. evansi* regardless of age and sexes. Blood samples were collected through jugular vein in vacutainer tubes containing ethylene di-amine tetraacetic acid (EDTA), and then divided into two parts. One was for microscopic examination and the remaining sample was preserved at -20 °C for DNA extraction of trypanosomes for PCR.

Microscopic detection for *Trypanosoma*

Thin blood smears were prepared from all the samples collected for parasitological examination, the thin blood smears were air dried, fixed in methanol, stained with a 10% solution of Giemsa, and then observed under the light microscope using 400X and 1000X magnification objectives, according to Zajac et al. (2021). Blood sample

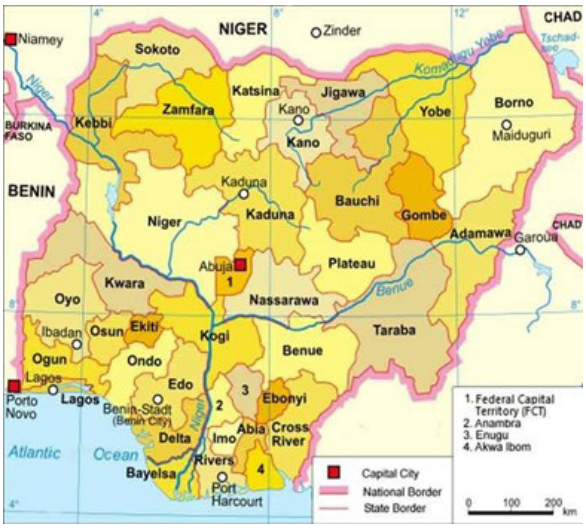

Figure 1 Map of Nigeria showing the 36 states, including Gombe and Borno States (blue star), Nigeria

Source: https://www.nigeriagalleria.com/Nigeria/States_Nigeria/Borno/ - Search Images (bing.com)

of cattle from the study areas were considered positive for *T. evansi* when stained blood smears revealed the presence of *Trypanosoma* parasite. Photomicrographs of *T. evansi* parasites were taken using Amscope digital camera for microscope version (3.0 China).

Isolation of trypanosome DNA

DNA extraction

From the 455 blood samples collected, a total of 50 randomly selected samples, with 25 each from Borno and Gombe States were subjected to molecular analysis for detection of *T. evansi* using standard technique according to Kyari et al. (2021). Briefly, genomic DNA was extracted from the whole blood samples using AccuPrep® Genomic DNA Extraction kit (K-3032, Bioneer, Germany), according to manufacturer's instructions. DNA quantity and purity were measured using a spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany) and stored at - 20 °C until further use.

PCR amplification

Forward and reverse primer sequences used at an amplification fragment of 250bp (base pair) are shown below:

F5'-GCGGGGTGTTTAAAGCAATA-3',
R5'-ATTAGTGCTGCGTGTGTTCG-3'
(Invitrogen®, USA)
(Barghash et al., 2016).

In whole reaction quantity of 25 µl having 50 ng of template DNA and 12.5µl of standard commercial PCR Mastermix (AccuPrep®, Germany), the PCR amplification reaction was prepared. A concentration of 10 pmol/µl was used for the primers (RoTat 1.2VSG gene). This was followed by PCR amplification in a Biometra thermocycler. The following circling conditions were used: 94°C/3min (1 cycle) for denaturation, annealing at 57°C/1 min, 72°C/1 min (40 cycles) for polymerization and 72°C/5 min for final extension (Barghash et al., 2016). Subsequently, the outcome of every sample was electrophoresed via 2% agarose, and for visualization of amplified DNA and comparison with standard DNA 100bp (Bioneer, AccuPrep®, USA), the voltage was set at 60V. Using a transilluminator, the amplicons were examined for expected size.

PCR products sequencing

The products of PCR were cleaned up, decontaminated and sent for direct sequencing in both directions (Bioneer, South Korea). All procedures involved were carried out according to standard protocols. The Basic Local Alignment Search Tool (BLAST) programs were used to align and compare the sequences obtained with others stored in the GenBank. A phylogenetic tree was constructed using the neighbour-joining (NJ) algorithms, version 3.6a 2.1 of Bioedit Software (Hall, 2013). The produced DNA sequences were flung into the GenBank databases in order to locate homologous sequences that had been documented. Also, the Maximum Parsimony (MP) method was used to infer the outcome of phylogeny from present study and other isolates from previous studies.

Data analysis

Prevalence, chi-square and odds ratio were used for the analyses. Contingency coefficient was used to determine association between the statuses of infection. The data were analyzed using JMP, version 11 software (SAS institute Inc. Cary, NC). Results from the analyses were considered significant at a p-value of less than 0.05.

RESULTS

Microscopic identification of *T. evansi* in cattle from Gombe and Borno States, Nigeria

It was revealed that microscopic identification of *T. evansi* had higher prevalence in Gombe (11.8%) than Borno State (9.0%) (Table 1).

Table 1 Microscopic identification of *T. evansi* in cattle from Gombe and Borno States, Nigeria

Study area	Sex of cattle	No of cattle examined	No of cattle infected (%)	Prevalence (%) 95% CI (LL - UL)	χ^2	p- value	Odd ratio
Gombe	Male	66	10 (15.2)	6.9 (0.0381 – 0.1231)	1.301	0.2540	1.6883
	Female	78	7 (9.0)	4.9 (0.0237 – 0.0969)			
Borno	Male	174	11 (6.3)	3.5 (0.0199 – 0.0623)	3.455	0.0631	1.9628
	Female	137	17 (12.4)	5.5 (0.0344 – 0.0858)			

CI = Confidence interval; LL = Lower limit; UL = Upper limit; χ^2 = Chi-square LGA = Local Government Area

Additionally, the results of *T. evansi* prevalence based on the LGAs from samples in Gombe State showed that the prevalence was higher in Yamaltu Deba (6.9%), followed by Dukku (2.8%) and Gombe (2.1%). The association between the statuses of infection in the Local Government Areas of Gombe State was not significant (Chi-square test = 2.39; p = 0.3034 with a contingency coefficient of 0.128, indicating weak association). However, the prevalence of *T. evansi* based on the LGAs in Borno State showed higher prevalence rate in Konduga (4.2%), followed by MMC (3.2%) and Jere (1.6%) LGAs. The association between the statuses of infection in the local government areas of Borno State was not significant (Chi-square test = 3.556; p = 0.1689 with a contingency coefficient of 0.128, indicating weak association). Also, the PCR technique revealed higher (42.0%) prevalence rate for *T. evansi* than the microscopy technique (12.0%) (Table 2).

Table 2 Comparison between microscopic and molecular (PCR) detection rate of *T. evansi* in cattle from Gombe and Borno States, Nigeria

Technique	No. of samples analyzed	*T. evansi* positive sample (% prevalence)
Microscopy	50	06 (12.0%)
PCR	50	21 (42.0%)

Keys: PCR = polymerase chain reaction, No.= number, *T. evansi* =*Trypanosoma evansi*, % = percentage

The phylogenetic tree created from the 5 *T. evansi* RoTat 1.2 gene isolates obtained from cattle in Borno and Gombe states using the neighbor joining method at 1000 bootstrap replicates indicated that 4 of the isolates (No 27, No 45, No 33 and No 39) formed a major clade with 2 subclades. However, in one of the subclades, one of the *T. evansi* isolates (No 39) further diverged from the other isolates, while one of the remaining isolates (No 50) distanced itself from all the other four isolates to form a major clade that looks like an outgroup (Figure 2).

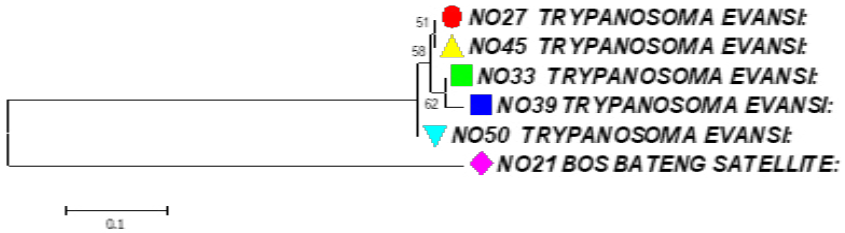

Figure 2 Phylogenic Tree of 5 *T. evansi* Partial RoTat 1.2 Gene Sequences obtained from cattle examined in Borno and Gombe States. Neighbor-joining was used to Inferred the tree, and 1000 Bootstrap Replicates were used.

The evolutionary divergence estimate calculated using the pairwise distance estimation method at four decimal places between the *T. evansi* partial RoTat 1.2 gene sequence from cattle in the studied areas is reported here. Using No 27 as reference isolate indicated that *T. evansi* No 27 and No 45 were the same at 1 standard error, while the other 3 appeared to be evolutionary different from each other. At 2 standard errors, isolates No 39 and No 45 appeared to be the same (0.0164) as seen in Table 3.

Table 3 Pairwise distance estimation (at 4 decimal places) between the *T. evansi* partial RoTat 1.2 gene sequences from cattle in Nigeria's Borno and Gombe States

	1	2	3	4	5	6
NO27__TRYPANOSOMA_EVANSI:		**0.0112**	**0.0161**	**0.0000**	**0.0082**	**0.1476**
NO33__TRYPANOSOMA_EVANSI:	**0.0164**		**0.0113**	**0.0112**	**0.0081**	**0.1456**
NO39_TRYPANOSOMA_EVANSI:	**0.0332**	**0.0164**		**0.0161**	**0.0140**	**0.1554**
NO45__TRYPANOSOMA_EVANSI:	**0.0000**	**0.0164**	**0.0332**		**0.0082**	**0.1476**
NO50__TRYPANOSOMA_EVANSI:	**0.0082**	**0.0082**	**0.0248**	**0.0082**		**0.1418**
NO21_BOS_BATENG_SATELLITE:	**0.8615**	**0.8615**	**0.9146**	**0.8615**	**0.8363**	

Note: Values on top represent the standard errors, while the bottom diagonal values are the actual variance estimates

The evolutionary divergence estimation of the 5 isolates obtained from this study and other geographically dispersed isolates calculated, using the pairwise distance estimation at 3 decimal places for the incomplete RoTat 1.2 *T. evansi* gene sequences, indicated that three of the isolates from this study (isolates No 27, No 33 and No 45) appeared to be the same at 1 standard error (Table 4). On the other hand, two isolates from Egypt and one from Kenya (isolates No JX888091, KF726106 and AF317914, respectively) with an estimation of 1.303 and 1.719 for isolates reported in this study are related to those from Egypt and Kenya, respectively.

The phylogenetic tree created from the 5 isolates based on this research and other isolates from geographically distant locations retrieved from the GeneBank reveals that all the isolates from this study distanced themselves from all other isolates obtained from other geographical locales to form a major clade. It is important to note that the Asian isolates from India appeared to distance evolutionarily and diverged themselves from the other African isolates (Figure 3).

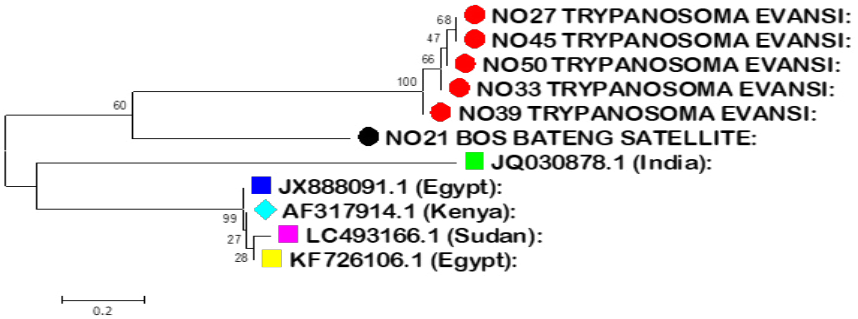

Figure 3 Nigerian RoTat 1.2 *T. evansi* Isolates Phylogenetic Tree obtained in present study were compared with other geographically dispersed *T. evansi* RoTat 1.2 isolates. The phylogentic tree was generated with 1000 Bootstarp Replicates and the Neighbor-joining technique.

Table 4 Pairwise distance estimation (at 3 decimal places) between the *T. evansi* partial RoTat 1.2 gene sequences from cattle in Borno and Gombe States compared with other geographically dispersed isolates

	1	2	3	4	5	6	7	8	9	10	11
NO21_BOS_BATENG_SATELLITE:		0.352	0.358	0.331	0.352	0.323	0.562	0.660	0.748	0.660	0.660
NO27_TRYPANOSOMA_EVANSI:	1.303		0.016	0.020	0.000	0.011	1.036	0.558	0.651	0.558	0.558
NO33_TRYPANOSOMA_EVANSI:	1.303	0.023		0.012	0.016	0.011	0.952	0.491	0.568	0.491	0.491
NO39_TRYPANOSOMA_EVANSI:	1.242	0.034	0.011		0.020	0.017	0.851	0.491	0.568	0.491	0.491
NO45_TRYPANOSOMA_EVANSI:	1.303	0.000	0.023	0.034		0.011	1.036	0.558	0.651	0.558	0.558
NO50_TRYPANOSOMA_EVANSI:	1.242	0.011	0.011	0.023	0.011		0.952	0.491	0.568	0.491	0.491
JQ030878.1_(India):	1.615	2.392	2.159	1.982	2.392	2.159		0.508	0.508	0.508	0.508

	1	2	3	4	5	6	7	8	9	10	11
JX888091.1_(Egypt):	1.719	1.615	1.524	1.524	1.615	1.524	1.524		0.011	0.000	0.000
LC493166.1_(Sudan):	1.839	1.719	1.615	1.615	1.719	1.615	1.524	0.011		0.011	0.011
AF317914.1_(Kenya):	1.719	1.615	1.524	1.524	1.615	1.524	1.524	0.000	0.011		0.000
KF726106.1_(Egypt):	1.719	1.615	1.524	1.524	1.615	1.524	1.524	0.000	0.011	0.000	

Note: Values on top represent the standard errors, while the bottom diagonal values are the actual variance estimate.

DISCUSSION AND CONCLUSION

Molecular diagnostic techniques represent essential tools for the detection of parasitic infection because of their high sensitivity, and are widely spread in the detection of surra globally because they are rapid, accurate and reliable (Sengupta et al., 2010).

Nonetheless, microscopy and PCR may exhibit extremely similar sensitivity in early stages of the infection. Conversely, during the chronic phase of infection, microscopic examination exhibits very low sensitivity, whereas PCR is 2-3 times more sensitive (Desquesnes and Davila, 2002). Out of the 50 samples tested using PCR amplification with RoTat 1.2, it is likely that the 21 *T. evansi* positively tested cattle were most likely infected during the active stage of illness. This finding may suggest that enough parasites were circulating in the blood of the infected cattle, allowing for their detection in both blood smear examination and PCR assay (Desquesnes et al., 2013). Compared with microscopic blood examination, the PCR technique is suggested for application in the diagnosis of trypanosomosis due to its greater sensitivity. Further molecular characterization and phylogenetic analysis of *T. evansi* isolates in naturally infected cattle in Gombe and Borno States showed PCR- RoTat 1.2 that amplified at 205 bp, followed by sequence analysis of this fragment. The choice of this gene is in accordance with a previous study by Claes et al. (2004) who reported a higher sensitivity by using the RoTat 1.2 in the PCR assay for diagnosis of *T. evansi* in bovine species compared to other related gene primers. Thus, this study confirms the greater sensitivity of PCR- RoTat 1.2 gene as a diagnostic primer tool for *T. evansi,* even at a very low infection. PCR used in this study for the detection of the presence of *T. evansi* RoTat 1.2 gene at genus and species levels, as expected, formed a major clade compared to other dispersed geographical isolates. More so, the results of the phylogenetic analysis in this study have also shown that all putative species/genus identified using BLAST searches of the GenBank clustered together (dendogram), indicating very close genetic relatedness. The RoTat 1.2 gene used here is a reliable marker for phylogeny, as it has been previously used to infer phylogenetic affinities and establish evolution and divergence of *T. evansi* in different species of bovine (Barghash et al., 2014). The phylogenetic tree constructed from the 5 *T. evansi* RoTat 1.2 gene isolates obtained from cattle in Borno and Gombe states using the neighbor joining method at 1000 bootstrap replicate indicated that 4 of the isolates formed a major clade with 2 subclades. However, in one of the subclades, one of the *T. evansi* isolate further diverged from the others, while one of the remaining isolates distanced itself from all the other four to form a major clade that looked like an outgroup. This finding is similar to previous reports by Barghash et al. (2016) in Camels from Egypt who observed variation between *T. evansi* isolates in their study and those earlier deposited in the GeneBank.

Moreover, evolutionary divergence calculated using the pairwise distance estimation method at four decimal places between the partial RoTat 1.2 gene sequence of *T. evansi* from cattle in Borno and Gombe states, using No. 27 as a reference isolate, indicated that *T. evansi* at 27 and 45 are the same at one standard error. In contrast, the other 3 appeared to be evolutionary different from each other. At two standard errors, isolates No 39 and No 45 appeared to be the same. This finding supported the reports

of Elhaig et al. (2016) and Elata et al. (2019) who also reported *T. evansi* in cattle and donkeys in Egypt and Sudan, respectively, using molecular phylogenecity. The phylogenetic tree created from the 5 isolates obtained, and compared with other isolates from other geographically dispersed locations in the GeneBank revealed that all the isolates from this study distanced themselves from all other isolates obtained from other geographical locales to form a major clade. It is important to note that the Asian isolates from India appeared to distance evolutionarily and diverged itself from the other African isolates. This agrees with the result of Urakawa et al. (2001) in Kenya who obtained similar isolates that claded closely with the isolate from this study. The estimation of evolutionary divergence of the 5 isolates here and other geographically dispersed isolates calculated using the pairwise distance estimation at 3 decimal places for the partial RoTat 1.2 gene sequences of *T. evansi* indicated that 3 of the isolates from this study (isolates No. 27, No. 33 and No. 45) appeared to be the same at 1 standard error, while two isolates from Egypt (Elhaig et al., 2016) and one from Kenya (Urakawa et al., 2001) were close to the isolates obtained in this study. A partial sequence of RoTat 1.2 VSG gene was reported to be identical to the *T. evansi* sequences reported from India and Kenya, but the varied similarity was seen when aligned with other isolates of *T. evansi* in Africa, which supported the findings of *T. evansi* sequences in Egypt by Witola et al. (2005). Using Maximum Parsimony (MP) method, the outcome of phylogenies in the present study suggested that the isolates of *T. evansi* from Kenya, Egypt, India and Sudan were more diverse than other isolates. This finding agrees with the reports of Elhaig et al. (2016) and Elata et al. (2019). A strong tree clustering of all known *T. evansi* stations that are circulating worldwide and that were retrieved from GenBank with values at pertinent nodes was shown by phylogenetic analysis. The length of the horizontal lines on the phylogenetic tree, which represented the evolutionary relationship between the sequences, was proportionate to the estimated genetic distance between the sequences.

In conclusion, RoTat 1.2 VSG molecular technique proved to be more sensitive and reliable as a diagnostic tool for *T. evansi* in cattle examined in Nigeria's states of Borno and Gombe. *T. evansi* detected in this study was found to be unrelated to some isolates from Africa and Asia in the GeneBank. It is, therefore, recommended that, since the gene used in this study is a protein coding gene, there is the need to further translate the nucleotide sequences obtained from this study into amino acids. There is also the need to look for the presence of tandem repeat sequences, which are useful for vaccine production.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTION

Concept and Design -AMB; Supervision - OE; AWM; MMB; Sample collection –JD; SMT; HIA; HMBM; Literature review and analysis and or interpretation of data- LA; Writing and Critical review- HA

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MOLEKULARNA DIJAGNOSTIKA *TRYPANOSOME EVANSI* KORIŠTENJEM (VSG) GENA KOD GOVEDA U DRŽAVAMA BORNO I GOMBE U NIGERIJI

SAŽETAK

Trypanosoma evansi (*T. evansi*) uzrokuje suru, važno hematoprotozoarno oboljenje od ekonomskog značaja koje ima širok spektar domaćina. Kod goveda smo proveli istraživanje sa ciljem dijagnosticiranja *T. evansi* korištenjem molekularne metode u državama Gombe i Borno, u Nigeriji. Prikupljeni su uzorci krvi od 455 naizgled zdravih goveda od mjeseca maja do oktobra, 2018. godine sa različitih područja vladinih uprava. Mikroskopiranjem je ustanovljena prevalenca od 11.8% za državu Gombe i 9.0% za državu Borno za vrijeme trajanja studije. Od prikupljenih 455 krvnih uzoraka, 50 randomiziranih uzoraka, po 25 iz država Borno i Gombe, su podvrgnuti molekularnoj analizi u svrhu identifikacije *T. evansi*. Kod 50 goveda je otkriven dvadeset i jedan uzorak pozitivan na *T. evansi* korištenjem PCR metode sa RoTat 1.2 genom za varijabilne površinske glikoproteine (VSG) kao metom. PCR-pozitivni uzorci su dalje analizirani filogenetički. Sekvencijska analiza Rotat 1.2 VSG gena je pokazala visoku varijabilnost između izolata iz ovog istraživanja i izolata ranije deponiranih u GenBank. Mikroskopski pregled je pokazao veoma nisku senzitivnost, dok je PCR uz korištenje RoTat 1.2 VSG gena pokazao veću senzitivnost u dijagnosticiranju *T. evansi* kod goveda. U zaključku, filogenetski rezultati dobiveni tehnikom pridruživanja pokazuju da izolat *T. evansi* iz našeg istraživanja nije povezan s izolatima iz Egipta, Kenije, Sudana i Indije.

Ključne riječi: Goveda, Nigerija, RoTat 1.2 gen, *Trypanosoma evansi*

RESEARCH ARTICLE

THE PROTECTIVE EFFECT OF DICLOFENAC SODIUM AND ENOXAPARIN ON THE FORMATION OF POSTOPERATIVE INTRA-ABDOMINAL ADHESIONS AND FIBROTIC CHANGES IN THE LARGE INTESTINE WALL IN AN EXPERIMENTAL RAT MODEL

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ABSTRACT

Postoperative intra-abdominal adhesions are a frequent complication following abdominal surgeries, potentially resulting in pain, bowel obstruction, and infertility. This experimental study aims to evaluate the protective effects of diclofenac sodium and enoxaparin in reducing intra-abdominal adhesions and fibrotic changes within the large intestine wall. Using a rat model, animals were divided into three groups: a control group, a diclofenac sodium group, and an enoxaparin group. Each group underwent a standardized surgical procedure to induce adhesion formation, with postoperative administration of respective treatments in the diclofenac and enoxaparin groups. On day 14 post-surgery, animals were euthanized, and intra-abdominal adhesions were assessed histologically. Both diclofenac sodium and enoxaparin treatment groups showed a statistically significant reduction in adhesion severity and large intestine wall fibrosis compared to the control group ($p<0.05$). Enoxaparin exhibited a slightly greater efficacy in minimizing fibrotic tissue formation. The findings suggest that diclofenac sodium and enoxaparin have a protective effect against postoperative intra-abdominal adhesions and fibrosis. Enoxaparin, in particular, shows the potential as a therapeutic option for adhesion prevention in clinical settings. Further studies are recommended to optimize dosage and evaluate long-term outcomes.

Keywords: Diclofenac, enoxaparin, fibrosis, postoperative complications, rats

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INTRODUCTION

Postoperative intra-abdominal adhesions are pathological fibrous connections that form between the visceral membranes of organs or between organs and the walls of surrounding body cavities after tissue trauma and ischemia (Arung et al., 2011). Surgical techniques have continuously evolved, yet adhesions remain a common postoperative complication, developing after 50-95% of all surgeries, regardless of the anatomical location of the surgical procedure (Lauder et al., 2010). Adhesions can vary from thin layers of connective tissue to thick vascularized and innervated fibrous bridges (Diamond et al., 2011). They significantly impair the quality of life for patients, increasing the risk of repeat and additional surgical procedures, leading to higher morbidity, mortality, and treatment costs (Sikirica et al., 2011).

In the process of forming postoperative peritoneal adhesions, a crucial role is played by the local balance between fibrin production and fibrinolysis, which determines whether normal peritoneal repair occurs or adhesions form. If fibrin is not degraded within 5-7 days post-peritoneal injury, intra-abdominal adhesions develop (Duron, 2019; Choi et al., 2011; Wei et al., 2018; Hasdemir et al., 2017). This process involves numerous types of cells, cytokines, and biological processes. Local trauma may cause exudation of bleeding into the tissue, triggering an inflammatory response, while the fibrogenesis system plays an important role in adhesion formation (Arung et al., 2016). Simply put, local trauma in the peritoneum can lead to exudation of blood into the local tissue, stimulating an inflammatory response, whereby local vascular permeability increases and fluid rich in fibrinogen is secreted, which may result in the formation of a local membrane structure at the wound site (Bello-Guerrero et al., 2016). Subsequently, the inflammatory response system and the fibrogenesis system play a significant role in the further development of adhesions (Beyene et al., 2015; Fatehi et al., 2021).

There is also significant research addressing the use of non-steroidal anti-inflammatory drugs (NSAIDs) to reduce adhesions. Although the mechanism of this effect is unclear, it is believed that it results from the inhibition of cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2), which reduces the production of prostaglandins and thromboxanes (Gómez and Betancourt, 2018). Based on studies, the application of sodium diclofenac has been shown to be effective in reducing granulation tissue and adhesions (Allahverdi et al., 2014; Bahadir et al., 2017).

Despite the knowledge gained, many unknowns remain about the formation of intra-abdominal adhesions. For instance, while we know that adhesions result from a complex process, the precise mechanisms of interaction among various cells and cytokines in the inflammatory response are still not fully understood. Additionally, further research is needed to understand the optimal conditions for preventing adhesion formation and the impact of various factors, such as the type of surgery and individual patient characteristics.

Moreover, while studies have shown the efficacy of heparin in reducing adhesions, the mechanisms by which heparin acts and the optimal doses for prevention remain unclear (Opitz et al., 2003). In this context, future research should focus on clarifying these unknowns to enhance strategies for the prevention and treatment of postoperative adhesions.

The aim of this study was to investigate the protective effect of sodium diclofenac and enoxaparin on the formation of postoperative intra-abdominal adhesions and fibrotic changes in the large intestine wall using an experimental rat model.

MATERIALS AND METHODS

The study was conducted at the Veterinary Faculty and the Faculty of Medicine at the University of Sarajevo as a randomized, controlled, interventional, prospective, experimental study using a rat model.

Ethics Committee Approval

Approval for conducting the experimental work was granted by the Ethics Committee of the Veterinary Faculty of the University of Sarajevo under the number 07-03-1253-4/23.

Animals

Twenty-one healthy albino rats (Wistar strain) were used in the study, housed in a vivarium under standard conditions (temperature 21°C ± 2°C; humidity 50% ± 10%; 12 hours light/12 hours dark). Food and water were available ad libitum, with a restriction on food and water intake for 12 hours prior to surgery. Each rat was marked for unique identification.

Methods

The animals were randomly assigned to two groups (n=7) as follows:

Group I (Control): Induction of adhesions

Group II: Induction of adhesions with the application of low molecular weight heparin intraperitoneally and sodium diclofenac intramuscularly 7 days post-surgery.

Surgical Procedure for Adhesion Induction

Surgeries were performed under general anesthesia and aseptic conditions. The abdominal cavity was opened via medial laparotomy. The cecum was identified, and the terminal ileum was localized. Point bleeding on the colon was induced with sterile gauze, taking care to avoid injuries. An incision of 3 mm was made on the bowel, which was closed with sutures (polypropylene 5/0).

Therapeutic Procedures

Anesthesia was induced with ketamine hydrochloride (80 mg/kg IM). The dose of low molecular weight heparin (enoxaparin) was 0.5 mg/kg/day, and sodium diclofenac was administered at 2 mg/kg/day, both intramuscularly and intraperitoneally.

Postoperative Monitoring

The rats were monitored for 2 weeks post-surgery, with the appropriate administration of low molecular weight heparin and sodium diclofenac for 7 days, according to the previously defined groups.

Procedure for Biological Material Collection and Euthanasia

The rats were euthanized on the 14th day of the experiment with ketamine hydrochloride. Samples for adhesion analysis were collected via laparotomy.

Quantitative Evaluation of Adhesions

The assessment of the number of adhesions was conducted according to the Nair Modified Scoring Adhesion System (Table 1).

Histopathological Analysis

A 2 mm thick part of the large intestine, located 5 cm distal to the ileocolic junction, was taken for the histopathological analysis. The large intestine samples were fixed in 10% buffered formalin, stained with H&E and Masson trichrome, and qualitative histopathological analysis was performed.

Table 1 Nair Modified Scoring system for adhesion classification

Adhesion grade
0 - No adhesions present
1 - Presence of a single thin adhesion: organ-organ, or organ-abdominal wall
2- Presence of a single thick adhesion: organ-organ or organ-abdominal wall
3 - Presence of two thin or thick adhesions: organ-organ or organ-abdominal wall
4 - Presence of more than two thin or thick adhesions: organ-organ or organ-abdominal wall or intestines forming a conglomerate with adhesions without adhesion to the abdominal wall

All relevant findings were documented/photographed using an iPhone 14 Pro Max (Figure 1).

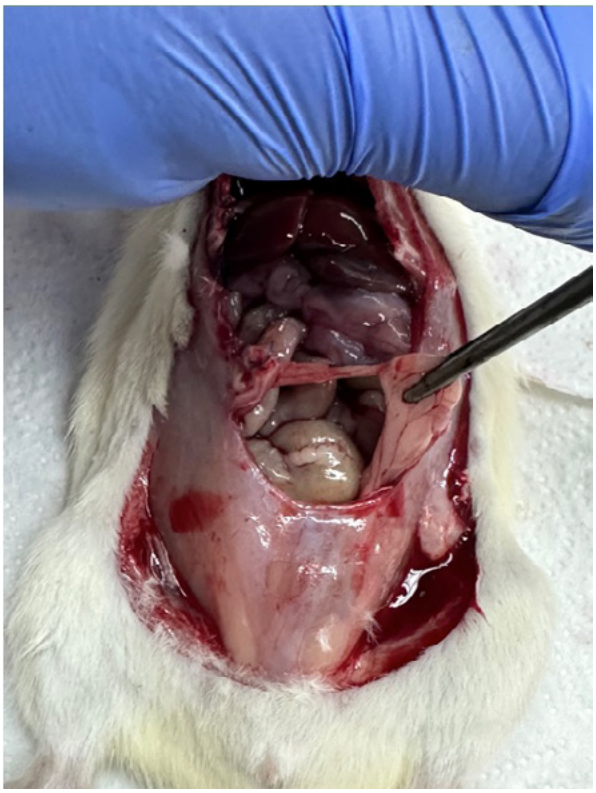

Figure 1 Grade 2 adhesions according to the Nair Modified Scoring system

Statistical Data Analysis

Collected data were statistically analyzed using R 4.4.2. (R Foundation for Statistical Computing, Vienna, Austria) and Rstudio 2024.04.2 (Posit Software, PBC, Boston, Massachusetts, USA). Numerical variables are represented by arithmetic means and standard deviations, and median, interquartile range (Q1-Q3) and range (min-max). Quantitative variables were tested for normal distribution using Shapiro-Wilk test and evaluated by QQ-plot and histograms.

RESULTS

The median value of the modified Nair scoring system in the control group was 3 (IQR=2-4), while in the group treated with intraperitoneal low molecular weight heparin and intramuscular diclofenac, it was significantly lower at 1 (IQR=1-2), which was statistically significant (p=0.01).

In the group of animals that did not receive treatment, a higher average intensity of adhesions was observed. Two animals (29%) had a Nair score of 2, while three animals (43%) had two adhesions present. Additionally, two animals (29%) had more than two adhesions.

In contrast, among the animals treated with intraperitoneal low molecular weight heparin combined with intramuscular diclofenac, one (14%) animal had no adhesions, four (57%) had one thin adhesion, one (14%) had one thick adhesion, and one (14%) had two adhesions (Table 2).

Table 2 Results of the Modified Nair scale by groups

Variable	Group		p-value
	Control N = 7	LMWH (IP) + DS (IM) N = 7	
Nair Modified Scoring system			**0.0101**
Mean (±SD)	3.00 (±0.82)	1.29 (±0.95)	
Median [Q1–Q3]	3.00 [2.00–4.00]	1.00 [1.00–2.00]	
Range (Min–Max)	2.00–4.00	0.00–3.00	
Modified Nair Scoring Scale (descriptive), n (%)			0.0792
No adhesions present (0)	0 (0%)	1 (14%)	
Presence of one thin adhesion (1)	0 (0%)	4 (57%)	
Presence of one thick adhesion (2)	2 (29%)	1 (14%)	
Presence of two thin or thick adhesions (3)	3 (43%)	1 (14%)	
Presence of more than two adhesions (4)	2 (29%)	0 (0%)	
1Wilcoxon rank-sum test			
2Fisher's exact test			

LMWH, Low Molecular Weight Heparin; DS, Diclofenac Sodium

Results of the Histopathological Analysis

Group 1

The histopathological analysis of the large intestine in animals from this group revealed marked alterations of the intestinal walls (Figures 2A and 2C). The mucosa was infiltrated with mononuclear cells, particularly pronounced beneath the surface epithelium. Mild oedema of the lamina propria was also present in some areas (Figure 2C). The mononuclear infiltrate was extending from the lamina propria into the superficial parts of the submucosa, leading to reduced visibility of the muscularis mucosae. Additionally, fibrous changes in the submucosa were observed in places. The subserosa was markedly thickened due to an increased amount of fibrous connective tissue, and occasionally, due to an increase in cellular connective tissue (Figure 2A and 2E).

Group 2

In this group of animals, the large intestine walls exhibitedmild alterations and a clear stratification (Figures 2B and 2D). The mucosa was also infiltrated with mononuclear cells but without pronounced accumulation beneath the epithelium (Figure 2D). Fibrosis and infiltration in the submucosa were rare, with a clear boundary between the mucosa and submucosa. The subserosa was slightly thickened, dominantly composed of cellular connective tissue (Figure 2B and 2F).

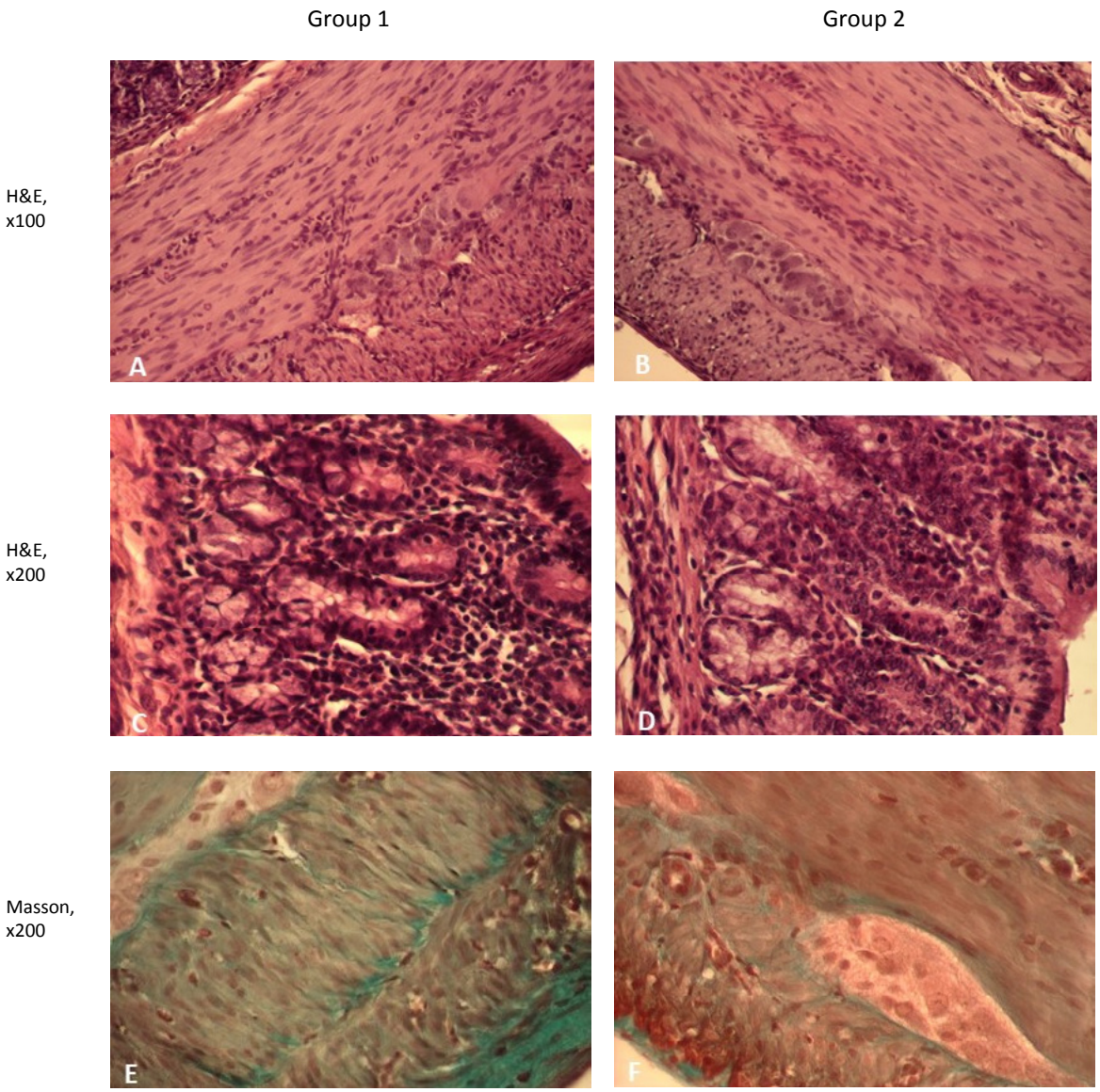

Figure 2 Representative photomicrographs of groups 1 and 2

DISCUSSION AND CONCLUSION

Abdominal postoperative adhesions represent a significant clinical problem, often causing numerous complications, readmissions, and even chronic abdominal pain. These pathological fibrous connections, which form between the membranes of visceral organs or between organs and the walls of surrounding body cavities after surgical procedures, can lead to subocclusive disturbances in patients. Literature reports that up to 80% of patients exhibit symptoms of these disturbances, further highlighting the seriousness of the issue (Arung et al. 2011; Sikirica et al., 2011; Choi et al., 2018; Wei et al., 2018).

Certain chronic conditions, such as hypertension, diabetes mellitus, and metabolic syndrome, have been shown to increase the risk of developing postoperative adhesions. These complications not only worsen the quality of life for patients, but

also significantly increase hospital treatment costs, estimated to be in the billions of dollars annually. Therefore, understanding the pathophysiology of adhesion formation and developing strategies for their prevention is essential (Vipond et al., 1990).

Many strategies, including mechanical barriers, chemicals, and pharmacological approaches, have been investigated to prevent the formation of postoperative adhesions. In our study, we focused on the effectiveness of low molecular weight heparin (enoxaparin) in preventing intra-abdominal adhesions by disrupting the coagulation cascade, thereby reducing the formation of fibrin bridges. Our results indicate a significant reduction in adhesions in the group treated with enoxaparin, which aligns with previous research suggesting that low molecular weight heparins have a prophylactic effect (Kement et al., 2011; Arikan et al. 2005; Kaptanoglu et al. 2008; Rosillo et al., 2011).

In addition to heparin, we investigated the effectiveness of sodium diclofenac, a COX-2 inhibitor, which may limit the inflammatory response of the peritoneum. Our study reveals that intramuscular administration of diclofenac resulted in reduced inflammation and fibrosis in the wall of the large intestine, supporting findings from other researchers who have reported similar effects (Girish and Pradhan, 2012; Lisete et al., 2005; Lardinois et al., 2004; Harvey et al., 2019).

What is particularly significant in this study is the synergistic effect of the combination of enoxaparin and diclofenac. Our results suggest that the application of this combination led to a statistically significant reduction in the incidence of postoperative adhesions and fibrotic changes compared to control groups. These findings support the hypothesis that inhibition of fibrinogen formation, along with limiting the inflammatory response, can effectively reduce adhesion formation.

In conclusion, our research demonstrates that low molecular weight heparin (enoxaparin) and sodium diclofenac significantly reduce the incidence of postoperative adhesions. The synergistic effect of this drug combination further contributes to the reduction of inflammation and fibrosis, suggesting that their application may effectively prevent complications following surgical procedures. These results underscore the need for further research to better understand the mechanisms of action and optimize their clinical use.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception: EK, EH, DK; Design: EH; Supervision: AB, DK; Materials: EK, MS; Data Collection and/or Processing: MS, EK; Analysis and/or Interpretation: ED, EH, MS; Literature Search: EK, EH, DK; Writing – Original Draft: EK; Critical Review: EH

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ZAŠTITNI EFEKAT DIKLOFENAK NATRIJUMA I ENOKSAPARINA NA FORMIRANJE POSTOPERATIVNIH INTRAABDOMINALNIH ADHEZIJA I FIBROZNIH PROMJENA U ZIDU DEBELOG CRIJEVA NA EKSPERIMENTALNOM MODELU STAHORA

SAŽETAK

Postoperativne intraabdominalne adhezije predstavljaju čestu komplikaciju abdominalnih operacija i mogu izazvati bol, opstrukciju crijeva i neplodnost. Cilj našeg eksperimentalnog istraživanja je evaluacija zaštitnih efekata diklofenak natrijuma i enoksaparina u smanjenju intraabdominalnih adhezija i fibroznih promjena koje nastaju u zidu debelog crijeva. Korištenjem modela štakora, životinje su podijeljene u tri grupe: kontrolnu, diklofenak natrijum grupu i enoksaparin grupu. Svaka grupa je podvrgnuta standardiziranoj operativnoj proceduri sa ciljem indukcije adhezija uz postoperativnu aplikaciju odgovarajuće terapije u diklofenak i enoksaparin grupi. Životinje su eutanazirane 14. dan postoperativno, a intraabdominalne adhezije su histološki analizirane. Diklofenak natrijum grupa i enoksaparin grupa su pokazale statistički signifikantno smanjenje adhezija i fibroze intestinalnog zida u usporedbi sa kontrolnom grupom. Enoksaparin je pokazao nešto bolji učinak u minimiziranju nastanka fibroznog tkiva. Rezultati pokazuju da i diklofenak natrijum i enoksaparin imaju zaštitno djelovanje kod nastanka intraabdominalnih adhezija i fibroze. Naročito enoksaparin pokazuje terapijski potencijal u prevenciji adhezija u kliničkim uvjetima. Preporučuju se dodatna istraživanja u svrhu optimiziranja doze i procjene dugoročnih rezultata.

Ključne riječi: Diklofenak, enoksaparin, fibroza, postoperativne komplikacije, stahori

RESEARCH ARTICLE

ASSESSMENT OF HAEMATOLOGICAL PROFILE AND OTHER PATHOLOGICAL CHANGES IN FARM CHICKENS NATURALLY INFECTED WITH CHICKEN ANAEMIA VIRUS IN MAIDUGURI

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ABSTRACT

The haematology and histopathological changes in Chicken anaemia virus (CAV) infection among apparently healthy village chickens in Maiduguri, Nigeria, were investigated using conventional PCR. A total of one hundred blood and tissue samples were obtained from the study area. Blood samples were collected in EDTA tubes for haematological analysis, while tissue samples (thymus, liver, and spleen) were collected and pooled from each of the birds in duplicates into 10% neutral buffered formalin for histopathology, and the other sample was frozen at -20oC for chicken anaemia virus DNA extraction. A total of 42/100 (42%) of the pooled tissue samples were positive for CAV DNA with the expected band size of 387 bp. Haematological findings showed no significant difference between CAV-positive and CAV- negative chickens in all the parameters examined. Microscopic changes observed in the CAV- positive samples were focal aggregations of inflammatory cells, primarily in lymphoid cells and binucleate hepatocytes in the liver, lymphoid depletion in the white pulp, arising as a result of lymphocytolysis of the lymphoid follicular cells of the spleen, and lymphoid depletion in the cortical area of the thymus. These findings are the first of their kind in this region, underscoring the need to further explore CAV epidemiology in Nigeria. The study highlights the necessity for ongoing development and implementation of reliable diagnostic methods, including molecular techniques, to inform appropriate preventive measures against this economically significant avian disease.

Keywords: Chicken anaemia virus, haematological parameters, histopathology, molecular diagnostic, village chicken

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INTRODUCTION

Diseases are the main factor limiting the viability of the village chicken production system in underdeveloped nations (Hamer et al., 2013; Sehgal, 2015). However, the studies on infectious poultry diseases in developing African nations have mostly concentrated on bacterial, viral, and protozoan infections; other studies have also examined ectoparasites and gastrointestinal parasites (Letebrhan et al., 2015; Weyuma et al., 2015). There has not been much focus about Chicken anaemia virus infections and their effects. In much of Nigeria, village chickens are usually raised under complex management systems (Opara et al., 2014). According to Chepkemoi et al. (2017) and Lawal et al. (2021), they have access to an environment where they hunt for food, even on unclean rubbish dumps and near unclean pools of water. Where the chickens are kept unconfined, disease control becomes difficult, and outbreaks of infectious diseases such as Chicken anaemia virus (CAV) cause substantial losses (Shettima et al., 2017).

Chicken anaemia virus disease is an economically significant poultry disease that causes infectious anaemia. It is one of the important poultry diseases in developing regions, including Nigeria (Fatoba and Adeleke, 2019). The virus has been classified as a single-stranded DNA virus with icosahedral symmetry initially belonging to the family *Circoviridae* (Alkateb and Gerish, 2019; Sreekala et al., 2020). However, CAV has recently been classified in the *Gyrovirus* genus of the *Anelloviridae* (Tanget al., 2016; Li et al., 2017a; Rosario et al., 2017). Chicken infectious anaemia, also known as blue wing disease or anaemia-dermatitis syndrome, is a viral infection of chicks mostly 2–4 weeks old (Gowthaman, 2019; Schatand Van Santen, 2020; Kamdi et al., 2020). The virus is known for its high mortality in chickens. It also causes immunosuppression, subcutaneous hemorrhage, and anaemia (Orakpoghenor, 2019; Chandrashekaraiah et al., 2020). Other reported clinical signs and lesions included lymphoid depletion, atrophy of the bursa of Fabricius and thymus, hemorrhages in thigh and breast muscles and yellowish to whitish bone marrow (Abdallah et al., 2022; Mounika et al., 2023).

Since its initial discovery in 1979, Chicken anaemia virus disease has become widely distributed and prevalent worldwide. The disease was first documented in Ibadan, Nigeria, by Oluwayeluet al. (2005) in Ibadan, Nigeria. The Chicken anaemia virus is thought to be ubiquitous in Nigeria because its presence has been identified in commercial flocks of birds (Oluwayelu, 2010; Schatand Van Santen, 2020; Adedeji et al., 2024), including village chickens, ducks, turkeys, and geese (Shettima et al., 2017).

To the best of our literature search, there is a paucity of information about the Chicken anaemia virus in the study area. Therefore, the purpose of this study was to determine the prevalence of CAV using conventional PCR techniques, and to evaluate the possible haematological and microscopic changes induced by the disease in village chickens.

MATERIALS AND METHODS

Ethical Consideration

All applicable national and international guidelines for the care and use of animals were followed. All procedures performed in the studied animals were following the ethical standards of the University of Maiduguri, Faculty of Veterinary Medicine, Committee on Animal Use and Care (AUP No.: AUP-R003/2023).

Study Area

This investigation was conducted in Maiduguri Metropolis, Borno State, Nigeria. Borno State is located in the northeastern part of Nigeria (Elijah et al., 2022). The state shares borders with three West African countries, namely, the Republic of Chad to the northeast, Niger Republic to the north and Cameroon Republic to the east. Within the country, it neighbors were Bauchi State to the south, Yobe State to the west and Gombe State to the southwest (Elijah et al., 2022).

Experimental Design

The study utilized a convenience sampling method. One hundred blood and tissue samples (including thymus, liver, and spleen) were collected from village chickens in Maiduguri. The study took into consideration gender (cocks and hens). Chickens sampled were classified as growers (3–4 months) and adults (over 5 months), according to Addass et al. (2012) age descriptions for chickens. Blood samples obtained at the point of slaughter were placed into sterile vacutainer tubes containing EDTA and transported to the Veterinary Pathology Laboratory at the University of Maiduguri for haematological analysis. The tissue samples were collected in duplicate, one aliquot was directly placed into sample bottles and stored at -20oC for subsequent investigation of CAV presence using a conventional method. The other aliquot was fixed in 10% buffered formalin for histopathological analysis. A Chicken anaemia virus (Cux-1 DNA)-positive control, indicative of Chicken anaemia virus, was sourced from a virus research laboratory at the National Veterinary Research Institute (NVRI) in Vom, Nigeria. This control was utilized for PCR optimization and viral replication monitoring.

Evaluation of Haematological Parameters

For haematological analysis, blood samples were collected into EDTA sample tubes from the jugular vein during slaughter. Blood samples were assessed for packed cell volume (PCV), hemoglobin concentration (HbC), total red blood cell (RBC) count, white blood cells (WBC) count and WBC differentials, according to Campbell (2010). The mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were calculated, based on the PCV, RBC count, and Hb, respectively.

Tissue Preparation for Histology

The 10% buffered formalin fixed thymus, spleen, and liver were dehydrated in graded alcohol (70, 80, 90, and 100%), while xylene and paraffin wax were used for clearing and embedding, respectively. Serial sections 5 µ thick were obtained using a rotator microtome. Deparafinised sections were stained with hematoxylin and eosin, as described by Majama and associates (2024). Slides were examined using light microscope at different magnifications. Photomicrographs of lesions were obtained using a digital camera (AmScope) mounted on the microscope.

PCR

Viral DNA extraction was carried out from homogenized pooled tissue samples using a high pure template preparation kit (innuPREP DNA Mini Kit 2.0), according to the manufacturer's instructions (Ajinnuscreen, GmbH, Berlin, Germany). The extracted DNA concentration was measured and kept at -20°C prior to use. Briefly, a general master mix (MyTaqTMMix, Bioline GmbH, Germany) was prepared, which contained all the reaction components in one cocktail without the DNA template. The PCR tubes were labeled according to the number of samples, one each of the negative and positive controls was included. The reagents were thawed and left on ice. The PCR tubes were transferred into the heating block of the thermocycler (Eppendorf Mastercycler Nexus, Hamburg, Germany), and the tubes were properly closed to prevent evaporation during PCR. According to the DNA polymerase provider, there was an initial denaturation phase, followed by 35 cycles of one denaturation, one annealing, and one extension step each. Denaturation was observed at 95°C for 30 seconds, annealing at 64°C for 45 seconds, and elongation was set at 65°C for 5 minutes.

Statistical Analysis

The data generated from the study were subjected to statistical analysis using SPSS version 16.0 statistical software and the data were expressed as mean±standard deviation. Chi-square test was used to perform categorical comparison and determine significance at 95% confidence interval. P-value of 0.05 or less was considered statistically significant.

RESULTS

Detection of CAV Infection by Conventional PCR

The conventional PCR used for the detection of CAV DNA from village chickens tissue samples in Maiduguri using the CUX-1 set of primers produced the expected band of 387bp on Agarose gel electrophoresis. Out of the 100 tissue samples analyzed by conventional PCR, 42 (42%) samples were positive for CAV nucleic acid.

The effects of natural CAV infection on haematological parameters of village chickens in Maiduguri

Table 1 summarizes the effects of natural CAV infection on the haematological parameters of village chickens in Maiduguri, Nigeria. No significant difference was seen in all the haematological parameters of CAV-positive birds as compared to the CAV-negative birds, with the exception of MCH, MCHC, and mean heterophil counts. The mean MCH, MCHC, and heterophil counts were significantly ($P < 0.05$) higher in CAV-positive chickens as compared to CAV-negative birds.

The mean values of the haematological parameters for CAV-positive and CAV-negative male and female village chickens are presented in Table 2. There were no significant differences between male and female village chickens, both CAV-positive and CAV-negative, in the mean values of the PCV, Hb, RBC, WBC, MCV, heterophil, lymphocyte and basophil counts. However, CAV-positive female chickens demonstrated significantly higher MCH and MCHC values ($P < 0.05$) compared to CAV-positive male village chickens and CAV-negative male and female village chickens. On the other hand, the CAV-positive male village chickens exhibited significantly higher mean monocyte and eosinophil counts ($P < 0.05$) than CAV- positive female and CAV-negative male and female village chickens.

The mean values of haematological parameters of CAV-positive and CAV-negative chicks and grower village chickens are presented in Table 3. No significant differences ($P > 0.05$) were observedbetween the chicks and growers of both CAV-positive and CAV-negative village chickens in the mean values of PCV, Hb, RBC, WBC, MCV, heterophil, lymphocyte, and basophil counts. However, CAV-positive grower village chickens demonstrated significantly higher MCH and MCHC values ($P < 0.05$) compared to CAV-positive chicks, as well as both chicks and grower CAV-negative village chickens. Additionally, mean monocyte and eosinophil counts were to be significantly higher ($P<0.05$) higher in CAV-positive village chicks as compared to CAV-positive grower chickens, and both CAV-negative chicks and grower village chickens.

Microscopic lesions

Histopathological examination of naturally infected CAV-positive chicken tissues confirmed by PCR assay showed focal aggregations of inflammatory cells primarily in lymphoid cells and binucleate hepatocytes in the liver (Figure 1). The spleen showed severe lymphoid depletion in the white pulp arising as a result of lymphocytolysis of the lymphoid follicular cells (Figure 2). The thymus also showed lymphoid depletion in the cortical area (Figure 3).

Table 1 Effect of natural CAV infection on haematological parameters of village chickens in Maiduguri, Nigeria

Parameters	CAV-Positive (n=42) village chickens	CAV-Negative (n=58) village chickens
PCV (%)	31.8±3.8a	31.2±3.6a
Hb(g/dl)	10.8± 5.1a	10.5 ± 3.2a
RBC(x106/µl)	2.6 ± 0.5a	2.5 ± 0.4a
WBC(x103/µl)	18.2 ± 5.7a	17.0 ± 5.3a
MCV(fl)	127.4 ± 15.6a	125.8 ± 18.8a
MCHC(g/dl)	35.8 ± 15.0a	32.0 ± 7.1b
MCH(pg)	45.8 ± 22.1a	39.8 ± 8.3b
Heterophil(x103/µl)	6.1 ± 5.7a	5.8 ± 2.3b
Lymphocyte (x103/µl)	10.4 ± 3.2a	9.8 ±3.1a
Monocyte (x103/µl)	0.9 ± 0.5a	0.8 ± 0.3a
Eosinophil(x103/µl)	0.8 ± 0.4a	0.6 ± 0.3a
Basophil (x103/µl)	0.2 ± 0.6a	0.2 ± 0.8a

a,bMeans±standard deviations with different superscripts are significantly different at $p<0.05$ along rows

Table 2 Effects of sex on the haematological parameters of village chickens naturally infected with CAV in Maiduguri Nigeria

Parameters	CAV-Positive		CAV-Negative	
	Male (n=20)	Female (n=22)	Male (n=24)	Female (n=26)
PCV(%)	32.5 ± 3.5a	31.2 ± 4.0a	30.6 ± 2.7a	32.5 ± 4.0a
Hb(g/dl)	11.0 ± 5.1a	10.6 ± 5.1a	10.7 ± 4.1a	10.8 ± 2.4a
RBC(x106/µl)	2.6 ± 0.5a	2.5 ± 0.5a	2.4 ± 0.4a	2.7 ± 0.4a
WBC(x103/µl)	19.1 ± 5.7a	17.4 ±5.6a	15.9 ± 4.8a	18.6± 5.6a
MCV(fl)	127.2±15.5a	127.6+16.0a	125.8±19.6a	124.5±17.6a
MCHC (g/dl)	28.3 ± 4.9a	42.5 ± 54.2b	31.2±6.0a	34.0±8.3a
MCH(pg)	35.8 ±5.9a	55.0 ±27.1b	38.7±6.1a	41.9±10.3a
Heterophil (103/µl)	6.4 ± 2.3a	5.9 ± 2.6a	5.5 ± 2.3a	6.2 ± 2.4a
Lymphocyte (103/µl)	10.9 ± 3.5a	10.1 ± 3.1a	9.1 ± 2.7a	10.7 ± 3.6a
Monocyte (103/µl)	1.0 ± 0.5b	0.8 ±0.4a	0.7 ± 0.3a	0.9 ±0.3a
Eosinophil(x103/µl)	0.9 ± 0.5b	0.7 ± 0.3a	0.6 ± 0.3a	0.7 ± 0.3a
Basophil (x103/µl)	0.3 ± 0.7a	0.0 ± 0.0a	0.2 ±0.8a	0.0 ± 0.1a

a,bMeans±standard deviations with different superscripts are significantly different at $p<0.05$ along rows

Table 3 Effect of age on the haematological parameters of village chickens naturally infected with CAV in Maiduguri

Parameters	CAV-Positive		CAV-Negative	
	(Chicks)	(Growers)	(Chicks)	(Growers)
PCV (%)	31.3 ± 4.4a	32.1 ± 3.5a	31.1 ± 2.9a	31.1 ± 4.2a
Hb(g/dl)	9.0 ± 1.7a	11.6 ± 5.9a	10.4 ± 2.7a	10.7 ± 3.6a
RBC(x106/µl)	2.7 ± 0.4a	2.5 ± 0.5a	2.5 ± 0.4a	2.5 ± 0.4a
WBC(x103/µl)	20.0 ± 5.0a	17.4 ±5.9a	17.3 ± 4.4a	16.7 ± 5.9a
MCV(fl)	126.1±15.0a	128.0+16.0a	127.0±11.6a	124.5±14.6a
MCHC (g/dl)	30.5 ± 6.3a	38.2 ± 17.1b	31.8±8.7a	32.3±5.2a
MCH(pg)	37.9 ±6.0a	49.4 ±25.6b	39.5±9.5a	40.0±7.0a
Heterophil (103/µl)	6.9 ± 2.3a	5.8 ± 2.6a	6.0 ± 1.9a	5.6 ± 2.6a
Lymphocyte (103/µl)	11.1 ± 3.0a	10.1 ± 3.4a	9.7 ± 3.0a	9.7 ± 3.3a
Monocyte (103/µl)	1.1 ± 0.5b	0.8 ±0.4a	0.8 ± 0.3a	0.8 ±0.3a
Eosinophil(x103/µl)	0.9 ± 0.5b	0.7 ± 0.4a	0.7 ± 0.4a	0.5 ± 0.3a
Basophil (x103/µl)	0.0 ± 0.1a	0.0 ± 0.1a	0.0 ±0.1a	0.0 ± 0.0a

a,b Means±standard deviations with different superscripts are significantly different at $p<0.05$ along rows

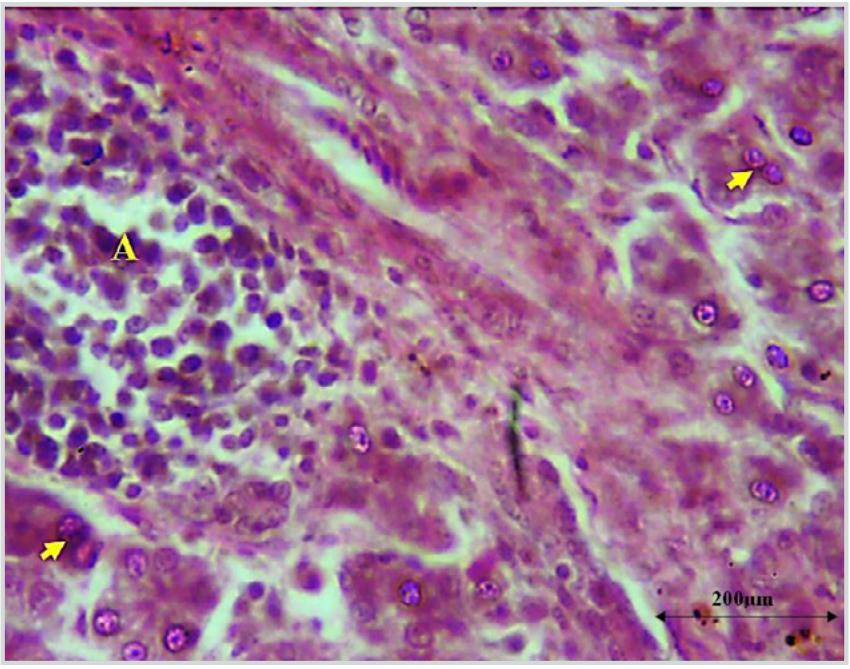

Figure 1
Photomicrograph of a section of the liver in CAV-positive village chicken showing focal aggregations of lymphoid cells (A) and binucleate hepatocytes (arrow heads), H & E X400

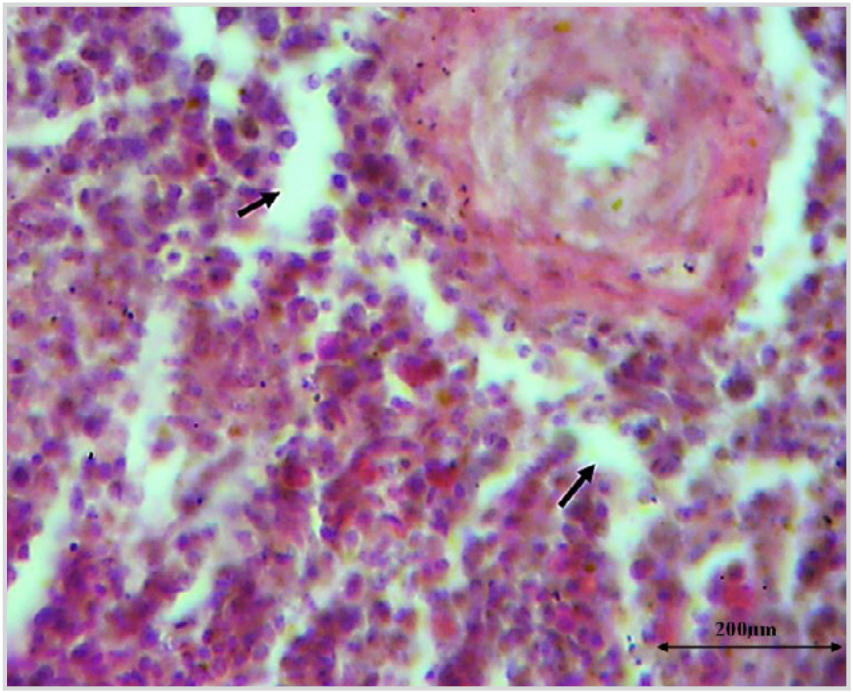

Figure 2

Photomicrograph of a section of the spleen in CAV-positive chicken showing severe depletion of the lymphoid follicle leaving empty spaces (black arrow), H & E, X400

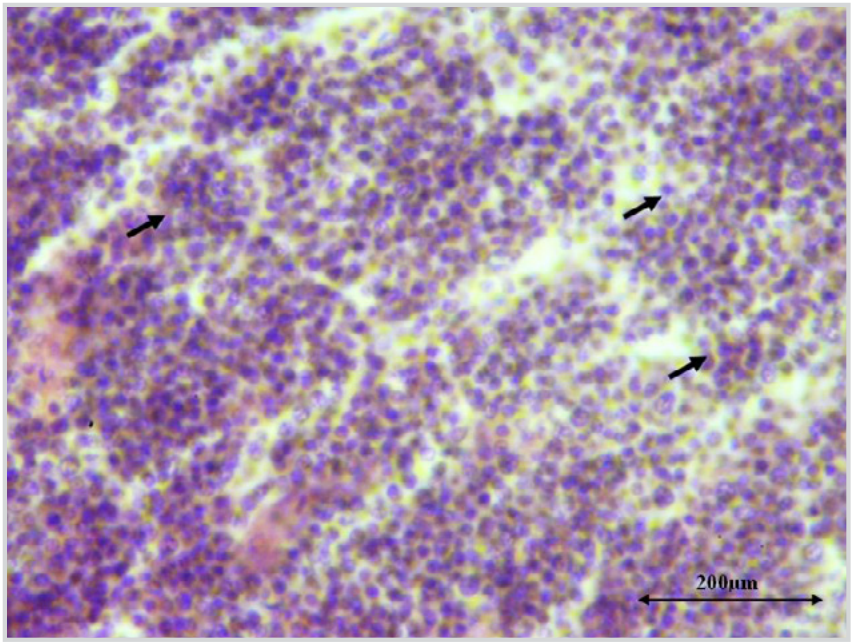

Figure 3

Photomicrograph of a section of the thymus in CAV-positive chickens showing cortical depletion of lymphocytes (black arrows), H & E, X400

DISCUSSION AND CONCLUSION

Chicken anaemia virus has been identified using different serological (Abdelwahab and Mansour, 2019; Fatoba et al., 2019) and molecular techniques (Yaoet al., 2019; Tan et al., 2020). Molecular techniques,such as polymerase chain reaction (PCR), offer significant advantages by providing faster and more specific identification of more fastidious viral pathogens (Mustafa et al., 2020). Therefore, the identification of CAV DNA in village chicken tissues by PCR in this investigation confirm the susceptibility of hens to CAV infections, as the presence of DNA indicates active infection. Given the vaccination against CAV is not commonly practiced among backyard chickens in Nigeria, the detection of CAV DNA in thee ostensibly healthy, free-roaming chickens suggests natural exposure to the virus and identifies them as potential reservoirs for transmission to commercial poultry. Furthermore, it was observed in this study that there was no statistically significant difference between the haematological values (PCV, Hb, RBC, WBC, MCV, heterophil, lymphocyte, monocyte, and basophil counts, MCH, and MCHC) of CAV-positive village chickens, between gender or across age groups. This aligns with the findings of Haridy et al. (2012), who reported that no significant difference between the PCV of CAV- experimentally- infected chickens and their uninfected controls. The higher mean values of WBC, heterophil, lymphocyte, observed in this study tallies with the findings of Wani et al. (2015) reported elevated heterophil and lymphocyte values in CAV-infected flocks compared to normal ones. Notably, heterophils significantly increased, whereas lymphocytes showed a significantly decreased in CAV-infected flocks compared to the normal flock. In contrast, eosinophils and monocytes did not significantly change except in some flocks (Krishan et al., 2015). The findings of the present study may be attributed to co-infection of the CAV with other pathogens. It is also considered that village chickens could act as carriers for facilitating transmission within the flocks (Oluwayelu et al., 2005; Pauly et al., 2019; Conteh et al., 2020).

The microscopic changes observed in the thymus, liver, and spleen of CAV-infected village chickens in the present study are in general agreement with earlier reports by Mounika et al. (2023). This observation also supports the report of Hegazy et al. (2014) that the Chicken anaemia virus leads to atrophy of the thymus gland. Hussein et al. (2016) and Govindhasamy (2022) reported a similar pattern of lymphoid reduction and proliferation of ellipsoidal reticular cells in the spleen and thymus in certain CAV-infected birds. It has also been observed that CAV produces significant shrinkage and loss of zonal architecture, with no clear distinction between the cortex and medulla of the thymus (Abdallah et al., 2022). CAV generates a protein (VP3 or apoptin) that triggers apoptosis, and it is this apoptotic mode of thymocyte death that most likely accounts for lymphoid depletion in the absence of severe inflammation (Hussein et al., 2016). The significant death of lymphocytes seen in all analyzed lymphoid organs in the current investigation is consistent with the findings of Hegazy et al. (2014), Lai et al. (2017) and Feng et al. (2020) all reported that the Chicken anaemia virus VP3 protein triggered apoptosis in vitro and in vivo. These show that apoptosis, a phenomenon that has been observed for a few other viruses, is also an important phenomenon during the pathogenesis of CAV (Hussein et al., 2016; Feng et al., 2020). Backyard chickens' hardiness, which is the outcome of natural challenge selection, shows that they are more disease resistant and less susceptible to CAV infection. Furthermore, these chicks may develop into healthy reservoirs, a theory that calls for additional research into the potential of subclinical chicken anaemia.

In conclusion, there were no statistically significant changes among the haematological parameters of CAV-positive and CAV-negative village chickens. Histopathological examination of the naturally infected tissues, which were positive for CAV DNA by PCR assay, confirmed microscopic lesions associated with CAV infections.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

CONTRIBUTION

YMS-Conception, Design, Funding and writing; HIG-Supervision, Data interpretation/Analysis, and critical review; TMH-Critical review; MUS-Data collection and Literature review; MBM-Supervision; YBM-Materials; AMW-Writing.

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PROCJENA HEMATOLOŠKOG PROFILA I DRUGIH PATOLOŠKIH PROMJENA KOD FARMSKIH PILIĆA PRIRODNO INFICIRANIH SA VIRUSOM INFEKTIVNE ANEMIJE PILIĆA U MAIDUGURIJU

SAŽETAK

Korištenjem konvencionalne PCR metode istraživali smo hematološke i histopatološke promjene kod infekcije sa virusom infektivne anemije pilića (CAV) naizgled zdravih seoskih pilića u Maiduguriju, u Nigeriji. Na istraživanom području smo prikupili ukupno stotinu krvnih i tkivnih uzoraka. Krvni uzorci za hematološku analizu su prikupljeni u EDTA epruvete, dok su uzorci tkiva (timus, jetra i slezena) prikupljeni dvostruko i stavljeni u 10% neutralni puferski formalin za histopatologiju, dok je drugi uzorak zamrznut na -20oC radi ekstrakcije DNA virusa infektivne anemije pilića. Ukupno 42/100 (42%) uzetih tkivnih uzoraka je bilo pozitivno na CAV DNA s očekivanom veličinom trake od 387 bp. Hamatološki nalazi nisu pokazali signifikantnu razliku između CAV-pozitivnih i CAV-negativnih pilića za sve ispitivane parametere. Mikroskopske promjene uočene na CAV-pozitivnim uzorcima su bile u obliku fokalnih nakupina upalnih stanica, prvenstveno kod limfoidnih stanica i binuklearnih hepatocita, limfoidna deplecija u bijeloj srži kao rezultat limfocitolize limfoidnih folikularnih stanica slezene te limfoidna deplecija kortikalnog područja timusa. Ovi rezultati su prvi ovakve vrste u regiji, naglašavajući potrebu za daljnjim istraživanjem epidemiologije CAV-a u Nigeriji. Istraživanje naglašava potrebu za stalnim razvojem i implementacijom pouzdanih dijagnostičkih metoda, uključujući i molekularne tehnike, kako bi se kreirale odgovarajuće preventivne mjere protiv ove ekonomski signifikantne ptičje bolesti.

Ključne riječi: Hematološki parametri, histopatologija, molekularna dijagnostika, seoski pilići, virus infektivne anemije pilića

RESEARCH ARTICLE

ANALYSIS OF SOME INTERLEUKINS AND LEUKOGRAM IN ISA BROWN COCKS EXPERIMENTALLY INFECTED WITH *SALMONELLA GALLINARUM*

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ABSTRACT

This research aimed to investigate the profile of IL- 1β, INFγ, and leukogram in cocks experimentally infected with *Salmonella* gallinarum. A total of 40 cocks, infected and control, 20 birds each were used for this study. Each bird in the infected group was administered orally 1ml inoculum 9.0 x 108 CFU/ml *Salmonella* gallinarum. Blood samples were collec ted and analyzed for leukogram, and the harvested serum samples were analyzed for IL-Iβ and INF γ. There were significant differences ($p < 0.05$) in the mean absolute TWBC, heterophil and lymphocyte counts in the infected and the control cocks almost at levels. The highest values of TWBC (14.5 ± 0.60 x 109/1), heterophil (4.4 ± 0.31 x 109/1) and lymphocyte (10.4±0.55 x 109/1) counts were observed in the infected group on Day 21 (p.i.). There were significant differences ($p < 0.05$) in the mean serum IL-1β and IFN-γ concentrations in the infected and the control cocks on Day 4 (185±46.0 pg/ml) and Day 7 p.i. (2634± 342 pg/ml), respectively. The highest and lowest values for mean serum IL-1β concentration kwere observed to be 695±1.0 pg/ml and 185±46.0 pg/ml in the infected group on Days 14 and 7 p.i., while that of IFN γ was 4134±217 pg/ml and 2634 ± 342 pg/ml in the infected group on Days15 p.i. and cocks may play a vital role in driving higher immune responses during *Salmonella* gallinarum infection.

Keywords: Cockerels, inoculum, ISA Brown, *Salmonella*

INTRODUCTION

Among poultry diseases, fowl typhoid, caused by *Salmonella g*allinarum, is one of the most important bacterial diseases that poses serious challenges to poultry production, worldwide (Saidu et al., 1990; Saidu et al., 1994; Majid et al., 2010). This is aside the fact that it also constitutes a source of food-borne and zoonotic transmission of the disease to humans (Hafez *et al*., 2020). Fowl typhoid is an acute disease, which affects primarily chickens and turkeys, but pheasants, quails, and guinea-fowl are also susceptible (Shivaprasad, 2000; Casagrande et *al*., 2014). Fowl typhoid is considered one of the most important septicaemic bacterial diseases of chickens with consequent huge economic losses (Evans, 2011). These losses are represented by variable morbidity and high mortality, with a severe septicemic disease, occurring primarily in adult birds (Gemechu et al., 2020). The most significant sources of the infection in the poultry industry are food, environment, or contaminated eggs from infected or carrier birds (Shivaprasad, 2000; Celis-Estupiñan et al., 2017, NVRI, 2020).

Infection with *Salmonella* has been reported to not only affect poultry but is also an emerging pandemic and threat to public health (Zeinab et al., 2020). *Salmonella* gallinarum is a host-specific pathogen causing systemic infection in poultry, which leads to significant economic losses due to high mortality (Ojima et al., 2021) and decreased egg production in layers (Haque et al., 2021).

The gross and histopathological changes induced by *Salmonella* gallinarum infection are majorly observed in liver, spleen, kidneys, heart, intestines and other organs and is characterised by depletion of lymphocytes, vascular congestion in various organs, especially liver, spleen and kidney; there is multifocal necrosis of hepatocytes with accumulation of fibrin and infiltration of heterophils mixed with a few lymphocytes and plasma cells in the liver (Kokosharov et al., 1997; Hossain et al., 2006; Dutta et al 2015., Anny et al., 2017).

Understanding the interplay of host factors in immune responses during the pathogenesis of fowl typhoid is pivotal not only in diagnosis of the disease but also in development of treatment protocol and in vaccines development. Among the host's inflammatory response factors aimed at surmounting diseases are cytokines. Cytokines are proteins or peptides that are secreted by cells that play a significant role in immune and inflammatory responses through the activation and regulation of other cell types and tissues. Cytokines are effective elements of the avian immune system that are capable of eliminating foreign antigens (Al-Khalaifah et al., 2018).

In mammals, the roles of cytokines are well known with a vast number of publications describing the structure of cytokines and their roles in health and disease (Wigley et al, 2003). IL-1β is produced by a range of cells following stimulation by microbes or microbial products (Dinarello, 1998), and its biological activity is highly inflammatory, with its main function being to activate the immune system in an acute phase response. IL-1β activates a range of cells such as macrophages and lymphocytes that may thus lead to production of other cytokines and chemokines. The production of IL-1β should be expected in many avian infections where a proinflammatory response occurs, as is the case with mammalian models of infection. The IFN-γ is known to play a vital role in immune response, and more involved in the inflammatory response of chickens, it is also known to play a role in macrophage activation (Horiuch et al., 2001). Interferons (IFN) are a group of cytokines that are produced by leukocytes and viral-infected cells because of stimulation of the immune system by viral infection and inflammation reactions.

This present study evaluated the profile of Interleukin 1 beta (IL 1β), Interferon gamma (INF γ) and leukogram in commercial ISA Brown cocks experimentally infected with *Salmonella* gallinarum.

MATERIAL AND METHODS

Ethical Clearance

Ethical approval for the experimental protocol was sought from Ahmadu Bello University Committee on animal use and care (ABUCAUC) with approval number ABUCAUC/2023/155.

Experimental Birds

A total of 40 Isa brown cocks weighing between 3-4kg were used for this study. They were unvaccinated against fowl typhoid, but vaccinated against other infectious diseases, such as Newcastle disease, infectious bursal disease and fowl pox and were purchased from a reputable farm in Jos and brooded for four weeks and reared to 18 weeks of age. They were then housed on the deep litter system and managed intensively. Throughout the experiment, standard commercial feed and water were provided to the birds *ad libitum*. The birds were acclimatized for a period of four weeks to get used to all the handling conditions and environment.

Experimental Design

The *Salmonella enterica* serovar gallinarum that was used for the research was obtained from the Department of Microbiology, National Veterinary Research Institute (NVRI), Vom, Nigeria. After the cocks attained reproductive age (23 weeks), the cocks were randomly allocated into two groups, infected and control of 20 birds each. Before infecting the experimental birds, cloacal swab was collected from individual bird and was then immersed in buffered peptone water, followed by plating them in MacConkey agar (MCA). Both cloacal swab and plates were incubated in a bacteriological incubator at 370C for 24 hours according to the standard laboratory methods (Wigley et al., 2001; Parmer and Davies, 2007). *Salmonella* gallinarum from the previously prepared slant was reactivated by inoculating it onto MacConkey agar (MCA). The colonies formed were then examined for their characteristic features, color and morphology and tested for the Gram stain reaction (Gram-negative). McFarland turbidity standards were made in the laboratory by preparing a 1% solution of anhydrous barium chloride and 1% solution of sulfuric acid, and they were mixed to obtain a barium precipitate. The volumes of the two reagents were adjusted to prepare standards of different turbidities that represent different concentrations of bacteria. The standards were used to visually compare the turbidity of a suspension of bacteria (McFarland, 1907). All the cocks in the infected group were inoculated orally at a dose of 1.0 ml inoculum containing 9x108 cfu/ml of *Salmonella* gallinarum, while cocks in control group were not challenged with the bacterium, but were given each 1 ml of distilled water only.

Clinical Examination

Following inoculation of the birds with the *Salmonella* Gallinarum, the infected group was observed daily for clinical signs of fowl typhoid and findings were recorded. All observations made during the six-week experiment were recorded, accordingly.

Blood Sampling

Blood samples were collected via wing vein, using 23-gauge sterile hypodermic needles and syringes. 2 to 3 ml of blood from each cock were collected in the morning (8:00 AM) from the infected and control groups. Blood collection were on Days 0, 4, 7, 14, 21, 28, 35, 42 post infection (p.i.). The blood was divided into two parts, one part of blood was dispensed into a tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and was used to determine total white blood cell count (WBC) and differential leukocyte count that were determined using methods described by Campbell and Ellis (2007). The second part of the blood was centrifuged for 10 minutes at approximately 1000×g. The harvested serum from each cock was then emptied into micro-vials and stored at -20oC, and the harvested serum was then assayed for serum IL-1β and IFN-γ using chicken-specific enzyme-linked immunosorbent assay (ELISA) kit (ELK Biotechnology CO., LTD), following the manufacturer's instructions.

Blood Serum IL-1 β and IFN- γ Concentrations

Cocks from each of the two groups were used at each time point post-infection Days 0, 4, 7, 14, 21, 28, 35 and 42 post infection (p.i.). The serum IL-1β and IFN-γ concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (ELK Biotechnology CO., LTD).

Statistical Analysis

Data obtained were subjected to statistical analysis using Graph Pad Prism Version 8.00 for Windows, GraphPad Software, San Diego, California, USA. Data from the two groups was compared using the student t-test, and values of $P<0.05$ were considered significant.

RESULTS

Clinical Manifestations of Fowl Typhoid in Infected Commercial ISA Brown Cocks

During the experimental period, neither morbidity nor mortalities were recorded in normal control ISA Brown cocks. However, in the infected group, starting from Day 8 post-infection up to Day 15 post-infection, the clinical signs observed in the infected ISA Brown cocks included: sudden death, depression, somnolence, decreased in feed and water consumption, greenish-yellow diarrhea, and some with blood stained, huddling, ruffled feathers, somnolence, and paleness of the comb and wattle. The morbidity rate recorded in the infected group was 50% (Table 1), while the mortality rate was 35% (Table 2).

Table 1 Morbidity rate in control and experimentally-infected ISA Brown cocks with Salmonella gallinarum during 42 days post infection

Days post- infection	Infected (N=20)	Control (N=20)
0	0	0
4	0	0
7	0	0
8-14	8	0
15-21	2	0
22-28	0	0
29-35	0	0
36-42	0	0
Total	10	0
MR	50%	0

Morbidity rate= Number of sick birds/ Number of birds inoculated x 100

Table 2 Mortality rate in control and experimentally-infected ISA Brown cocks with Salmonella gallinarum during 42 days post infection

Days post- infection	Infected (N=20)	Control (N=20)
0	0	0
4	0	0
7	0	0
8-14	5	0
15-21	2	0
22-28	0	0

Days post- infection	Infected (N=20)	Control (N=20)
29-35	0	0
36-42	0	0
Total	7	0
MR	35%	0

Mortality rate = Number of dead birds/ Number of birds inoculated x 100%

Effect of *Salmonella Enterica* Serovar Gallinarum Infection on Leukogram of ISA Brown Cocks

Absolute Mean White Blood Cell Count

The absolute mean white blood cell values of the *Salmonella enterica* serovar gallinarum infected and uninfected control groups are presented in Figure 1. The absolute mean WBC value in the infected (7.00 ± 0.28 x109/L) and control broiler cocks (6.90 ± 1.30 x109/L) was not significantly different (P>0.05) on Day 0 p.i.. A decrease in WBC value was observed in the infected group from Day 4 p.i. up to Day 7 p.i.. Thereafter, WBC value in the infected group was found to be significantly higher (p<0.05) in the infected cocks when compared to the control counterpart, and reaching its peak value on Day 14 p.i. (9.2±0.46 x109/L) than that of the control group (7.10 ± 1.20 x109/L). This was followed by a gradual decrease from Day 21 p.i. until termination of the experiment.

Absolute Mean Heterophil Value

The absolute mean heterophil values of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 2. The absolute mean heterophil value followed similar pattern of changes as that of the WBC (x109/L) value in the two groups of experimental birds up till the end of the study. However, starting from Day 14 p.i., a significant (P<0.05) increase in heterophil value was recorded in the infected group, which reached its maximum value of 4.4±0.31 x109/L on Day 21 p.i., which then followed by gradual decrease from Day 28 p.i. till the termination of the study.

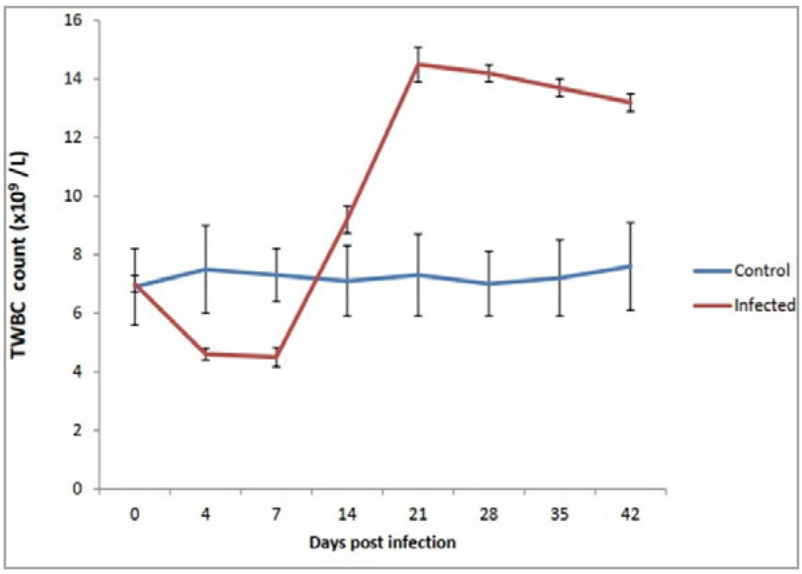

Figure 1

Absolute mean (± SEM) of white blood cell value of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

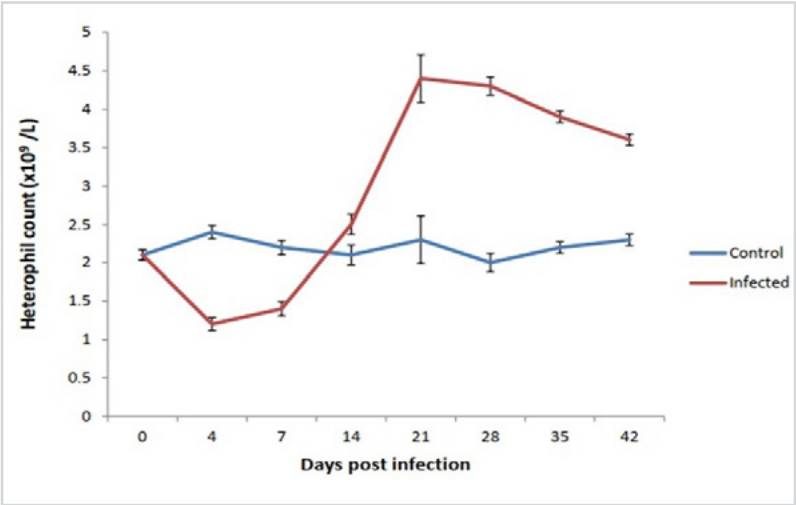

Figure 2

Absolute mean (± SEM) of heterophil value of ISA Brown cocks experimentally- infected with *Salmonella enterica* serovar gallinarum, and control cocks

Absolute Mean Lymphocyte Value

The absolute mean lymphocyte values of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 3. The absolute mean lymphocyte value (x109/L) was not significantly different ($p>0.05$) between the infected (4.90 ± 0.24 x109/L) and control (4.70 ± 0.20 x109/L) groups on Day 0 p.i. until Day 4 and 7 p.i., where slight reduction in mean lymphocyte value was recorded in the infected cocks. Thereafter, a significant rise ($p<0.05$) in this index was recorded in the infected group from Day 14 p.i., reaching its maximum value on Day 21 p.i. (10.4±0.55 x109/L). Following this, a slight drop was recorded on Day 28 p.i. in the infected group (9.6±0.19 x109/L). The mean lymphocyte value in the infected group during this period was still significantly higher ($p<0.05$) than in the control group.

Effect of *Salmonella Enterica* Serovar Gallinarum Infection on Serum IL-1β and IFN-γ of ISA Brown Cocks

Mean Interleukin-1beta Concentration

The mean IL-1β concentration of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 4. The mean IL-1β concentrations in the infected (74.00 ± 65.00 pg/ml) and control broiler cocks (74.00 ± 13.00 pg/ml) were not significantly different ($P>0.05$) on Day 0 p.i.. However, on Day 4 p.i., the value in the infected group increased to 185.00 ± 64.00 pg/ml, reaching its peak on Day 14

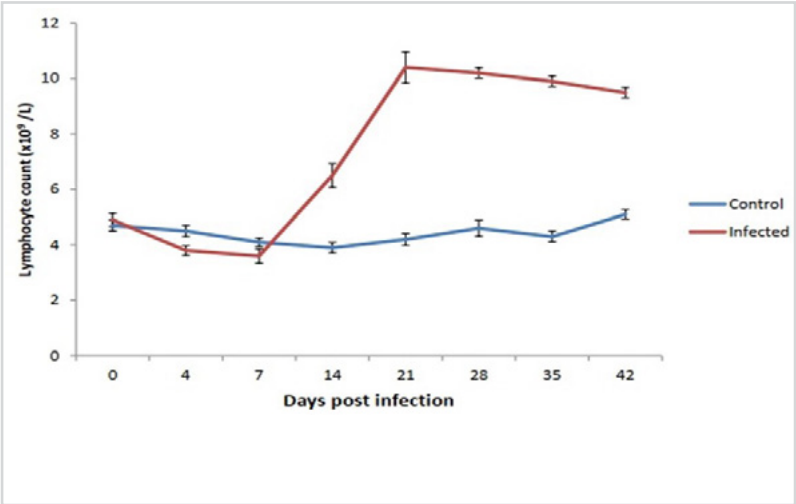

Figure 3

Absolute mean (± SEM) of lymphocyte value of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

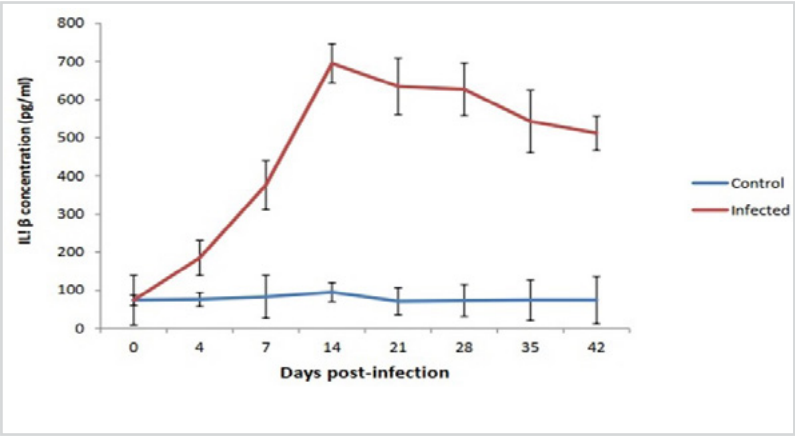

Figure 4

Mean (± SEM) of IL-1β concentration of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

p.i. and, thereafter, started declining on Day 21 p.i. up to the end of the experiment.

Mean Interferon-γ Concentration

The mean INF—γ concentration of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 5. The mean INF--γ concentration followed the same pattern as mean IL-1β. The mean INF--γ concentration in the infected group started increasing from Day 7 p.i., reaching its peak on Day 14 p.i. (4134.00 ± 217.00 pg/ml), and in the uninfected control ISA Brown cocks (325.00 ± 210.00 pg/ml). Thereafter, the mean INF—γ concentration value of the infected group started declining from Day 21 p.i. up to the end of the experiment.

DISCUSSION AND CONCLUSION

The clinical findings observed in the present study indicate that the infected ISA Brown cocks developed severe fowl typhoid infection, which was marked by depression, ruffled feathers, huddling, reduction in body weight, emaciation, somnolence, loss of appetite, blood stained greenish-yellow diarrhoea, paleness of the comb and wattle. The clinical signs observed in the infected ISA Brown cocks in this present study were consistent with the findings of previous authors (Garcia et al., 2010; Soufy et al.,2016). The incubation period observed in the infected ISA Brown cocks in this present study was 8 days, which contrasts 3 days reported by Garcia et al. (2010) and 7 days by Chiroma et al. (2018). The difference in the incubation period could be due to several factors, which include differences in the infective dose of the bacteria, the pathogenicity of the bacteria, the nature of the host-pathogen interaction, virulence

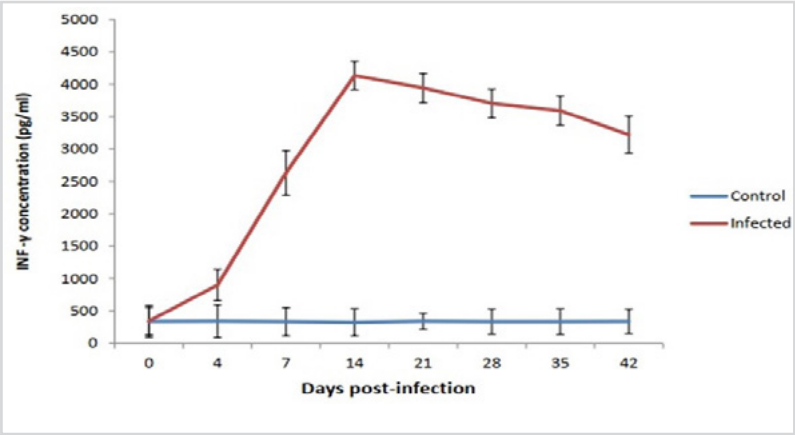

Figure 5

Mean (± SEM) of INF—γ concentration of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

of the organism, the capacity of the host to build adequate immune response against the pathogen (Lahiri et al., 2010; Sreekantapuram et al.,2021). The mortality rate recorded in the infected ISA Brown cocks in this study was 35%, which is in accordance with those of previous reports made by other authors (Paiva et al., 2009; Sannat et al., 2017; Chiroma et al., 2018). The mortality started 8 days p.i. with the range of 10-100%. Invasion of the organism via the gastrointestinal tract, thereby establishing systemic infection, might have accounted for the recorded mortalities. The ability of *Salmonella* to invade macrophages and probably dendritic cells and their translocation to the spleen and liverwhere multiplication occurs, is responsible for the clinical signs of the disease (Chappell et al., 2009).

The significant reduction in heterophil, lymphocyte and total white blood cell count observed initially in the infected group when compared with the control counterparts may be a result of cytopathic effect of *Salmonella* gallinarum lipopolysaccharides (LPS) on leukocytes of the infected ISA Brown cocks, thereby inducing cell lysis. This finding is in accordance with the reports of Chiroma et al. *(*2017), as cited earlier by Lam and Munn (2002). The significant reduction in heterophil value in the *Salmonella* gallinarum-infected ISA Brown cocks may be due to interaction of heterophils with the bacteria (Genovese et al., 2013; Sreekantapuram et al.,2021). While the significant increase *(*$p<0.05$) in total white blood cell, heterophil and lymphocyte counts observed on Day 14 post infection in the infected group contributed to leukocytosis as earlier reported by Berchieri (2000), and may be a result of fast multiplication of *Salmonella* gallinarum inside the phagocytes, with subsequent cell lysis and release of the bacterium into the extracellular compartment, which evoked strong immune response, thereby inducing antigenantibody reaction that is responsible for the clinical signs of fowl typhoid. The findings in this current study support those of previous authors Brar et al., 2000; Morguli, 2002., Freitas Neto et al., 2007. Abou Zeid et al., 2020).

The significant increase in mean serum concentration of IL-1β and IFNγ in the infected gr oup, which occurred on Days 4 and 7 p.i., respectively, and reaching its maximum level on Day 14 post infection as observed in this study, coincided with increase in heterophils, lymphocytes and total white blood cell value on Day 14 post infection. The IL-1β responded significantly to the infection on Day 4 post infection which was then followed by IFN-γ, which responded on Day 7 p.i.. The increase in serum concentration of these proinflammatory cytokines in the infected group, when compared with the control group, may probably be due to the fact that these cytokines play an important role in the immune response to *Salmonella* gallinarum infection. The significant increase in the serum concentration of IL-1β and IFN-γ in the infected ISA Brown cocks observed in this study is in part similar to the findings of Ojima et al. (2021) who reported upregulation of IFN-γ in the spleen of chickens infected with *Salmonella* gallinarum between Days 4 and 6 p.i..

A similar suggestion was made in Newcastle disease in which an increase in both IL 1β and IFN-γ was observed in the testes of roosters experimentally-infected with Newcastle disease virus (Rehman et al., 2020). Thakur et al., (2020) also reported significant increase *(*$P<0.05$) in both IL-1β and IFN-γ, following an oral infection of chicken with oocysts of *Eimeriaadenoeides* on Day 7 p.i.. Additionally, Zhang et al. (2012) also recorded a significant increase in IL-1β and IFN-γ genes in the day-old chickens 3-hour post infection. Similar findings were also reported by Al-Idreesi et al. (2013) who observed a significantly higher level of IFN-γ in the serum samples of ISA Brown chicken infected with *Eimeriatenella*. However, these findings conflict with findings in the reports of Ying et al. (2020) who observed decrease expression of both IL-1β and IFN-γ in the caecal tonsil and spleen of chicken infected with *Salmonella* gallinarum when compared with the control group. In this study, the period of increase in the serum concentrations of IL-1β and IFN-γ coincides with the period of increase of heterophil and lymphocyte values in the infected

cocks. In the chicken intestine, IL 1β and IFNγ are produced as a result of microorganism invasion-attracting heterophils to the site of infection (Ijaz et al., 2021). Upon contact with pathogens, heterophils are activated through the interaction of Toll-Like Receptors with bacterial ligands such as as lipopolysaccharide and peptidoglycan (Kogut et al., 2005). This activation of heterophils results in a sequence of events including phagocytosis, oxidative burst, degranulation, and IL-1β production. Both IL-1β and IFN-γ play a critical role in promoting immune responses, which are essential for promoting protective responses against invading pathogens (Eckmann et al., 2001). In the present study, the *Salmonella* gallinarum- infected cocks increased the IL-1β, and IFN-γ blood serum concentrations during the infective cycle. The increase in hetero- phil, lymphocyte and white blood cell value observed in the infected group on Day 14 p.i. in this present study may be possibly associated with the level of IL-1β and IFN-γ blood serum concentrations at Day 14 post infection. In general, the heterophil, lymphocyte and white blood cell value in the challanged cocks in this present study were negatively correlated with IL-1β and IFN-γ blood serum concentrations at Day 0 up to Day 7 p.i., but positively correlated with IL-1β and IFNγ blood serum concentrations at Day 14 p.i., indicating that chickens with high values of heterophils, lymphocytes and white blood cells have a more robust and appropriate inflammatory response to *Salmonella* gallinarum infection. It is possible to suggest that chickens with the low heterophil, lymphocyte and white blood cell value display a reduced heterophil cells number but with an enhanced function of this particular avian immune cell.

In conclusion, this study confirmed that the increase in the serum concentrations of IL-1β and IFN-γ in the cocks played a very critical role in protection against *Salmonella* gallinarum.

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COMPLIANCE WITH ETHICAL STANDARDS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

CONTRIBUTION

Concept and Design- AS, OBS, and PHM; Supervison;-AS, OBS, and PHM; Funding- CMA; Materials and Data collection and or processing CMA, JJG and AH; Literature review and Analysis and or interpretation of the data- CMA, JJG and EI; Writing and Critical review- CMA, AS, OBS, and PHM.

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ANALIZA POJEDINIH INTERLEUKINA I LEUKOGRAMA KOD ISA BROWN PIJETLOVA EKSPERIMENTALNO ZARAŽENIH SA *SALMONELLOM GALLINARUM*

SAŽETAK

Cilj istraživanja jeste ispitati profile IL- 1β, INF-γ i leukogram kod pijetlova eksperimentalno zaraženih sa *Salmonellom* gallinarum. Za istraživanje je korišteno ukupno 40 pijetlova, 20 inficiranih i 20 kontrolnih. Svakom pijetlu iz inficirane grupe je oralno apliciran 1 ml inokuluma 9.0 x 108 CFU/ml *Salmonelle* gallinarum. Nakon što su prikupljeni krvni uzorci izvršena je analiza leukograma, a serumski uzorci su analizirani na IL-Iβ i INF γ. Postojale su statistički signifikantne razlike ($p < 0.05$) u srednjem apsolutnom broju leukocita, broju heterofila i broju limfocita između inficirane i kontrolne grupe. Najviše vrijednosti leukocita (14.5 ± 0.60 x 109/1), heterofila (4.4 ± 0.31 x 109/1) i limfocita (10.4±0.55 x 109/1) su zabilježene u inficiranoj grupi 21. dana (p.i.). Postojale su statistički signifikantne razlike ($p < 0.05$) u srednjim koncentracijama IL-1β i IFN-γ u serumu između inficirane i kontrolne grupe 4. dana (185±46.0 pg/ml) i 7. dana p.i. (2634± 342 pg/ml). Najviše i najniže zabilježene srednje vrijednosti koncentracije IL-1β u serumu su iznosile 695±1.0 pg/ml i 185±46.0 pg/ml u inficiranoj grupi 14. i 7. dana p.i., a IFN γ 4134±217 pg/ml i 2634 ± 342 pg/ml u inficiranoj grupi 15. i 7. dana p.i. U zaključku, povišene vrijednosti serumskog IL 1β i INF gamma kod inficiranih pijetlova može igrati odlučujuću ulogu u ispoljavanju pojačanog imunološkog odgovora kod infekcije *Salmonellom* gallinarum.

Ključne riječi: Inokulum, ISA Brown, pijetlovi, *Salmonella*

RESEARCH ARTICLE

MORPHOMETRICS OF HEART OF THE FOETAL DROMEDARY

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ABSTRACT

The present study provides an anatomical account of the morphometry of heart of prenatal camel. For this purpose, 15 normal fresh hearts were randomly obtained from different fetuses collected from Maiduguri central abattoir. The fetuses were divided into three different growth periods i.e., first (2-4 months), second (4 -7 months), and third (7-10 months) based on their body weight and crown-rump length. The prenatal dromedary heart was grossly observed to be coned-like with flattened base and nearly sharp apex. The subepicardial blood vessels showed corresponding development with each quarter of the pregnancy. The heart weight showed no significant increase during the second growth period ($p>0.05$), while an extremely significant increases ($p<0.001$) were observed during the third growth period. The dimension of the prenatal dromedary heart presented a significant increase ($p<0.05$) during the second growth period, while an extremely significant increase ($p<0.001$) was observed during the third growth period in all the fetuses. This increase indicates that there was more embryogenesis of the heart during the third growth period of the prenatal dromedary. It was concluded that the length of posterior border base to apex of prenatal dromedary is higher in length than anterior border base.

Keywords: Dromedary**,** foetal, heart, grooves, morphometry

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INTRODUCTION

The heart is the muscular central organ of the blood-vascular system, that pumps blood by rhythmic contractions through the blood vessels (Dyce et al. 2017). It is mainly comprised of the heart muscle that forms a sac divided into four chamberes consisting of the right and left atrium and ventricle (Konig et al., 2004). The two atria and ventricles are separated by an internal septum, but the atrium and ventricle of each side communicate through a large opening (Dyce et al., 2017). The right side of the heart pumps deoxygenated blood to the lungs for oxygenation, whereas the left side delivers oxygenated blood for systemic circulation (Pasquini et al., 1999). This cardiovascular function is altered during fetal life by a shunt system of foramen ovale and ductus arteriosus that closes shortly before or immediately after birth (Hyttel et al., 2009). The heart is fairly variable among domestic species, but generally resembles a pointed, bilaterally flattened cone enclosed by the pericardium (Schummer et al., 1981). There are various disorders and congenital defects affecting the heart of camel (Tharwat et al., 2012) and diagnosis presents rather a challenging task due to the absence of clinical signs (Aref et al., 2024). Heart diseases manifest as changes in size and weight in proportion to its severity (Queiroz et al., 2018). In camels, diagnosis usually occurs in slaughterhouses, or happens by chance during a post-mortem examination (Fowler, 2010). There is rarely published data regarding the standard dimensions of the fetal dromedary heart. Sarmad and Anas (2010) reported on the morphometry of some splanchnic organs, including the heart in dromedary camel calves. Recently, Aref et al. (2024) reported on normal cardiac dimension of the heart of adult camel by magnetic resonance imaging and topographic anatomy. This study aims to add new knowledge of the developmental anatomy of the heart with focus on the morphometry of the heart, with the hope that will benefit science and practitioners.

MATERIAL AND METHODS

The studies involved 15 apparently normal fresh hearts of one-humped camel fetuses obtained from Maiduguri central abattoir over a period of six months. The fetuses were divided according to four periods of development based on their body weight (BW) and crown-rump length (CRL) measurement, as described previously (Jaji et al., 2011). Thus, they were divided into 2 – 4, 4 – 7, 7 – 10, and 10 – 13 months growth periods. Fetal hearts from the first three growth periods (1st, 2nd and 3rd) were immediately weighed and fixed in 10% buffered formalin for 48 hours. Then, the hearts were reweighed and the following parameters were measured:

i. Length of anterior border base to the apex
ii. Length of posterior border base to the apex
iii. Circumference at the coronary grooves
iv. Distance between the right coronary grooves to the apex
v. Distance between the left coronary grooves to the apex

The whole-body weight (kg) of each fetus was determined using (spring flatform scale), whereas electronic precision balance (METRA TL – 300) was used to weigh the heart (g). The hearts were carefully removed from the thoracic cavity with the aid of scalpel, and the surrounding pericardial tissue was freed from the heart using thumb forceps. Measuring tape (Butterfly brand) and thread were used to measure the crown-rump length of the fetuses and all the aforementioned heart parameters.

Statistical analysis

The fetal heart dimensions at different phases of prenatal growth were presented as means ± SD in the Table. The differences across the phases were further analyzed using ANOVA test and Duncan's follow-up test at a 95% confidence level using statistical software GraphPad Prism version 5.00 for Windows.

RESULTS

In the prenatal dromedaries the heart was reddish – brown in color. It occupied the greater part of the middle mediastinal space. Its shape was irregular and somewhat flattened cone. It was attached to its base by the vena cavae, otherwise entirely free within the fibrous sac pericardium. It was asymmetrical in position. The long axis (from the middle of the base to the apex) was directed ventrally and caudally. The hearts presented an apex, base, two surfaces and two borders. The base was directed dorsally, and its highest part laid approximately at the junction of the dorsal and middle thirds of the dorsoventral diameter of the thorax. It was formed by the right and left atria; the cranial and caudal vanae cavae and the pulmonary veins entered through the base. The apex laid centrally, dorsal to the sternum. The caudal (left) border was much shorter and nearly vertical. The surfaces, atrial (right, diaphragmatic) and auricular (left, sternocostal) were convex and marked by grooves, which indicated the division of the heart into four chambers. The coronary groove, partly filled with adipose tissue, divided the atria from the ventricles at the base of the heart, two atria dorsally and two ventricles ventrally (Figure 1). The camel heart at the second quarter of pregnancy at left auricular presentation showed the convex cranial border belonging to the right ventricle. The coronary groove was much developed at the second quarter, clearly separating the right from the left ventricle. The paraconal interventricular branch of the left coronary artery and left ventricular branches emanating from the circumflex branches, and the intermediate groove that runs at the margin of the left ventricle were all discernable (Figure 2,3), At the fourth quarter, the subepicardial distribution of adipose tissue was wider, covering much of the surface of the right ventricle and the interventricular groove up to the apex of the heart. The conically shaped conus arteriosus at the proximal portion of the right ventricle and the pulmonary trunk were conspicuous. The branch of the great cardiac vein accompanying the paraconal interventricular branch can be seen at the fat-filled cardiac groove (Figure 4).

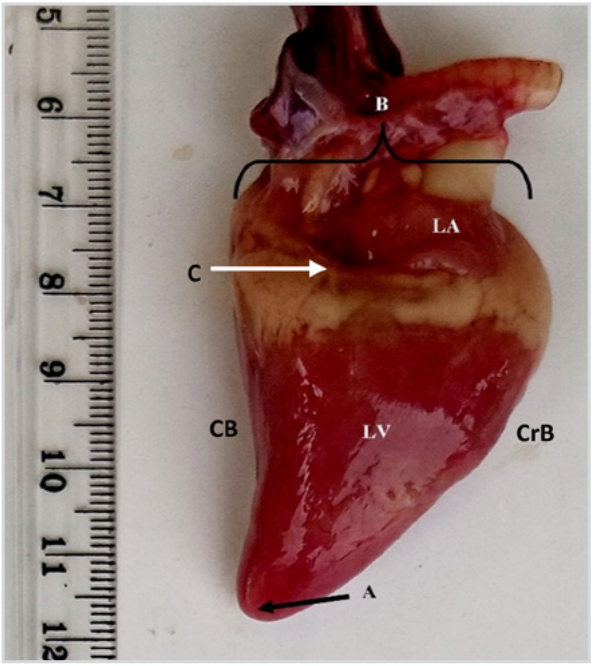

Figure 1

(Left view) A one-humped camel fetal heart of a 1st quarter pregnancy, showing the Base (B), Left Atrium (LA), Fat-filled Coronary Sulcus (C), Left Ventricle (LV), Apex (A), Cranial Border (CrB) and Caudal Border (CB)

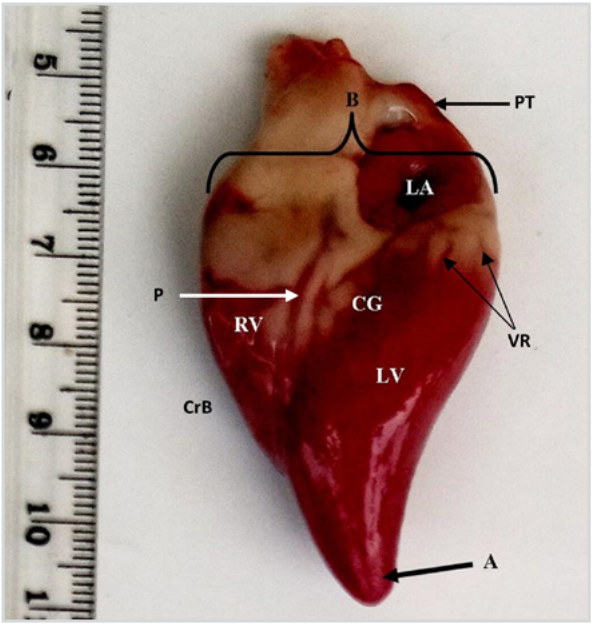

Figure 2

(Left auricular view) A one-humped camel fetal heart of a 2nd quarter pregnancy, showing the Base (B), Pulmonary Trunk (PT), Left Atrium (LA), Ventricular Rami of Coronary artery (VR), Left Ventricle (LV), Coronary Groove (CG), Paraconal Interventricular Branch of Left Coronary Artery (P), Right Ventricle (RV), Cranial Border (CrB), and Apex (A)

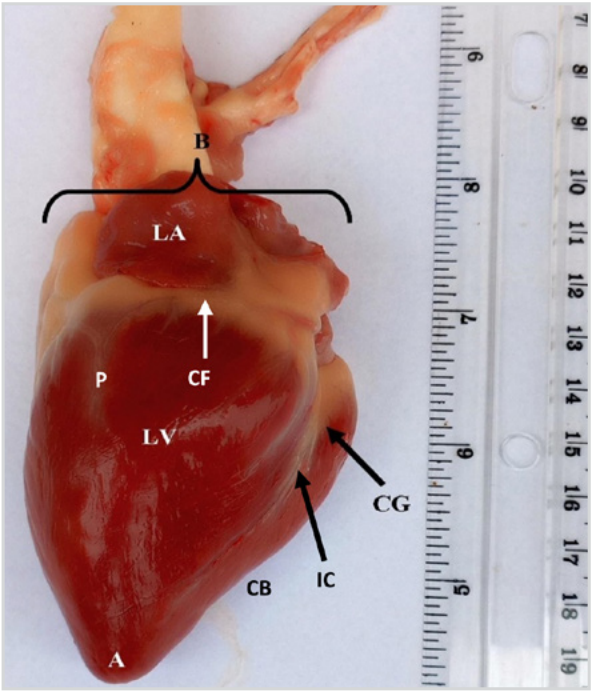

Figure 3

(Left auricular view) A one-humped camel fetal heart of a 3rd quarter pregnancy, showing the Base (B), Left Atrium (LA), Circumflex Branch of Left Coronary Artery (CF), Paraconal Interventricular Branch (P), Left Ventricle (LV), Coronary Groove (CG), Intermediate Branch of Circumflex Branch of Left Coronary Artery (IC), Caudal Border (CB) and Apex (A)

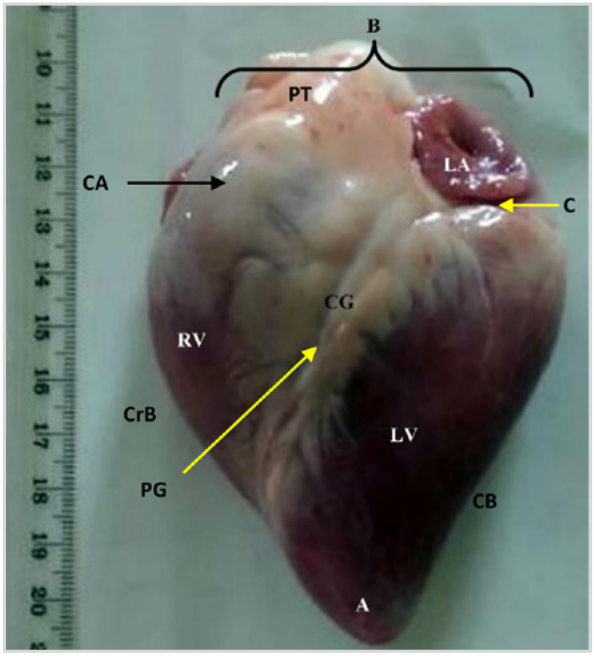

Figure 4

(Left auricular view) A one-humped camel fetal heart of a 4th quarter pregnancy, showing the Base (B), Pulmonary Trunk (PT), Left Auricle (LA), Conus Arteriosus (CA), Circumflex Groove (C), Fat-filled Coronary Groove (CG), Right Ventricle (RV), Left Ventricle (LV), Paraconal Interventricular Branch of Left Coronary and Great Cardiac Vein (PG), Cranial Border (CrB), Caudal Border (CB), and Apex (A)

Biometry of heart of foetal dromedary

The weight of heart of camel foetus

The weight of the heart of the dromedary foetus was 13.52 ± 8.74 g during the first growth period. It showed a level of significance increase along the four prenatal growth periods, as shown in Table 1.

The length of anterior border base to apex of heart of camel foetus

The length of the anterior border base to the apex of the heart of the dromedary foetus was 3.94 ± 0.78 cm during the first growth period. It showed the levels of significant increase along the four prenatal growth periods, as shown in Table 1.

The length of posterior border base to apex of heart of camel foetus

The length of posterior border base to apex of the heart of camel foetus was 3.5 ± 0.91 cm during the first growth period. It showed a level of significance increase along the four prenatal growth periods, as shown in Table 1.

The circumference at coronary grooves of heart of camel foetus

The circumference at coronary grooves of heart of camel foetus was 7.71 ± 0.80 cm during the growth period. It showed the levels of significant increase along the four prenatal growth periods, as shown in Table 1.

The distance between right and left coronary grooves to apex of heart of camel foetus

The distance between right and left coronary grooves to apex of the heart of camel foetus were 3.32 ± 0.65 cm and 3.4 ± 0.49 cm, respectively during the first growth period. The showed levels of significant increases along the four prenatal growth periods are shown in Table 1.

Table 1 Morphometrics of heart of foetal dromedary

Heart Dimensions (Mean±SD)	Prenatal Growth Period (n=6 per phase)			
	1st Period	2nd Period	3rd Period	4th Period
Weight (g)	13.52±1.74	29.44±3.28***	69.39±7.04***	77.73±6.72
Length of anterior border base to apex (cm)	3.94±0.28	5.45±0.34***	6.87±0.45***	7.51±0.33*
Length of posterior border base to apex (cm)	3.58±0.41	5.38±0.49***	7.13±0.55***	8.63±0.44***
Circumference at coronary groove (cm)	7.71±0.70	11.32±0.74***	15.17±1.04***	17.44±0.1.31**
Distance between right coronary groove to apex (cm)	3.32±0.40	5.04±0.50***	6.63±0.70***	7.70±0.37**
Distance between left coronary groove to apex (cm)	3.46±0.29	4.98±0.43***	6.63±0.70***	8.37.37±0.61***

* = significant ($p<0.05$), ** = significant ($p<0.005$), *** = significant ($p<0.001$), n = Number of observations

DISCUSSION AND CONCLUSION

The prenatal dromedary heart was observed to have occupied the greater part of the middle mediastinal space, having an irregular, somewhat flattened cone shape and attached base by the great vessels, but otherwise entirely asymmetrical in position, common to most domestic mammals. The wedge-shaped sternum, particularly in ungulates, which is bilaterally flattened influences the position of the heart of the domestic mammals within the thoracic cage (Schummer et al., 1981).

The weight of the prenatal dromedary heart showed no significant increases during the second growth period. An extremely significant increase was, however, observed during the third growth period. Leinder (1996), Mohan and Prakash (1997) and Miglino and Amorim (1998) observed that the postnatal buffalo heart showed an average weight to be ranged from 1800 – 2500 g. Miller (2004) recorded the mean weight of the heart of buffaloes ranging from 5 – 7 lb. The breed, resultant size and body weight affects both absolute and relative weight of the heart. It has been stated that the significance of measurements will depend on diastolic and systolic state or whether the heart was in rigor at the time of measurement (Schummer et al., 1981). The length of anterior border base to apex of the prenatal dromedary heart showed a very significant increase during the second growth period. An extremely significant increase was observed for the length of anterior border to the apex during the third growth periods, though Mohan and Praskash (1997), and Miller (2004) recorded a relative dearth existing on the biometrical value of the mean length buffaloes.

The length of posterior border to apex of the prenatal dromedary heart showed a very significant increase during the second growth period. An extremely significant increase was observed for the length of posterior border base to the apex during the third growth period. Report from Miller (2004) indicated the average length of posterior border base to apex of buffaloes as 13.50cm.

The circumference at the coronary grooves of prenatal dromedary heart showed a very significant increase during the second growth period. An extremely significant increase was observed for the circumference at coronary grooves during the third growth periods. According to Mohan and Prakash (1997), the circumference within the coronary groove averaged 28.95cm in buffaloes and 38.75cm in cows, while Braun et al. (1999) recorded 37cm for the buffalos' heart.

The distance between the right and left coronary grooves to apex of prenatal dromedary heart showed a very significant increase during the second growth periods. An extremely significant increase was observed for the distance between the right and the left coronary grooves to the apex during the third growth periods. It is difficult to compare the present result regarding the right and left coronary grooves of prenatal growth periods of dromedary to the result of other workers because, to the best of our knowledge, no such kind of studies have been carried out on camel before. However, Braun et al. (1999) studied and measured the distance between the right and left coronary grooves to apex in cows and obtained average of 15 cm.

It was discovered that the length of posterior border base to apex of prenatal dromedary was longer than anterior border base. The left coronary artery gives off the paraconal interventricular branch and circumflex branch and runs caudally via the intermediate groove to give the intermediate branch. Thus, the dromedary, like the ruminants and carnivores, is a species that possesses a left coronary type of supply.

More research is required to determine the morphometry of the internal structures of dromedary heart so as to have a record of the full term. So, also, histogenesis of the dromedary heart needs to be studied so as to have enabled the complete elucidation of the prenatal development of the dromedary heart.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

CONTRIBUTION

Conception, Design, Supervision: AZJ; Materials, Data collection and Processing: ASS, FD; Analysis and Interpretation of the Data: AZJ, IAG; Literature Review: SMA, BGG; Writing: IAG; Critical review: AZJ, IAG, AY

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MORFOMETRIJA SRCA FETUSA JEDNOGRBE KAMILE

SAŽETAK

Ovo istraživanje pruža anatomski uvid u morfometriju srca prenatalne kamile. U tu svrhu smo randomizirali 15 svježih srca iz fetusa prikupljenih u centralnoj klaonici Maidugurija. Fetusi su podijeljeni prema tri različita perioda rasta: prvi (2-4 mjeseca), drugi (4-7 mjeseci) i treći (7-10 mjeseci) na osnovu tjelesne težine i dužine krunice. Prenatalno srce dromedara je makroskopski honičnog oblika, zaravnjene baze i skoro oštrog apeksa. Subepikardijalne krvne žile su u svakom kvartalu trudnoće bile odgovarajuće razvijene. Težina srca se nije signifikantno povećavala u drugom periodu rasta ($p>0.05$), dok se u trećem periodu ekstremno povećala ($p<0.001$). Dimenzije prenatalnog srca fetusa jednogrbe kamile su u drugom periodu rasta pokazale signifikantno povećanje ($p<0.05$), dok je u trećem periodu kod svih fetusa zabilježeno ekstremno povećanje ($p<0.001$). Ovo povećanje pokazuje da je u trećem periodu rasta prenatalnih fetusa jednogrbe kamile embriogeneza najintenzivnija. Zaključak je da je dužina od posteriornog ruba baze do apeksa kod prenatalnih fetusa jednogrbe kamile veća od dužine anteriornog ruba baze.

Ključne riječi: Fetus, jednogrba kamila, morfometrija, srce, žljebovi

RESEARCH ARTICLE

INCIDENCE AND RISK FACTORS OF HORSE KICKS IN HORSES AND THEIR HANDLERS IN SELECTED STABLES IN LAGOS STATE, NIGERIA: A TWO-YEAR PROSPECTIVE STUDY (2021-2023)

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ABSTRACT

Horses, with their powerful hindquarters and defensive kicking mechanisms, present a considerable risk of severe injury to both humans and fellow equines. The lack of comprehensive data on horse kicks underscores the need for increased awareness of potential hazards. We aimed to prospectively study the incidence and risk factors of horse kicks in horses and handlers in selected stables in Lagos State, Nigeria from 2021 to 2023. A total of 200 horses and 55 handlers were included. The incidence of horse kicks was 12.5% in horses and 29.1% in handlers. For horses, personal space invasion (52%) was the most common situation leading to kick incidents, while for handlers, fear (44%) and startle response (31%) were the primary triggers. Injuries sustained included skin abrasions (32% in horses, 69% in handlers), hematomas (16% in horses), muscle/nerve damage (20% in horses, 13% in handlers), and fractures (32% in horses, 19% in handlers). Significant risk factors for horse kicks in horses were old age (>15 years) and being a stallion. In handlers, significant risk factors included young age (24–44 years), occupation as a farrier, and less than 5 years of experience. Horse kicks occur in both horses and their handlers. The findings highlight the need for targeted safety measures and training, particularly for high-risk groups, to reduce the risks associated with horse kicks for both horses and handlers in Lagos State stables.

Keywords: Horse kick, horse handler, incidence, prospective study

INTRODUCTION

Horses naturally employ kicking as a defensive action. They engage in kicking behaviour for several motives, such as fear, frustration, pain, aggression, and to establish dominance (Benthien et al., 2020). The formidable strength and power of a horse's legs make a horse's kick highly perilous, capable of inflicting severe injuries on both humans and horses. Horses generate the force for their kicks through robust muscles in their hindquarters, enabling them to deliver powerful and swift kicks (Majeedkutty and Khairulanuar, 2017). A single hoof kick can generate a force of up to 9807 Newtons (equivalent to 1 tonne), and steel horseshoes can cause even greater peak forces, increasing the risk of severe injury (Kriss and Kriss, 1997; Piskoty et al., 2005).

Horses are large, robust animals, and a kick from one could have negative effects. The sequelae of horse kick encompass many physical injuries, such as skin lacerations, hematomas, muscle, tendon, or nerve damage, or bony trauma, including fractures, damage to the outer layer of bones, and bone infections (Schroeder et al., 2013). Depending on the region that receives the kick, different types of traumas may occur. For instance, areas that have muscle and soft tissues protecting them are less likely to sustain bone-related injuries. However, parts of the body like the medial radius and medial tibia, as well as the metacarpal bones, metatarsal bones, and calcaneus, only have a thin layer of skin and very little soft tissue to protect them. This means that when there is an impact, these bones are exposed to strong forces (Swinebroad et al., 2003).

To the best of the authors' knowledge, there is a dearth of information on the incidence and risk factors of horse kicks in horses and their handlers. Horse handlers, especially farriers and grooms, face the potential hazard of being kicked by horses, highlighting the importance of their awareness regarding this risk. This study will fill this knowledge gap and contribute valuable insights towards the creation of evidence-based approaches for reducing the incidence of horse kicks, thereby enhancing safety within equine environments.

The present study aimed to determine the incidence and risk factors associated with horse kicks among horses and their handlers in selected stables in Lagos State, Nigeria, over a two-year period.

MATERIALS AND METHODS

Ethical Consideration

Ethical approval for this study was obtained from The Lagos State Ministry of Agriculture, Nigeria (reference number: MOA/LS/RDE/334/12) that approved this study. All procedures adhered to established ethical guidelines and principles for research involving animals. Informed consent was obtained from the stable owners and managers for the participation of their horses in the study. Additionally, all handlers involved in the study provided written informed consent prior to their participation. The welfare of both horses and handlers was prioritized throughout the study period, and all participants were informed of their right to withdraw from the study at any time, without consequence.

Study Area

This study was conducted in Lagos State, located in southwestern Nigeria. Lagos is the most populous city in Nigeria and is known for its vibrant equestrian scene. The city hosts various polo tournaments throughout the year, attracting both local and international participants. This active polo culture contributes to the presence of numerous stables and a significant population of polo horses in the area, making it an ideal location for this study on horse kicks in stable environments.

Study Design

A 2-year prospective study on horse kicks was conducted between August 2021 and September 2023, involving horses and horse handlers in selected stables in Lagos State.

Horse Management and Care

All horses were kept in individual stalls measuring approximately 3.6 x 3.6 meters (12 x 12 feet). The stalls were constructed of concrete blocks with metal bars, allowing visual contact between

horses. Each stall was bedded with wood shavings and saw dust, which were changed daily. Horses were turned out in paddocks for 2-4 hours daily in small groups of 3-5 horses, allowing for socialization and free movement. The groupings were kept consistent to minimize stress.

The horses were primarily used for polo, with training sessions occurring 5-6 days per week. Exercise regimes included daily riding (1-2 hours), stick-and-ball practice, and weekly practice chukkas. During the polo season, horses participated in tournaments and matches. Horses were fed two times daily, with a diet consisting of high-quality grass hay (approximately 1.5-2% of body weight per day), concentrated feed (0.5-1% of body weight), and free access to fresh water. Feeding times were consistent, occurring at 8:00 AM and 6:00 PM. All horses underwent regular health checks by para-veterinarians and veterinarians every two weeks. These checks included physical examinations, dental care, and hoof inspections. A farrier visited every 4-6 weeks for routine hoof care and shoeing. The preventive health management program included: annual vaccinations against tetanus, equine influenza, and African horse sickness; bi-annual deworming with rotating anthelmintics; and daily temperature checks to monitor for early signs of illness. At the start of the study, all horses were deemed healthy and fit for polo. Any horses developing health issues during the study period were noted, and their participation in the study was re-evaluated based on the nature and severity of the condition.

Inclusion Criteria

Horses: Horses stabled in the selected facilities in Lagos State during the study period (August 2021 - September 2023).

Handlers: Those employed as farriers or grooms in the selected stables during the study period (August 2021–September 2023).

Exclusion Criteria

Horses: Those expected to be moved out of the selected facilities in Lagos State during the study period (August 2021–September 2023).

Handlers: Temporary or casual workers not regularly employed as farriers or grooms in the selected stables during the study period (August 2021–September 2023).

Data Collection

Baseline data was collected on all 200 horses included in the study, comprising 146 Argentine Polo Ponies and 54 Sudan Country-Bred Horses with ages ranging from 5 to 20 years. Of these, 166 were mares, and the remaining 34 were stallions. Baseline data was also collected on all 55 handlers included in the study, consisting of farriers (18) and grooms (37). The farriers were exclusively male, aged between 25 and 60 years, while the grooms included 7 females and 30 males, also aged between 25 and 60 years.

An observation form to document all incidents of horse kicks involving horses and handlers was made, which included details such as the situation at the time of incidence, body regions affected, and sequelae. For each reported incident, the research team conducted an initial assessment within 24–48 hours, during which the kicked body regions, for both horses and handlers, were categorised into head, neck, chest, abdomen, and limbs; also, the sequelae of horse kicks were classified as skin abrasion, hematoma, muscle or nerve damage, and fractures. The situation at the time of the incident was noted and recorded. Pictures were also taken using a digital camera.

Risk factors for horse kicks in both horses and handlers were separately observed using on-the-spot assessment forms. For horse kicks in horses, potential risk factors considered included age, breed, and sex. Age was classified as adult (5–15 years old) and old (>15 years old) (Akinniyi et al., 2023). The horse breeds were Argentine Polo Pony and Sudan Country-Bred, and sex was classified as stallion and mare. For horse kicks in the horse handlers, considered risk factors included occupation, age, and sex. Occupations considered were farriers and grooms. Age was categorised as young (25–44 years old) and middle age (44–60 years old) (Dyussenbayev, 2017), and sex was classified as male and female. Years of experience

were grouped into less than 5 years and 5 years and above.

Data Analysis

The collected data were summarised and presented in tables using descriptive statistics. The odds ratio was determined through multivariate logistic regression to assess the strength of the identified risk factors. Confidence values of 95% were calculated, and $P \leq 0.05$ was considered significant. The Statistical Package for Social Sciences (SPSS®, version 26) was employed for data analysis.

RESULTS

Study Population

Baseline data was collected on all 200 horses included in the study, comprising 146 (73%) Argentine Polo Ponies and 54 (27%) Sudan Country-Bred Horses with ages ranging from 5 to 20 years. Of these, 166 (83%) were mares, and the remaining 34 (17%) were stallions. Baseline data was also collected on all 55 handlers included in the study, consisting of 18 (32.7%) farriers and 37 (67.3%) grooms. The farriers were exclusively male (100%), aged between 25 and 60 years, while the grooms included 7 (18.9%) females and 30 (81.1%) males, also aged between 25 and 60 years.

Incidence of Horse Kicks

During the two-year study period (2021-2023), we observed a total of 200 horses and 55 horse handlers. The incidence of horse kicks was 12.5% (25/200) among horses and 29.1% (16/55) among handlers (Table 1).

Situations leading to kicks, the body regions affected, and the sequelae for both horses and handlers

For horses, the most common situation leading to kicks was personal space invasion (52%, 13/25), followed by mating and hierarchy situations (24% each, 6/25). For handlers, fear was the primary cause (44%, 7/16), followed by startle response (31%, 5/16) and pain procedures (25%, 4/16) (Table 2).

The body regions most frequently affected in horses were the limbs (40%, 10/25), followed by the abdomen (33%, 8/25) and head (28%, 7/25). In handlers, the head and neck were equally affected (31% each, 5/16), followed by limbs (25%, 4/16) and chest (13%, 2/16) (Table 3).

The most common sequelae in horses were skin abrasions and fractures (32% each, 8/25), followed by muscle or nerve damage (20%, 5/25) and hematoma (16%, 4/25). In handlers, skin abrasions were predominant (69%, 11/16), followed by fractures (19%, 3/16) and muscle or nerve damage (13%, 2/16) (Table 4).

Risk Factors for Horse Kicks

Among horses, significant risk factors for being kicked included older age and being a stallion. Old horses (>15 years) were 6 times more likely to be kicked than adult horses (5–15 years) (32.5% vs. 7.5%, OR 6, $P < 0.05$), while stallions were 5.2 times more likely to be kicked than mares (32.4% vs. 8.4%, OR 5.2, $P < 0.05$). Although not statistically significant, Sudan Country-Bred Horses showed a higher likelihood of being kicked compared to Argentine Polo Ponies (18.5% vs. 10.3%, OR 2, $P > 0.05$).

For handlers, significant risk factors included younger age, being a farrier, and having less experience. Handlers aged 24-44 years were 4.6 times more likely to be kicked than those aged 45-60 years (53.3% vs. 20%, OR 4.9, $P < 0.05$). Farriers faced a notably higher risk, being 16.7 times more likely to be kicked than grooms (66.7% vs. 10.8%, OR 16.7, $P < 0.05$). Additionally, handlers with less than 5 years of experience were 9.8 times more likely to be kicked compared to those with 5 or more years of experience (61.9% vs. 14.7%, OR 9.8, $P < 0.05$). While not statistically significant, male handlers showed a higher likelihood of being kicked compared to female handlers (31.3% vs. 14.3%, OR 2.7, $P > 0.05$).

Detailed data for all risk factors analysed can be found in Tables 5 and 6.

Table 1 A prospective study spanning two years (2021-2023) on the incidence of horse kicks in both horses and horse handlers

Horse kicked			Horse handlers kicked		
No. of horses	No. of horses kicked	Incidence (%)	No. of horse industry professionals	No. of horse handlers kicked	Incidence (%)
200	25	12.5	55	16	29.1

Table 2 Situation at the time of incidence of horse kick in horses and their handlers

Horses kicked			Horse handlers kicked		
Situation	**Number**	**Percentage (%)**	**Situation**	**Number**	**Percentage (%)**
Mating	6	24	Painful procedure	4	25
Personal space invasion	13	52	Startle response	5	31
Hierarchy	6	24	Fear	7	44
Total	25	100	Total	16	100

Table 3 A two-year (2021-2023) prospective study summarizing the body regions affected by horse kicks in both horses and horse handlers

Horses kicked			Horse handlers kicked		
Body region	**Number**	**Percentage (%)**	**Body region**	**Number**	**Percentage (%)**
Head	7	28	Head	5	31
Neck	0	0	Neck	5	31
Chest	0	0	Chest	2	13
Abdomen	8	33	Abdomen	0	0
Limbs	10	40	Limbs	4	25
Total	25	100	Total	16	100

Table 4 A two-year (2021-2023) prospective study summarizing the sequelae of horse kicks in both horses and horse handlers

Sequelae	Horse kicked		Horse handlers kicked	
	Number	**Percentage (%)**	**Number**	**Percentage (%)**
Skin abrasion	8	32	11	69
Haematoma	4	16	0	0
Muscle or nerve damage	5	20	2	13
Fracture	8	32	3	19
Total	25	100	16	100

Table 5 A two-year (2021-2023) prospective study on the risk factors associated with horse kicks in horses

Risk factor	No. of horses	No. of horses kicked	Incidence (%)	OR (95% CI)	P value
AGE					
Adult (5-15 years old)	160	12	7.5	Reference	
Old (> 15 years old)	40	13	32.5	6.0 (2.5, 14.4)	< 0.001*
BREED					
Argentine Polo Pony	146	15	10.3	Reference	
Sudan Country-Bred	54	10	18.5	2 (0.8, 5.0)	0.145
SEX					
Stallion	34	11	32.4	5.2 (2.1, 13.1)	< 0.001*
Mare	166	14	8.4	Reference	

*= Statistically significant

Table 6 A two-year (2021-2023) prospective study on the risk factors associated with horse kicks in horse handlers

Risk factor	No. of horse handlers	No. of horse handlers kicked	Incidence (%)	OR (95% CI)	P value
AGE					
Young age (25-44 years old)	15	8	53.3	4.6 (1.3, 15.5)	0.015*
Middle age (45-60 years old)	40	8	20	Reference	
OCCUPATION					
Farrier	18	12	66.7	16.7 (4.2, 66.4)	< 0.001*
Groom	37	4	10.8	Reference	
SEX					
Male	48	15	31.3	2.7 (0.31, 24.4)	0.362
Female	7	1	14.3	Reference	
HANDLER EXPERIENCE					
< 5 years	21	13	61.9	9.8 (2.7, 35.1)	0.001*
5 years and above	34	5	14.7	Reference	

DISCUSSION AND CONCLUSION

The aim of this study was to assess the incidence and factors contributing to horse kicks among horses and handlers in Lagos State stables during a two-year period. Our findings highlight the significant risk of horse kicks for both equines and humans in stable environments, with notable differences in incidence, contributing factors, and consequences.

The higher incidence of horse kicks among handlers (29.1%) compared to horses (12.5%) underscores the elevated risk for humans working closely with these animals. This aligns with previous studies identifying kicks as a primary cause of equine-related injuries to humans (Hawson et al., 2010). The incidence rate among handlers is comparable to that reported in equestrians (21%) by Exadaktylos et al. (2002), suggesting a consistent level of risk across different equine-related activities.

Our study revealed distinct situations leading to kicks in horses versus handlers. For horses, incidents primarily occurred on pasture, often due to personal space invasion, mating, or hierarchy disputes. This corroborates Derungs et al.'s (2004) findings and highlights the importance of proper pasture management and understanding equine social dynamics. For handlers, fear and startle responses in horses were primary triggers, emphasizing the need for handlers to recognize and mitigate potential stressors, as suggested by Hausberger et al. (2008).

The distribution of affected body regions differed between horses and handlers, with implications for protective measures. In horses, limb injuries were most common, consistent with Derungs et al.'s (2004) observations. Handlers experienced more upper body injuries, aligning with findings from Iba et al. (2001), Exadaktylos et al. (2002), and Jagodzinski and DeMuri (2005). This suggests that protective gear and handling techniques should be tailored differently for horses and humans.

Regarding sequelae, our findings of skin abrasions and fractures as common outcomes in both horses and handlers are consistent with previous research. Derungs et al. (2004) reported similar patterns in horses, while Williams and Ashby (1992) noted comparable injury types in humans. The higher proportion of skin abrasions compared to fractures in handlers may be attributed to kicks often occurring in muscle-covered areas, as suggested by Schroeder et al. (2013).

Our study identified age and sex as significant risk factors for horses, with older horses and stallions at higher risk. This may reflect changes in temperament with age and hormonal influences on behaviour, as noted by Rios and Houpt (1995) regarding stallion aggression. For handlers, younger age, being a farrier, and less experience was associated with higher risk. The increased risk for farriers aligns with Exadaktylos et al.'s (2002) observation that those cleaning horses' hooves are at elevated risk of kicks.

While our findings on breed-related risk were not statistically significant, they contrast with Knubben et al.'s (2008) results, suggesting the need for further investigation into breed-specific risks.

The study's main limitation is the small sample size, especially for horse handlers, which impacts statistical power and generalizability. Future research should expand the sample size and explore specific circumstances surrounding kick incidents in greater detail.

In conclusion, our results emphasize the intricate interaction of various factors leading to horse kick accidents. To reduce risks, it is suggested to create detailed safety training programs, particularly targeting at-risk individuals like young handlers, farriers, and inexperienced staff. It is essential to implement tactics for effective pasture management and enhance knowledge of equine social behaviour. It is crucial to create specific protective measures according to the various injury patterns seen in horses and handlers. Furthermore, raising awareness of possible stress factors and triggers for horses in stables can greatly help in prevention. By comprehensively addressing these areas, we can aim to decrease the incidence and severity of horse kick incidents, ultimately improving safety for both horses and humans in stable environments. Additional investigation with larger sample sizes and a deeper look into specific circumstances of kicking incidents will help improve our understanding and prevention tactics.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept – NR; Design – OOA; Supervision – NR; Resources – TRL; Materials – PWH, REE; Data Collection and Processing – OOA, TRL; Analysis and Interpretation – OOA, TRL, PWM, REE; Literature Search – OOA, NR; Writing Manuscript – OOA, REE; Critical Review – PWM, NR.

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INCIDENCA I FAKTORI RIZIKA RITANJA KONJA ZA KONJE I VODIČE U ODABRANIM ŠTALAMA U DRŽAVI LAGOS U NIGERIJI: DVOGODIŠNJA PROSPEKTIVNA STUDIJA

SAŽETAK

Konji sa svojim snažnim stražnjim dijelovima i defanzivnim mehanizmima ritanja predstavljaju znatan rizik od teških povreda za ljude i ostale konje. Nedostatak sveobuhvatnih podataka o ritanju kod konja naglašava potrebu za boljim poznavanjem potencijalnih opasnosti. Imali smo za cilj prospektivno istražiti incidencu i rizične faktore ritanja na ostale konje i vodiče u odabranim štalama u državi Lagos u Nigeriji, u periodu od 2021. do 2023. godine. U istraživanje je uključeno ukupno 200 konja i 55 vodiča. Incidenca ritanja i udaranja drugih konja je iznosila 12.5%, a vodiča 29.1%. Najčešća situacija koja je dovodila do ritanja i udaranja drugih konja je bila invazija privatnog prostora (52%), dok su kod udaranja vodiča primarni okidači bili strah (44%) i odgovor na prepad (31%). Nastale povrede su uključivale abrazije kože (32% kod konja i 69% kod vodiča), hematome (16% kod konja), oštećenje mišića/živaca (20% kod konja, 13% kod vodiča) i frakture (32% kod konja i 19% kod vodiča). Signifikantni rizični faktori za udaranje konja su bili starost (>15 godina) i status pastuha. Za vodiče su signifikantni rizični faktori uključivali mladost (24–44 godine), zanimanje potkivača i manje od 5 godina radnog iskustva. Ritanjem su udarani i drugi konji i vodiči. Dobiveni rezultati naglašavaju potrebu za postojanjem ciljanih mjera sigurnosti i obukom, posebice visoko rizičnih grupa, kako bi se smanjili rizici povezani sa ritanjem i udaranjem drugih konja i vodiča u štalama u državi Lagos.

Ključne riječi: Ritanje, incidence, prospektivna studija, voditelj konja

CASE REPORT

ASSESSING POST-SURGICAL BONE DEFORMATIONS: INTEGRATING ANATOMY WITH COMPUTERIZED 3D MORPHOMETRY

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ABSTRACT

Geometric morphometrics, a powerful method for analysing structural shapes, has become increasingly significant in various disciplines, including anatomy, morphology, and clinical medicine. Unlike traditional morphometric approaches, geometric morphometrics offers a comprehensive understanding of shape structure, particularly when enhanced by 3D imaging techniques. In this study, we explored the potential of this method in a clinical setting, applying it to post-operative evaluations of surgically treated bones. Utilizing computerized tomography (CT) scans, we compared the shapes of surgically treated bones to their healthy, symmetrical counterparts, focusing on the changes that occurred during the healing process. The study involved CT images from patients who had previously undergone bone surgery, sourced from the archives of Istanbul University-Cerrahpaşa Veterinary Faculty Animal Hospital. Advanced software tools, including Slicer and the ALPACA module, were employed to generate 3D models and facilitate precise comparisons between operated and non-operated bones. Our findings revealed that while shape deformations in control group bones were minimal, surgically treated bones exhibited more pronounced changes. These deformations were often localized to regions affected by surgery, suggesting that the method is effective in detecting post-operative alterations. The computerized approach used in this study not only allowed for detailed morphological analysis but also demonstrated the potential for future applications in clinical practice. As technology continues to advance, integrating computerized methods with anatomical studies can enhance the accuracy of surgical evaluations and improve patient outcomes. This study highlights the importance of combining anatomical knowledge with computer-assisted techniques to develop more effective clinical tools for assessing surgical success.

Keywords: Computerized analysis, point cloud analysis, shape analysis, surgical outcomes

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INTRODUCTION

Geometric morphometrics is a contemporary method that enables the analysis of structural shapes across various disciplines, including anatomy, morphology, palaeontology, and biology (Mitteroecker and Gunz, 2009; Klingenberg, 2016). Unlike traditional morphometry, which primarily provides information on distances, angles, and ratios between points, geometric morphometrics offers a more detailed and comprehensive understanding of shape structure (Slice, 2007; Gündemir et al., 2023). Recently, the advent of 3D geometric morphometrics has further refined shape analysis, allowing researchers to explore new research questions with greater precision.

The foundation of geometric morphometrics lies in the placement of landmarks on structures (Slice, 2007). These landmarks, when mapped onto a Cartesian plane, form the basis for statistical analysis (Klingenberg, 2016; Boz et al., 2023). Landmarks can be identified manually or automatically through specialized software. One such tool, the ALPACA (Automated Landmarking through Point Cloud Alignment and Correspondence Analysis) module, facilitates automatic landmarking (Porto et al., 2020). This method achieves comparable landmark accuracy by relying on a single reference sample and less frequent sampling of the structure's surface. ALPACA uses a point cloud-based approach to find the optimal parameter set that best aligns a source model (with predefined landmarks) to a target model (where landmarks are to be transferred), allowing for the geometric comparison of two different objects.

In recent years, the integration of computer technologies into clinical skill evaluation has gained momentum. For instance, one study investigated the assessment of laparoscopic surgical skills using computer simulations (Grantcharov et al., 2001). Similar studies have employed artificial intelligence and computer-based software to evaluate clinical skills or provide objective measures of surgical success (Aucar et al., 2005; Reiley et al., 2011; Khalid et al., 2020). Another study highlighted the potential of explainable artificial intelligence in medicine, serving as a reference for both medical experts and AI scientists in designing medical AI applications (Zhang et al., 2022). As these studies suggest, advancing technology can serve as a valuable tool for evaluating clinical skills.

In this study, we utilized computer-based landmarking software to evaluate surgically treated bones. By employing geometric morphometrics, we compared the shape of the surgically treated bone with that of its healthy, symmetrical counterpart, monitoring changes after the healing process. The objective is to investigate the potential of geometric morphometrics as a tool for assessing the success of bone surgeries.

MATERIALS AND METHODS

The study utilized computerized tomography (CT) images obtained from patients who had previously sustained fractures in long bones and received surgical treatment after recovery. These images were sourced from the archives of Istanbul University-Cerrahpaşa Veterinary Faculty Animal Hospital (Table 1). Specifically, the study focused on samples that had undergone bone surgery and were re-imaged after healing, with the data collected between 2021 and 2023. CT scans were conducted using Siemens Somatom Scope vc30b and Siemens Somatom Sensation 16 systems. The scanning parameters for all samples were consistent: a slice thickness of 0.6 mm, 110 kV, and 28 mA, with a total scan time of 14 seconds. Segmentation of the images was performed using Slicer software (version 5.3.0) (Dyaksa et al., 2023), and soft tissues were removed to isolate the bone structures. Models of both surgically treated bones and symmetrical, untreated bones were generated and saved in ply format.

Table 1 Samples

Sample	Species	Surgically Treated Bone	Clinical Diagnosis	Surgical Treatment
Nr1	Dog (French Bulldog)	Left Humerus	Humerus distal metaphyseal comminated fracture	A 2.5 mm thick 2.7 mm screw compatible 8-hole locking anatomic plate was applied. Screw thickness was 2 x 24 mm and 6 x 22 mm long. Mineralized callus formation was seen on the 60th day control radiographs.
Nr2	Dog (French Bulldog)	Right Humerus	Right distal humerus intercondylar and supracondylar fracture	Osteosynthesis was performed with 1 intercondylar pin, 1 pin from the lateral condyle to the humerus medulla, and 1 pin from the lateral condyle to the humerus medulla. Mineralized callus formation was detected on the 90th day radiographs.
Nr3	Jackal	Left Femur	Left femur mid-diaphyseal oblique fracture, comminated fracture of left ischia and pubis, and right coxofemoral dislocation	It was treated with a 10-hole locked compression plate compatible with 2.7 screws of 2 mm thickness. Mineralized callus formation was detected on the radiographic controls on the 90th day after the operation. The plate was removed 15 days later under general anaesthesia.

In the study, bones were compared as surgically treated and non-operated. However, the bones were anatomically symmetrical to each other, with structures on opposite sides. For example, when viewed from behind, the head of the humerus is on the left side of the right humerus but on the right side of the left humerus. To ensure accurate comparison, mirror images of the non-operated bone samples were created using the Slicer program (Figure 1A). This allowed the bones to be aligned in shape, enabling a more precise comparison between the treated and non-operated bones.

To ensure consistent orientation, a 3D image of one symmetrical sample was first obtained. For automatic landmark placement, a preliminary draft landmarks were created using non-surgical samples as references (Figure 1B). This draft landmark set was generated using the 'PseudoLMGenerator' module in Slicer (version 5.5.0) with a 10% range tolerance. The draft landmark set was then applied to the symmetrical samples one by one using the ALPACA module. This method created point clouds of the solid samples based on the reference landmarks, which were subsequently superimposed (Figure 1C and 1D). After superimposition, the point clouds were solidified, and the bone shapes were compared (Figure 1E). Protruding areas were evaluated to determine the exact location and extent of shape differences, which were then examined morphologically.

RESULTS

In the study, 3D images of two opposing bones were superimposed to evaluate shape differences morphologically. The unoperated, healthy bone (shown in bone colour in the Figures) served as

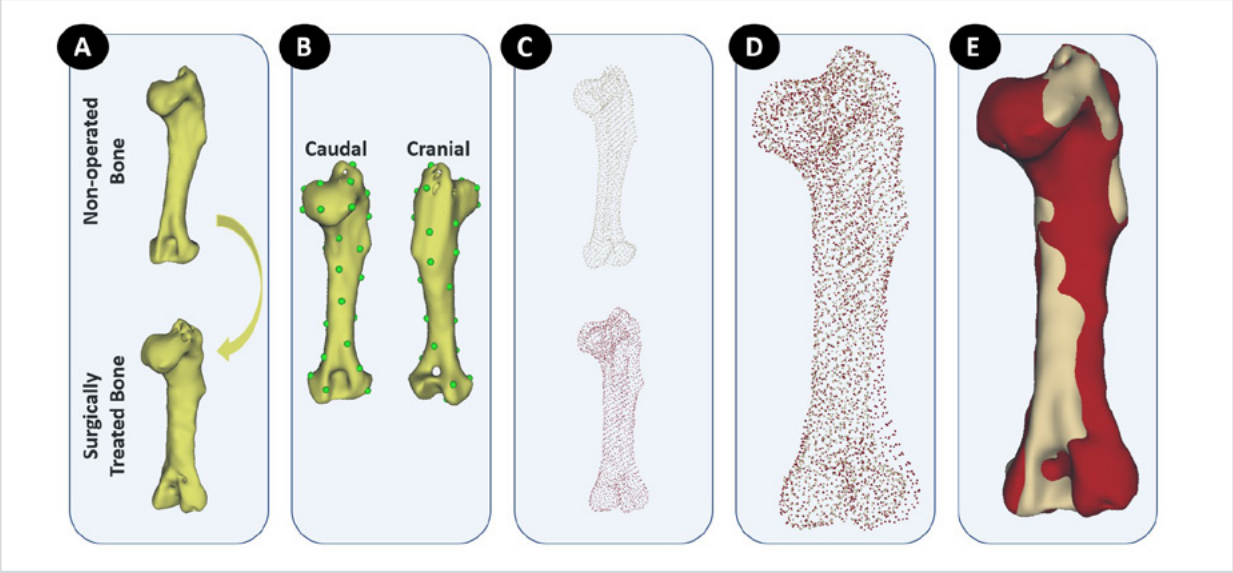

Figure 1A: The mirror images; **B:** The draft landmarks; **C:** The point clouds; **D:** The point clouds

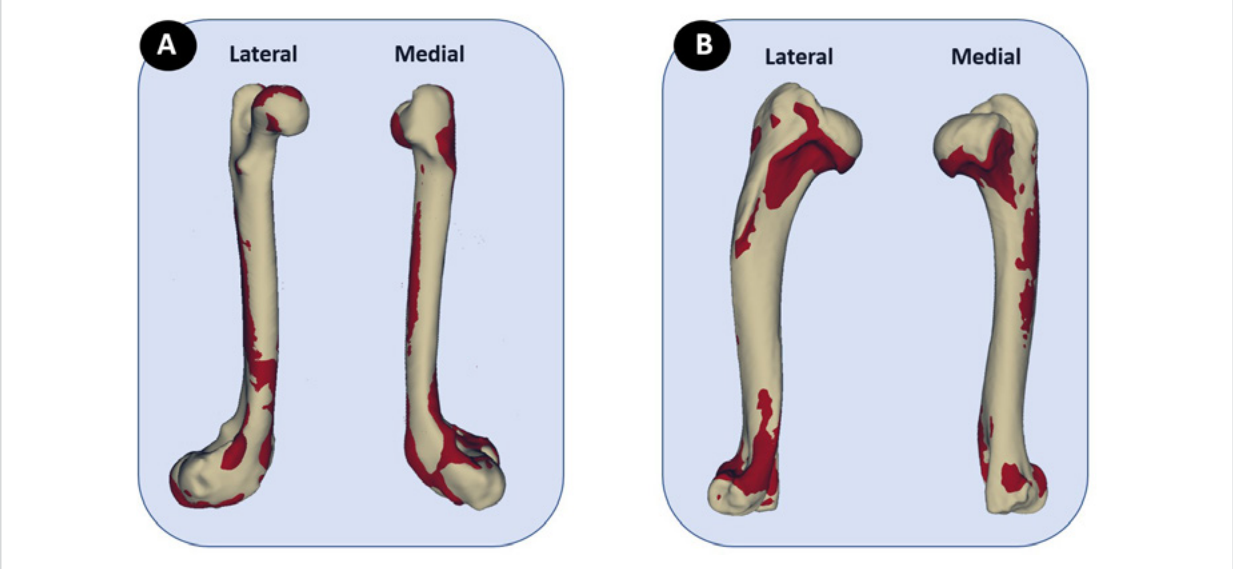

Figure 2 Control group. **A:** Femur models of a healthy dog. **B:** Humerus models of a healthy dog. Bone colour represents the left bone, red colour represents the right bone

the source bone, and the shape deformations of the surgically treated bone were compared against this reference. Deformed areas on the surgically treated bone were highlighted in red in the Figures, allowing us to assess the extent of overhangs and deformations relative to the healthy bone.

As a result of the study, bone pairs were superimposed in 3D, and shape differences were evaluated morphologically. Initially, the control group was assessed, revealing that the shape differences between the right and left bones in the control group were less pronounced than those in the surgically treated bones (Figure 2). The changes

in the control group were not concentrated in any specific area, although a slightly more noticeable difference was observed in collum humeri. Despite this, the shape changes resulting from the surgical operations were significantly more pronounced compared to the control group.

Significant morphological differences were observed when comparing the surgically treated bone with its healthy counterpart (Figure 3). In the case of Nr1, where the distal metaphysis of the left humerus had been fractured, the models were analysed after healing. The shape deformation in the surgically treated bone was quite evident, particularly towards the lateral side. This deformation began at the caput humeri, extended along the lateral surface of the bone, and continued to the lateral condyle.

In Nr2, the distal humerus fracture was treated. As a result of healing, the shape deformations of the surgically treated bone compared to the healthy bone were evident in the distal region of the humerus.

In the case of Nr3 (Jackal), fractures were present in the coxae and femur. A particularly notable finding was the upward deformation of the caput femoris. Additionally, post-healing, there was a noticeable expansion in the body of the bone, which was likely a result of the healing process. Overall, the changes in the surgically treated bone were significantly more apparent compared to the unoperated sample.

DISCUSSION AND CONCLUSION

A method based on shape analysis was employed in this study to evaluate the post-operative outcomes of surgically treated bones. Notably, no prior clinical application of this method was found in the literature, making this study a pioneering effort. The results were promising, demonstrating

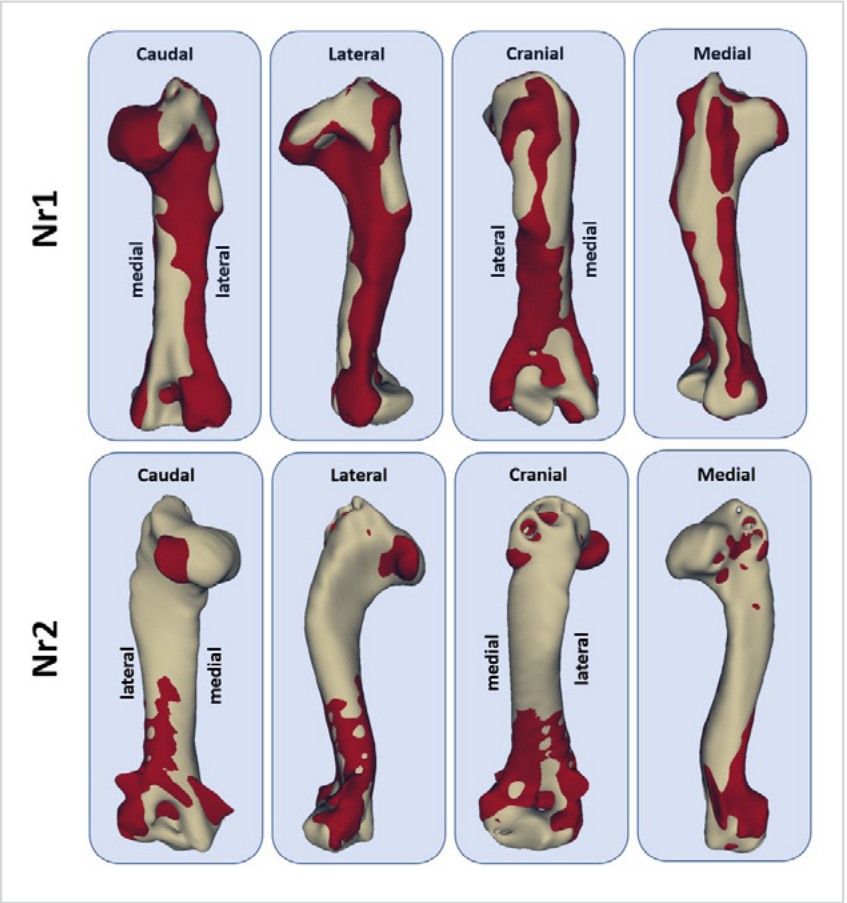

Figure 3
Superimposed models of healthy bone (shown in bone colour) and surgically treated bone (shown in red colour)

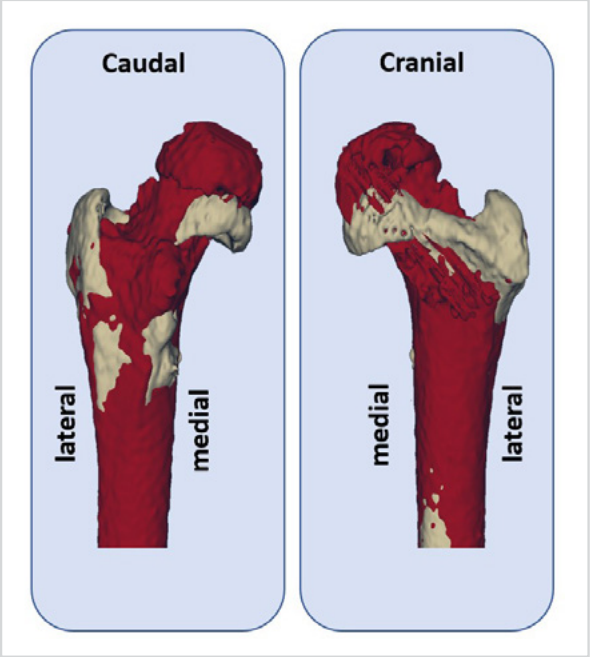

Figure 4

Superimposed models of healthy bone (shown in bone colour) and surgically treated bone (shown in red colour) of Jackal

that 3-dimensional visual analyses could be effectively utilized for morphological evaluations. This innovative approach allowed for the precise comparison of bone structures, revealing the extent of deformations that occurred as a result of surgical intervention.

The study utilized CT images to analyse recovery outcomes in two control groups and three animals that had undergone bone fracture surgery. In the control group, where no surgical intervention had taken place, deformations between the right and left bones were minimal and limited. However, in the surgically treated bones, deformations were significantly more pronounced. Importantly, these deformations corresponded closely to the regions of the bone that had been operated on, suggesting that this technique has strong potential for future clinical applications.

For instance, in the case of Nr1, the deformation occurred in areas where the brachialis muscle, one of the strongest arm muscles, attaches. This observation suggests that during the healing process, deformations may be influenced by the surrounding muscle groups, leading to shifts in bone structure towards these strong muscle attachments. Similarly, in Nr2, the distal humerus fracture showed a broader healing pattern than normal, which could be indicative of compensatory bone growth or remodelling during recovery. In the Jackal case, the significant positional change in the caput femoris due to healing raises concerns about potential long-term lameness or mobility issues, highlighting the importance of monitoring such changes post-surgery.

Despite these promising observations, the study faced limitations in drawing definitive conclusions regarding the accuracy of the applied technique or the success of the surgical interventions. The primary aim of the study was to explore the applicability of this novel shape analysis method and assess its potential for yielding meaningful results. Future studies with more homogeneous samples could further validate the technique and allow for the evaluation of surgical success based on post-operative bone healing.

One of the key limitations of this study is the inability to perform statistical evaluations of the results. Since each operated bone could only be compared to its healthy symmetrical counterpart, obtaining statistically significant data from these binary comparisons proved challenging. This limitation hinders the ability to make broader

scientific conclusions from the data. Consequently, the current method allows for primarily visual and morphological assessments, which, while insightful, may be best suited for clinical evaluation rather than comprehensive statistical analysis.

In conclusion, this study demonstrates that shape analysis methods can provide valuable insights into post-operative bone healing. Although statistical validation remains a challenge, the visual and morphological differences observed in this study suggest that this approach could become a useful tool in clinical settings, offering a new way to assess surgical outcomes and guide future treatment strategies.

In conclusion, the use of shape analysis for post-operative evaluation of surgically treated bones proved to be a promising approach, offering detailed insights into bone deformations that are otherwise difficult to quantify. The method allowed for precise 3D comparisons between operated and healthy bones, highlighting significant morphological changes that corresponded with surgical intervention sites. While this study primarily focused on demonstrating the applicability of the technique, the findings suggest that shape analysis could become a valuable tool in clinical settings for assessing surgical outcomes and guiding future treatments. However, the limitations regarding statistical evaluation emphasize the need for further research with larger, more homogeneous sample sizes to validate these preliminary findings and enhance the method's clinical relevance. Despite these challenges, the study opens new avenues for integrating advanced imaging techniques into veterinary and possibly human orthopaedic practices.

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CONTRIBUTIONS

Concept and Design: SO, GS; Supervision: OG; Fundings: GS; Materials: YA, GS; Data collection and Processing: GS, OG; Analysis and Interpretation: YA; Writing and Critical review: SO, OG

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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PROCJENA POSTOPERATIVNIH KOŠTANIH DEFORMACIJA: INTEGRACIJA ANATOMIJE I KOMPJUTERIZIRANE 3D MORFOMETRIJE

SAŽETAK

Geometrijska morfometrija, moćna metoda u analizi strukturnih oblika, naglo poprima sve veći značaj u različitim disciplinama, uključujući anatomiju, morfologiju i kliničku medicinu. Za razliku od tradicionalnih morfometrijskih pristupa, geometrijska morfometrija nudi sveobuhvatno razumijevanje strukture oblika, posebno ako je potpomognuta sa 3D tehnikama snimanja.

U našem istraživanju smo ispitali mogućnosti ove metode u kliničkim uvjetima, primjenjujući je kod postoperativnih evaluacija operativno tretiranih kostiju. Korištenjem snimaka kompjuterizirane tomografije (CT), izvršili smo usporedbu oblika operativno tretiranih kostiju sa kontralateralnim zdravim kostima, fokusirajući se na promjene koje nastaju u procesu cijeljenja. U istraživanju su korišteni CT snimci pacijenata koji su prethodno bili podvrgnuti koštanim operacijama, čiji izvor je arhiv Univerziteta u Istanbulu – Bolnica za životinje Veterinarskog fakulteta Cerrahpaşa. Korištene su napredne softverske alatke, uključujući module Slicer i ALPACA, kako bi se generirali 3D modeli i olakšale precizne komparacije operiranih i neoperiranih kostiju. Naši rezultati su pokazali da operativno tretirane kosti su pokazale izraženije promjene, dok su deformacije oblika u kontrolnoj grupi kostiju bile minimalne. Ove deformacije su često bile lokalizirane u operiranim područjima, što ukazuje na uspješnost metode u otkrivanju postoperativnih promjena. Kompjuterizirani pristup korišten u ovom istraživanju nije omogućio samo detaljnu morfološku analizu, nego je i pokazao potencijal za buduće aplikacije u kliničkoj praksi. Sa nastavkom napredovanja tehnologije, integriranje kompjuteriziranih metoda i anatomskih studija će povećati preciznost operativnih evaluacija i poboljšati ishode za pacijente. Ovo istraživanje naglašava značaj kombiniranja anatomskog znanja sa kompjuteriziranim tehnikama kako bi se razvile učinkovitije kliničke alatke za procjenu operativnog uspjeha.

Ključne riječi: Analiza oblika, kompjuterizirana analiza, operativni ishodi, point cloud analiza

CASE REPORT

CAN NAIL DAMAGE BE FATAL? A CASE REPORT OF TETANUS IN A GERMAN BOXER

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ABSTRACT

Tetanus is a neurologic disease caused by the action of tetanus toxin (TeNT) produced by the Gram-positive, ubiquitous, sporulating, anaerobic bacterium *Clostridium tetani*. It is a rare disease in dogs due to their resistance to the toxin. A 1-year-old, male German Boxer presented with a three-day history of lethargy, facial abnormalities, dyspnea, ptyalism, dysuria and constipation. The owners also reported that the dog's nail had broken two weeks ago. Clinical examination revealed ptyalism, hyperthermia, abnormal facial expressions (wrinkled forehead, *risus sardonicus*) and eyes' position (ventrolateral strabismus), trismus, dysuria, and muscle rigidity, which progressed to seizures and generalized spastic tetraplegia. Complete blood count showed increased reticulocyte count, low reticulocyte hemoglobin content, and eosinopenia. The biochemistry profile revealed decreased amylase values, hypochloremia, and creatine kinase was significantly elevated. A presumptive diagnosis of generalized tetanus was based on the history, wound presence, and characteristic clinical signs. The initial treatment was based on previously published recommendations, and it included: IV fluids, sedation and muscle relaxation, antibiotic (metronidazole), tetanus antitoxin, and supportive care. Unfortunately, during an episode of spastic muscle rigidity, apnea occured, leading to the dog's death.

Keywords: Dog, muscle spasm, nail, tetanus

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INTRODUCTION

Tetanus is a neurologic, potentially life-threatening disease caused by tetanus toxin. It is a potent neurotoxin produced by the Gram-positive, ubiquitous, anaerobic, sporulating bacterium *Clostridium tetani* (Popoff, 2020). The most common source of infection is a penetrating wound, but it can also be a damaged or broken tooth, umbilical infection in newborns, or *C. tetani*-contaminated surgical wounds (Popoff, 2020; Fawcett and Irwin, 2014). In an anaerobic environment, the spores germinate and the toxins are released. Tetanus is a rare disease in carnivores due to their relative resistance to the TeNT, and it can appear in generalized or localized forms (Greene, 2012; Maksimović et al., 2016). TeNT inhibits the release of inhibitory neurotransmitters gamma-aminobutyric acid (GABA) and glycine, leading to muscle spasms and characteristic clinical manifestations such as trismus, *risus sardonicus*, muscle rigidity, hyperextension of the limbs, ocular and facial abnormalities, urethral and anal sphincter hypertonicity, hyperthermia in generalized tetanus (Popoff, 2020; Fawcett and Irwin, 2014; Rhee et al., 2005), and painful, spastic muscles in the infected region in localized tetanus (Popoff, 2020). There is no available diagnostic test for tetanus; diagnosis is typically based on the patient's history, the presence of a wound and characteristic clinical signs (Greene, 2012; Adamantos and Boag, 2007). Complete blood count and biochemistry typically reveal non-specific abnormalities, though the muscle enzyme creatine kinase may be significantly elevated. Treatment of tetanus consists of the wound management, tetanus antitoxin, sedation and muscle relaxation, antibiotics, and supportive care (Acke et al., 2004). Possible complications may include: aspiration pneumonia, hiatal hernia, laryngeal spasm, respiratory arrest, and urinary tract infections (Adamantos and Boag, 2007). Untreated and neglected cases are usually fatal (Acke et al., 2004).

This is a case report of the diagnosis, treatment and outcome of generalized tetanus in a one- year-old male dog.

Case description

A 1-year-old male German Boxer, weighing 24 kg, was presented with a three-day history of lethargy, difficulty in breathing and eating, facial abnormalities, ptyalism, dysuria, and constipation. The first clinical sign that owners noticed was a change in facial expression. The local veterinarian suspected an allergic reaction and treated the dog with antihistamines. Due to the lack of visible improvement, the dog was referred to the Clinic for Internal Diseases, Oncology and Emergency Medicine of the University of Sarajevo - Veterinary Faculty for further investigation. The owners reported that the dog's nail had broken on the rails during a walk two weeks earlier. On the same day, the wound was treated with normal saline and chlorhexidene. According to the owners, the dog did not experience any head or spinal trauma. On admission, clinical examination revealed dyspnea, hyperthermia (39.9 °C), ptyalism and dysphagia. Neurological examination revealed generalized stiffness, retracted facial muscles and lips (*risus sardonicus*), wrinkled forehead, ventrolateral strabismus (Figure 1), trismus, and rigidity of the limb and tail muscles. This condition rapidly progressed throughout the day, leading to seizures and generalized spastic tetraplegia (Figure 2). Based on characteristic clinical signs and the history of an old wound, generalized tetanus was suspected. Diazepam was used as a muscle relaxant (0.5 mg/kg rectally) to enable intravenous cannulation and blood sampling. In order to achieve sedation to relieve distress, intravenous propofol was administered at a dose of 2 mg/kg. Diazepam administration achieved only mild muscle relaxation. On the other hand, propofol successfully achieved adequate sedation level (Youngblood et al., 2018). During the sedation, basic vital parameters were monitored using an electronic sphygmomanometer and pulse oximeter (Contec Medical System, China). A cuff for non-invasive BP measurement was placed on the upper front limb of the laterally recumbent animal, and

the SpO2 sensor was connected to the upper lip. SpO2 revealed the heart rate of 95 beats per minute, and oxygen blood saturation was between 94-96% on spontaneous ventilation. Blood pressure was 170/70 mmHg with the mean arterial pressure of 110 mmHg, with the body temperature of 38.6 °C during sedation. The history, clinical examination, and sedation were followed by blood sampling from the cephalic vein into an IDEXX EDTA KE/1.3 microtube for hematology and an L-heparin LH/1.3 microtube for heparinised plasma biochemistry panel (Chem 17 CLIP), electrolytes (Lyte 4 CLIP), and creatine kinase blood tests. Complete blood count was performed using a ProCyte® Hematology Analyzer (Idexx Laboratories Inc.), and biochemistry panel, electrolyte and creatine kinase using a Catalyst One® Chemistry Analyzer (Idexx Laboratories Inc.). Complete blood count revealed increased reticulocyte count, low reticulocyte hemoglobin content, and eosinopenia. Biochemistry profile showed decreased amylase values, hypochloremia, and CK was significantly elevated (774 U/L (reference ranges: 10-200 U/L)). With the exception of creatine kinase, which was abnormal, no other laboratory parameters were clinically relevant.

Based on characteristic clinical signs and the history of the old wound, generalized tetanus was suspected. Differential diagnoses for the dog included immune-mediated polymyositis, strychnine toxicity, spinal trauma, hypocalcemia, and meningoencephalitis. Immune-mediated polymyositis and meningoencephalitis were ruled out due to the lack of specific symptoms. Spinal trauma and strychnine toxicity were ruled out based on the history, and hypocalcemia could be ruled out based on the normal calcium values on the biochemical panel.

Intravenous fluid therapy with balanced isotonic crystalloid solutions (3 mL/kg/h of 0.9% normal saline) was used to maintain adequate hydration. Metronidazole (Fresenius Kabi, Austria (15 mg/kg IV)) was the antibiotic of choice. Initially, the patient received 60 IU of anti-tetanus equine serum (Clotean, Bioveta, Czech Republic) subcutaneously. Thirty minutes later, after verifying the absence of hypersensitivity to the serum, the full dose (12 000 IU) was given as a bolus intravenously over 30 minutes. Catheterization was performed due to the inability to urinate voluntarily.

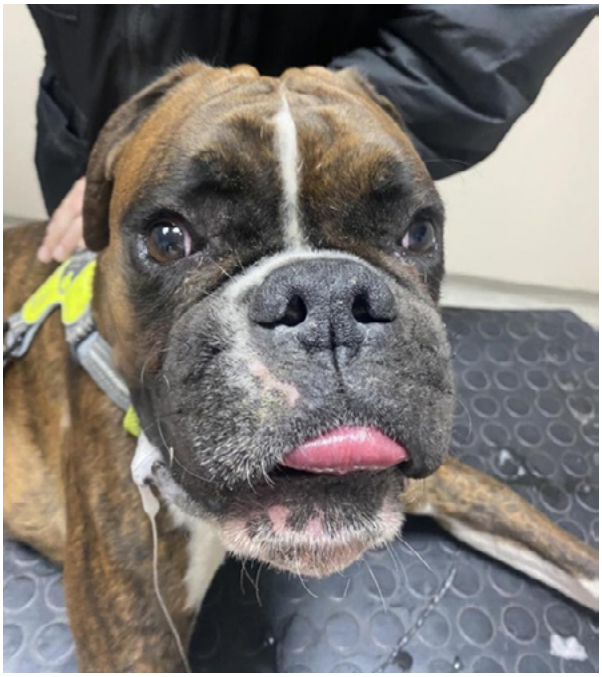

Figure 1

Abnormal facial expression and ptyalism in the dog with tetanus

On the same day, the dog was sent to the primary veterinary clinic for continued intensive care. Unfortunately, although the dog was stable during the day, its condition worsened in the evening. Due to a spastic muscle rigidity episode, apnea occurred, and it resulted in the dog's death.

DISCUSSION AND CONCLUSION

Tetanus is a rare neurological disease in dogs due to their species' inherent resistance to the TeNT. It is believed that their peripheral nervous tissue is insensitive to penetration by the toxin, anyhow, cases of tetanus have been reported in dogs (Adamantos and Boag, 2007; Acke et al., 2004; Matthews and Forbes, 1985; Bandt et al., 2007; Burkitt et al., 2007; Sprot, 2008). An anaerobic environment is necessary for bacteria to produce

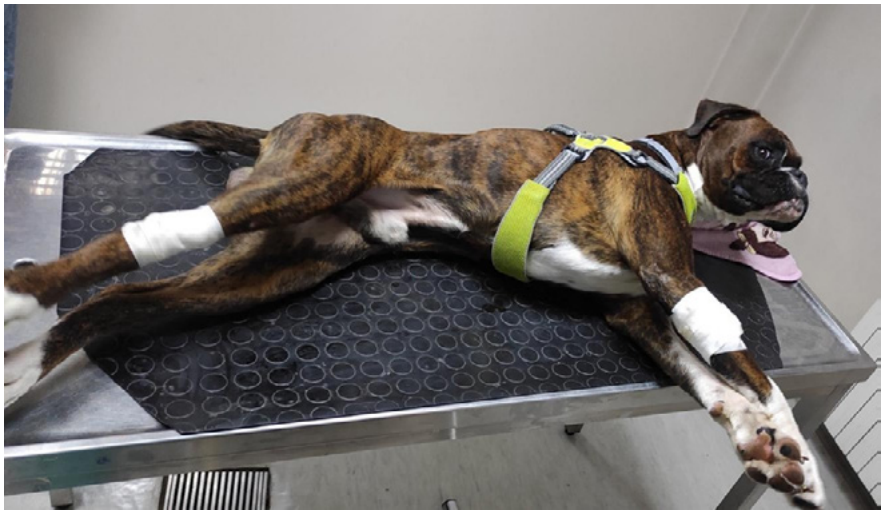

Figure 2

Spastic tetraplegia in the dog with tetanus

the neurotoxin. Toxin enters the axons of the peripheral nerves and migrates to the neuronal cell body in the spinal cord and the brain. From that point, TeNT is transported to the interneurons with which they irreversibly bind and inhibit the release of GABA and glycine, leading to muscle spasm (Greene, 2012). The most common form of tetanus in dogs is the generalized form; the first noticed clinical signs before progression to the generalized form are usually ocular and facial abnormalities (Ives, 2014). The clinical signs of the dog in this case were in typical progression, as it was described by Ives (2014). Clinical signs may develop 3-18 days after wounding, as was found in this case. Due to the aforementioned toxin resistance of dogs, disease onset may occur even 3 weeks after an injury. Incubation time varies, and it is also dependent on the distance of the wound from the central nervous system. The incubation period is longer when the wound is farther from the head, but usually, it lasts between 5 and 10 days (Greene, 2012; Burkitt et al., 2007). It is also well known that younger animals may be more likely to develop more severe clinical signs due to their immature natural immunity (Greene, 2012). The head-wound distance and the dog's age significantly corroborate with our case report in which the nail of the one-year-old dog had been broken two weeks ago.

There are no available antemortem diagnostic tests for tetanus, and the diagnosis is usually based on the history, the presence of a wound, and characteristic clinical signs (Greene, 2012; Adamantos and Boag, 2007). There are some potentially helpful parameters such as muscle enzymes creatine kinase and aspartate aminotransferase (AST) whose values are elevated due to continuous muscle contractions. Elevated creatine kinase further increased the suspicion of tetanus in this case. Even though thoracic radiography may be useful to rule out the presence of complications such as aspiration pneumonia or hiatal hernia (Ives, 2014), it was not taken in this case. Radiological examination was postponed to avoid increased muscle spasm due to manipulation, since it is known that it is usually triggered by stimulations such as touch, noise or light and that over-stimulation may just worsen the degree of spasm (Popoff, 2020; Ives, 2014). Anaerobic bacterial culture and serum antibody titers to tetanus toxin theoretically may be used. The reason why these tests are not widely used in practice is the small possibility of *C. tetani* culturing. Furthermore, serum antibody titers to tetanus toxin tests require comparing values to control animals (Ives, 2014). Both of these tests would be time-consuming, and the therapy in cases of tetanus needs to be started as soon as possible. For this reason, these tests were not performed in this case. Differential diagnoses for the dog included immune-mediated polymyositis, strychnine toxicity, spinal trauma, hypocalcemia, and meningoencephalitis (Sprott, 2008). Immune-mediated polymyositis and meningoencephalitis

were ruled out due to the lack of specific symptoms (Sprott, 2008; Morozumi et al., 1991). Spinal trauma and strychnine toxicity were ruled out based on the history, and hypocalcemia could be ruled out based on the normal calcium values on the biochemical panel. Tetanus treatment is not specific, but rather based on recommendations such as wound debridement, tetanus antitoxin, sedation and muscle relaxation, antibiotics, and supportive care (Popoff, 2020; Acke et al., 2004). In this case, the therapy was mainly based on previously published recommendations. Tetanus antitoxin usage in carnivores is controversial because the optimal dose and the efficacy of the antitoxin have not been clearly defined (Adamantos and Boag, 2007). Its role is to neutralize unbound toxins, and it is not effective in neutralizing already bound toxins. The dose ranges from 100 units/kg up to a maximum dose of 20.000 units (Sprott, 2008). Its half-life is approximately two weeks, so only one dose is needed (Adamantos and Boag, 2007). Although penicillin has been recommended for many years, metronidazole is the drug of choice for the treatment of dogs with tetanus recently. According to Ahmadsyah and Salim (1985), its use has been associated with a shorter recovery time and lower mortality rates. Besides that, dogs treated with metronidazole needed smaller doses of muscle relaxants and sedatives (Farrar et al., 2000). Penicillin is effective against anaerobic Gram-positive bacteria, but it is also a GABA antagonist so it may potentiate the effects of the TeNT (Adamantos and Boag, 2007). The addition of magnesium seemed to be beneficial in the early and latter stages of tetanus. Magnesium was used to increase muscle relaxation and to increase the time interval between a sedative administration (Papageorgiou and Anagnostou, 2021). As stated by Sun et al. (2019), there is a possible negative impact of wound debridement in cases of moderate or severe tetanus where the wound have already healed. In that case, debridement should be done after stabilization.

As it was explained by Burkitt et al. (2007), a canine tetanus severity classification system is derived from the human classification system, and it included 4 classes. Class I included dogs with facial signs of tetanus; class II included dogs with dysphagia and generalized rigidity with or without facial signs; class III included dogs with signs described in class I and/or class II along with recumbency and seizures; and class IV included dogs with some of the previously mentioned signs along with abnormal heart and respiratory rate or abnormal blood pressure measurements. Most of the 38 dogs evaluated in the retrospective case series by Burkitt et al. developed more severe clinical signs after the first evaluation. In this case, the dog was initially graded as class II due to the presence of facial abnormalities along with generalized rigidity and dysphagia, and it progressed during the day to class III with recumbency and seizures. In such cases, the prognosis is poor with a survival rate of 58% (Burkitt et al., 2007).

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MOŽE LI OŠTEĆENJE NOKTA BITI KOBNO? PRIKAZ SLUČAJA TETANUSA KOD NJEMAČKOG BOKSERA

SAŽETAK

Tetanus je neurološko oboljenje nastalo djelovanjem tetanusnog toksina (TeNT) kojeg proizvodi gram-pozitivna, ubikvitarna, sporogena anaerobna bakterija *Clostridium tetani*. Tetanus je kod pasa rijetko oboljenje zahvaljujući njihovoj rezistenciji na toksin. Jednogodišnji mužjak njemačkog boksera primljen je sa slikom trodnevne letargije, facijalnih abnormalnosti, dispnejom, ptijalizmom, disurijom i konstipacijom. Prema riječima vlasnika, pas je prije dvije sedmice slomio nokat. Klinički pregled je pokazao ptijalizam, hipertermiju, patološke facijalne ekspresije (nabrano čelo, *risus sardonicus*) i položaj očiju (ventrolateralni strabizam), trizmus, disuriju i mišićnu rigidnost koji su uznapredovali do konvulzija i generalizirane spastične tetraplegije. Kompletna krvna slika je pokazala povećan broj retikulocita, nizak sadržaj hemoglobina u retikulocitima i eozinopeniju. Biohemijska analiza krvi pokazala je snižene vrijednosti amilaze i klora, dok je kreatin kinaza bila signifikantno povišena. Presumptivna dijagnoza generaliziranog tetanusa je postavljena na osnovu istorije bolesti, prisustva rane i karakterističnih kliničkih znakova. Terapija se zasnivala na prethodno objavljenim smjernicama i uključivala je infuziju, sedaciju i mišićnu relaksaciju, antibiotik (metronidazol), tetanusni antitoksin i potporu. Nažalost, za vrijeme epizode spastične mišićne rigidnosti, došlo je do apneje koja je dovela do uginuća psa.

Ključne riječi: Mišićni spazam, nokat, pas, tetanus

CASE REPORT

PHYSICAL EXAMS AND SONOGRAPHY IN DIAGNOSIS OF SEVERE RESPIRATORY CONDITIONS IN CATTLE: A CASE REVIEW

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ABSTRACT

Auscultation and percussion are complementary and essential tools in the veterinary physical examination of the respiratory system, but they are ineffective in assessing the nature and extent of pulmonary pathology. In this clinical study, we have followed up on four cases of severe bovine respiratory disease. A general clinical examination was carried out, beginning with inspection, measurement of body temperature, and examination of the mucous membranes. Auscultation and percussion were the two key methods of physical examination. After that, the entire pulmonary field on both sides of the thorax underwent a sonographic examination. Results have shown that LUS combined with physical examination enabled a fairly accurate diagnosis and prognosis to be made in adult cows suffering from pulmonary disorders. The discussion of the results also provided an overview of some of the concepts involved in LUS and how they may relate to the findings of an ordinary clinical examination.

Keywords: Auscultation, percussion, lung sonography, pulmonary condition, cattle

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INTRODUCTION

Clinical examination of the respiratory system, whether by conventional means such as auscultation and percussion (Scott, 2013; Pardon et al., 2019; de Mattos et al., 2020) or by assessing a respiratory score (Buczinski et al., 2014; Cuevas-Gómez et al., 2021), is the main approach used to diagnose the bovine respiratory diseases and to establish criteria for treatment. However, these methods are limited in that they fail to detect subclinical respiratory conditions, courses of disease, and subsequent respiratory system damage. Lung ultrasography (LUS) has answered many diagnostic questions; it is a good method to screen and assess pleural and lung disorders. It can also reliably predict the viability of treated animals (Scott, 2013; Adams and Buczinski, 2016). Today, it is well known that LUS is better than radiography for detecting pleural effusion, pneumonia, and pulmonary edema (Marini et al., 2021). But LUS has its limitations, the main being the reflection of sound waves by the interface between the pleura and the pulmonary air (Marini et al., 2021). With less air in the lungs and alterations near the pleura, LUS gives a clear view of the subpleural abnormality (Tharwat and Oikawa, 2011). Likewise, consolidation processes and the comet tail artifact (CTA) phenomenon are other limiting factors that can lead to different diagnoses due to their various interpretations (Ali et al., 2014; El-Zahar et al., 2021). This study aims to highlight the importance of ultrasound in confirming a diagnosis of respiratory conditions when physical examination has been exhausted. In addition, this work will contribute to enriching the available ultrasound database on this topic.

MATERIAL AND METHODS

Physical examination

Four dairy cows were presented to the ruminant clinic of the Institute of Veterinary Sciences of Tiaret due to decreased appetite and respiratory signs. A routine clinical examination was done, except for the last case; it was shortened. The clinical examination began with inspection of the animal, measurement of body temperature, and examination of the mucous membranes. Auscultation and percussion then assessed the entire respiratory field, from the 4th to the 9th intercostal space (ICS), from the dorsal to the ventral part of the thorax, on both sides. Particular emphasis was given to auscultation and percussion with regard to exploration of the pulmonary system.

Sonographic examination

Both sides of the thorax were shaved. The skin was cleaned with alcohol, then ultrasound gel was applied. An ultrasound scan of the pulmonary field was then performed, based on standardized examination techniques (Flock 2004; Tharwat and Oikawa, 2011), from a dorsoventral plane holding the transducer parallel to the ribs with 3.5 MHz and 5 MHz convex transducer connected to a real-time B-mode portable ultrasound device (iScan 2 multi, Draminski S.A., Poland). The observed anomalies were the presence of CTA or B-line artifact (BLA), pleural fluid accumulation, pleural irregularity and thickening, consolidation, abscesses and emphysema.

Case 1

A 3-year-aged Holstein cow had been in inapetance for 8 days, with a body condition score (BCS) of 1.5. The cow presented with the head extended, a lethargic appearance, and respiratory signs (tachypnea and expiratory dyspnea, dilated nostrils and open-mouthed breathing with foaming (Figure 1). A putrid-smelling nasal discharge was noted. The rectal temperature recorded (37°C) was slightly below normal. The mucous membranes of the eyelids were slightly purplish, while those of the vulva were more so (Figure 2). Cardiac examination revealed tachycardia with a heart rate of 116 beats per minute. Pulmonary auscultation revealed fine crepitations (wet rales), and percussion of the caudodorsal lung on both sides showed dullness.

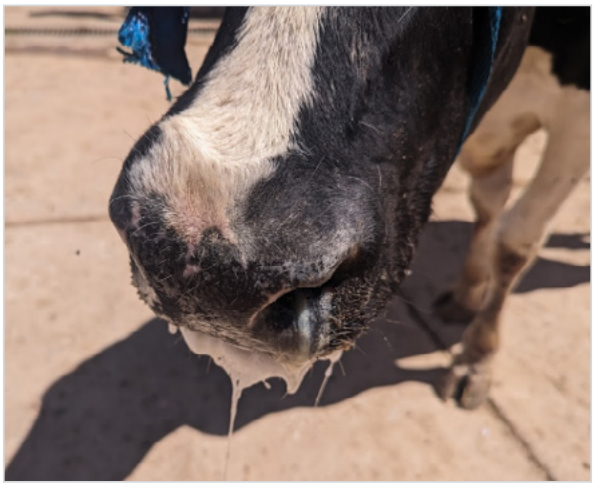

Figure 1 Foamy mouth with nasal discharge

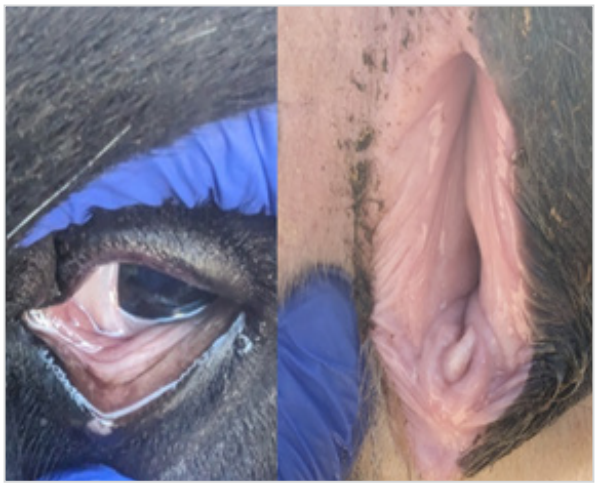

Figure 2 Purplish appearance of ocular and vulvar mucosa

Sonography

The sonographic exam of the caudodorsal region of the left lung shows a bright line of pleura but no modified structure under it. The numerous CTAs born on the pleura and extending deep into the lung (Figure 3) suggest an interstitial syndrome. Lesions appeared more extensive in the right lung. The pleural line being thickened and the small hypoechoic areas on the surface of the lung represent "superficial liquid alveolograms" and subpleural consolidation, with more CTAs (Figure 4). Ultrasound images are mainly from the mid-dorsal and caudodorsal areas (between the 6th and 8th ICS).

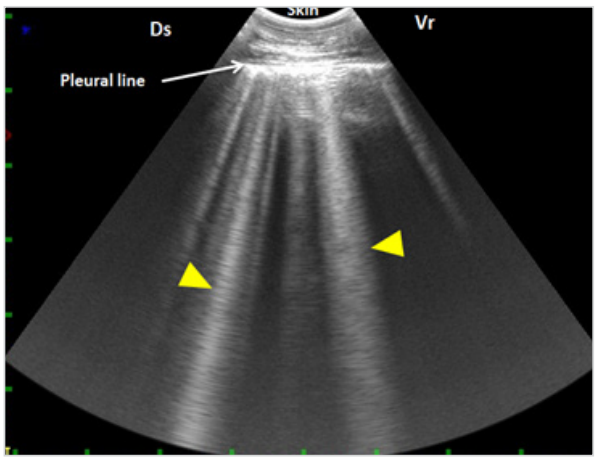

Figure 3 Sonogram of the left lung showing a bright line of thickened pleura, comet-tail artifacts fanning out from the lung-pleura interface and propagating to the image edge (arrowhead)

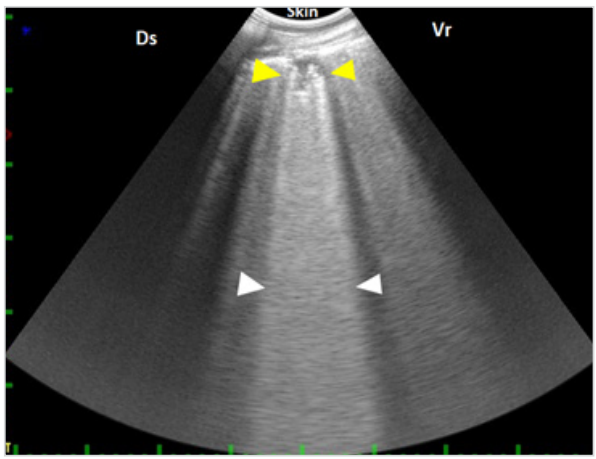

Figure 4 Sonogram of the right lung with small hypoechoic zones on the surface of the lungs represents superficial fluid alveolograms and bronchograms (yellow arrows) with multiple comet-tail artifacts (white arrows)

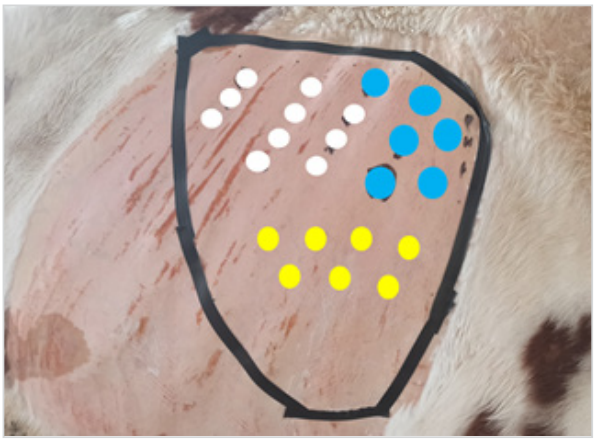

Figure 5 Sound types from right-side percussion: dull (blue), clear (white), hyper-resonant (yellow)

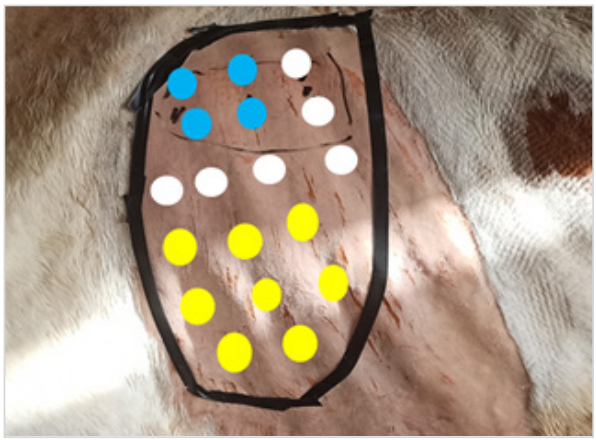

Figure 6 Left-side percussion sound type: limited dullness zone (blue), clear (white), hyper-resonant (yellow)

Sonographic findings, in addition to decreased resonance on percussion and wet rales on auscultation, point to pulmonary edema.

Case 2

It is about a 10-year-old crossbred Red Piebald cow with a poor BCS (1.5). The reason for the consultation was the cow's decreased appetite and coughing. Rectal temperature was normal (39.0 °C), as was the appearance of mucosa. The animal had an expiratory dyspnea, and auscultation revealed dry crackling rales over the entire right respiratory zone but on the left, rales were discrete. On percussion, the animal reacted to pain and coughed loudly. The dorsal and middle areas of the right lung showed dullness (Figure 5). On the

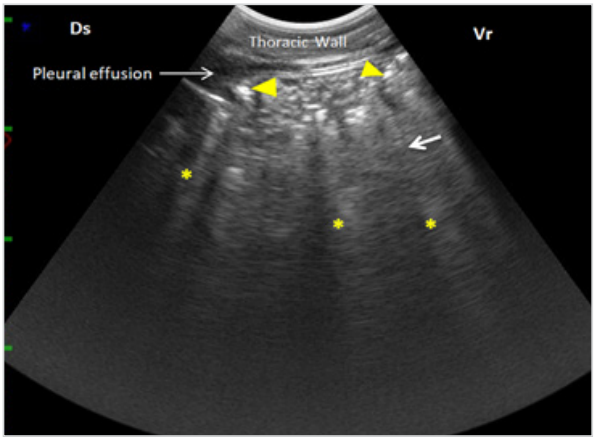

Figure 7 Lung consolidation: hyperechoic zones correspond to inclusions of air bronchograms (arrowheads), heterogeneous hyperechoic zone with comet-tail artifacts (asterisk), lower parynchial hepatization (white arrow) (left side)

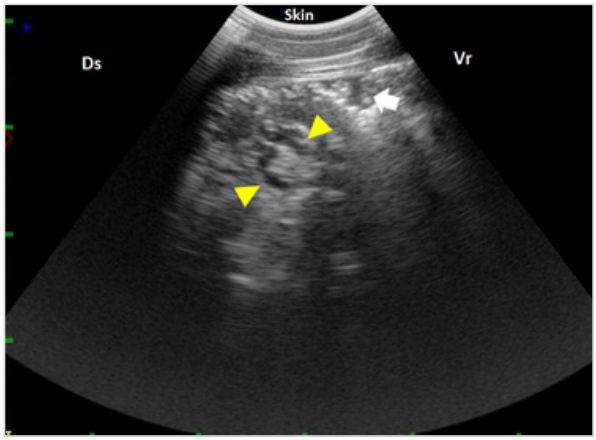

Figure 8 Extensive subpleural consolidation (white arrow); fluid bronchograms (yellow arrowheads) (right side)

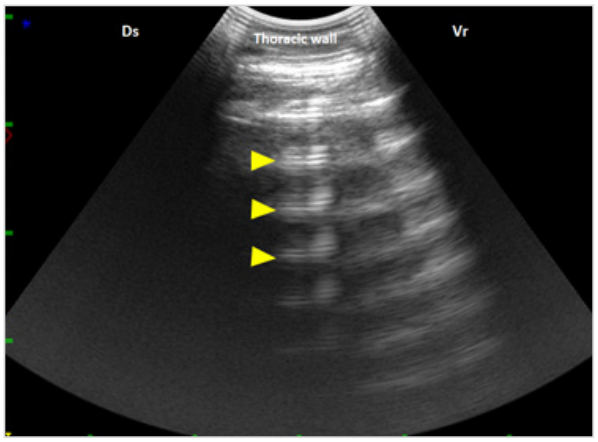

Figure 9 Emphysematous lung: multiple A-lines, rising from the pleura line and going deeper (arrowheads)

left lung, tympanum was dominant in the middle and lower parts. Dullness was limited to the craniodorsal region (Figure 6).

Sonography

Ultrasound examination of the left lung revealed signs of effusion between the pleural layers. The alveolograms appear as hyperechoic air inclusions, and parenchymal hepatization in the deep regions of the lung reveals a consolidation phenomenon (Figure 7). On the right side, examination of the area between the upper and middle thirds of the respiratory field at the level of the 7th ICS showed extensive consolidation, with anechoic liquid bronchograms (Figure 8). Beside, the multiple A-line artifacts observable, propagating from the subpleural lungs to distant parts of the image in Figure 9, are indicative of pulmonary emphysema.

Case 3

A 6-year-old crossbred cow presented with decreased appetite, recurrent coughing and bloating. The BCS of the cow was low (1.0). Rectal temperature was normal (38.9°C), as was the appearance of the mucous membranes. Heart rate and respiration were within normal limits. However, the respiratory type was markedly abdominal with mixed dyspnea, and a shortness of breath becoming more marked on the following days. Auscultation revealed dry crackles in the dorsal part of the right lung, but in the ventral part no sound was heard. In the dorsoventral parts of the left lung, bullous wheezes were heard over a

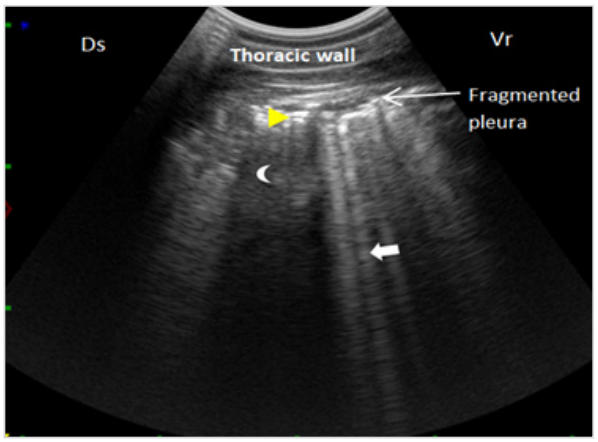

Figure 10 Fragmented pleura line with lung consolidation: hyperechoic zones correspond to inclusions of air bronchograms (arrowhead) with comet-tail artifacts (white arrow), lower parynchial hepatization (cressent)

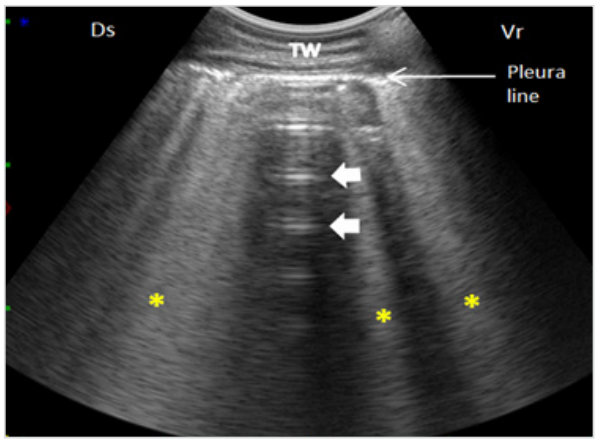

Figure 11 Emphysematous lung: multiple B-lines (asterisks) and A-lines (arrows), rising from the pleura line and going deeper on screen

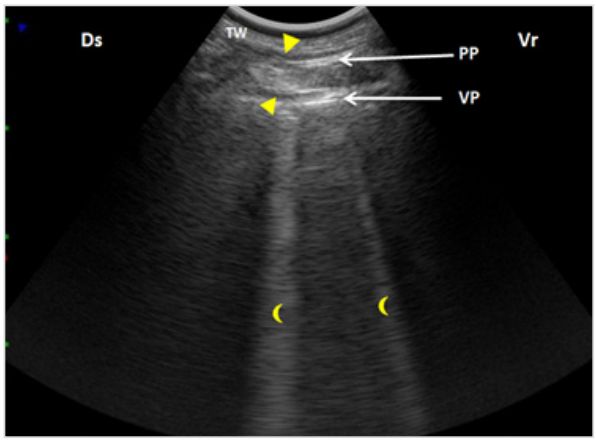

Figure 12 Thickened pleura (arrowhead) PP: parietal pleura, VP: visceral pleura. Comet-tail artifacts (crescent)

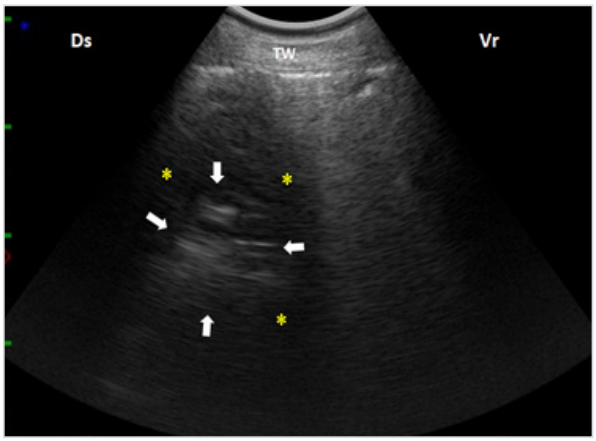

Figure 13 A large abscess pointed by white arrows, surrounded by anechoic liquid collection (asterisks) in the pulmonary parenchyma

wide area. Percussion had revealed no pathological sound in the dorsal areas of the right side, but the tympanum was clear in the cranio-ventral part of the thorax, with a characteristic ping resonance. On the left side, percussion revealed dullness in the dorsal area and a slight tympanum in the middle of the thorax.

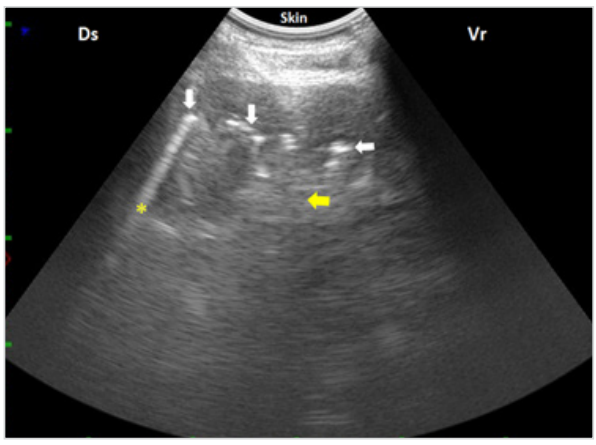

Figure 14 Echogenic structures, granular in shape (white arrows), comet-tail artifact (asterisk), and consolidation (yellow arrow), on the right side

Sonography

In Figure 10, the ultrasound image shows wide consolidation in the right caudodorsal region (8th ICS). The pleural line appears fragmented, with hyperechoic subpleural bronchograms accompanied by CTAs. The sonogram taken ventrally over the 6th and 7th ICS of the right lung (Figure 11) showed both B-lines and A-lines, propagating from the subpleural lungs to distant parts of the image with a sliding lung, clearly indicating pulmonary emphysema.

Case 4

A 5-year-old red Holstein cow presented with anorexia, coughing, and polypnea. The cow's BCS was poor (1.5) and clinical examination revealed a fever of 39.9°C, polypnea, and mixed dyspnea with head tense. Palpation of the external lymph nodes revealed significant hypertrophy of the prescapular and retropharyngeal.

Sonography

The left lung ultrasound showed pleural thickening with CTAs (Figure 12). Sononography had also revealed a large subpleural abscess with a hypoechoic center, surrounded by an anechoic collection (Figure 13). On the right side, we

observed pleural thickening with subpleural hyperechoic granules, which may be tuberculosis (TB) micronodules (Figure 14). Besides, ultrasound of the liver also showed the existence of nodular structures.

DISCUSSION AND CONCLUSION

Acute respiratory distress syndrome (ARDS) is a condition observed in humans and animals. This syndrome is characterized by pulmonary hypertension responsible for intravascular neutrophil aggregation, diffuse alveolar lesions, permeability edema, and acute respiratory distress (López and Martinson, 2017; Soldari et al, 2019). In veterinary medicine, acute bovine pulmonary edema and emphysema (ABPEE) is a pathophysiological condition (Muhammad et al., 2010; Hananeh and Ismail, 2018) comparable to ARDS. This syndrome is better known as acute interstitial pneumonia (AIP), which is a sporadic and often fatal respiratory condition in feedlot cattle (Woolums, 2015; Haydock et al., 2022; Bortoluzzi et al., 2023). Interstitial pneumonia (IP) is characterized by the acute onset of severe respiratory distress and a combination of pulmonary lesions, including pulmonary edema and congestion, interstitial emphysema, and alveolar epithelialization (Arnold and Lehmkuhler, 2015). On ultrasound, the respiratory distress syndrome has compact, extensive B-lines, subpleural consolidations, and a thickened, irregular pleural line (Liu et al., 2023a). CTAs seen in AIP may result from unventilated areas created by small accumulations of exudate, blood, mucus, and edema (Tharwat and Oikawa, 2011). In interstitial pneumonia, CTAs or BLAs are long, well-defined, laser-like hyperechoic artifacts originating from the pleural line (Hussein et al., 2018; Manolescu et al., 2018). Soldati et al. (2019) have, therefore, described the ultrasound appearance of IP as a mixture of spared (normal) lungs and areas with diffuse fan-shaped vertical artifacts originating from the lung-wall interface, known as the "white lung pattern". These reports are in perfect agreement with our observations in Figures 3 and 4. In view of our sonograms, IP appears to be primary and acute, given the absence of advanced and diffuse consolidation. In general, IP is characterized by diffuse lesions affecting all lung lobes; but, sometimes they are more pronounced in the caudodorsal part of the lung (Woolums 2015; López and Martinson, 2017), as we have seen. That said, primary or chronic bronchopneumonia (CBP) can lead to secondary AIP (Woolums, 2015; Haydock et al., 2022). In such cases, large areas of consolidation with alveolograms and bronchograms (Copetti et al., 2008; Carvallo and Stevenson, 2022) as well as the possible presence of abscesses (Panciera and Confer, 2010) will help to recognize the origin. Sometimes, bronchopneumonia with interstitial pneumonia is considered a variant of AIP rather than a separate disease (Haydock et al., 2023). However, there was no evidence of emphysema on clinical and ultrasound examination. Indeed, ultrasound cannot always distinguish edema from emphysema because of their common features, notably the presence of BLAs or CTAs in both cases (Buczinski et al., 2014; El-Zahar et al., 2021). Furthermore, diffuse BLA can be associated not only with edema but also with lung hemorrhage (Woolums, 2015).

On the other hand, AIP primarily affects the left side of the heart, leading to left heart failure (Hananeh and Ismail, 2018); that can lead to confusion with primary left congestive heart failure or pericarditis. What is more, differentiating cardiogenic or hydrostatic pulmonary edema from AIP is a real challenge, particularly in the early stages of the disease (Soldati et al., 2019). In cardiogenic pulmonary edema (CPE), the ultrasound image shows no intermittent white areas with spared zones; also, alveolar collapse and air bronchograms are absent. But in late-onset CPE, after alveolar flooding has occurred, the ultrasound image will be confused with that of early-onset AIP, often showing spared areas (Soldati et al., 2019). In addition, diffuse interstitial diseases may be associated with CPE, adding further uncertainty to the diagnosis (Soldati et al., 2019). In the case of inhalation pneumonia, the ultrasound will show a pleura separated by a large

anechoic zone containing multiple hyperechoic dots due to abscesses or pyothorax (Scott, 2012). Finally, Table 1 presents the ultrasound signs that guide differential diagnosis with AIP.

Bronchopneumonia describes both suppurative and fibrinous consolidation of the lungs (López and Martinson, 2017). In chronic pneumonia, diffuse alveolar damage defines the fibrosing stage with alveolar collapse, basement membrane fusion, and fibroblast proliferation (Carvallo and Stevenson, 2022). Consolidation often affects the posterior fields, particularly the bases, with an evidence of static or dynamic bronchograms (Copetti et al., 2008). In our case study, consolidation was extensive, with air bronchograms (Figure 7) suggesting complete lobar consolidation. Pulmonary consolidation in animals was reported to occur, most commonly, cranioventrally with bilateral distribution (López and Martinson, 2017). This fact is explained by inhalation as a possible cause of the pulmonary infectious pathway (Bayoumi et al., 2022). However, Tharwat and Al-Sobayil (2017) speak rather of a predominantly caudodorsal consolidation in the right lung, suggesting a hematogenous infection. So, several authors have agreed that consolidation of the entire lung lobe is associated with bacterial BP (Ollivett and Buczinski, 2016; Hoffelner et al., 2023). On LUS, consolidation shows a relatively hypoechoic heterogeneous echotexture (Berman et al., 2019), which describes the filling of bronchioles and alveoli with fluid, pus, blood, or cells (Grune, 2020). Consolidations due to pneumonia may also contain multiple lens-sized hyperechoic spots due to air trapped in the small airways, with associated CTAs (Nazerian et al., 2015). Bronchograms can accompany consolidations, appearing hyperechoic when aerated and rather as hypoechoic structures on lung ultrasound if fluid-filled (Grune, 2020). When structures are echogenic, consolidation appears isoechoic with the liver; this is known as pulmonary "hepatization" (Copetti et al., 2008).

The descriptions of consolidation processes given above by Nazerian et al. (2015), Grune (2020), and Copetti et al. (2008) correspond perfectly to what we have observed in Figures 7 and 8. In cases of CBP, consolidation has a more lumpy distribution; there is more obvious purulent bronchitis, bronchiectasis, and abscess formation (Panciera and Confer, 2010; Scott, 2013). In both animals and humans, consolidation is due to densification of lung tissue and leads to complete deaeration (Laursen et al., 2021); a wide consolidation is, therefore, linked to poor outcomes (Berman et al., 2019). However, early administration of antibiotics and anti-inflammatory drugs may reduce subsequent pathological effects (Fiore et al., 2022). In addition, physical examination revealed emphysema due to decreased intensity of breath sounds with tympany, as well as expiratory dyspnea seen during inspection. In Figure 9, emphysema appears as "multiple A-lines" due to the increased amount of subpleural air, as illustrated by Manolescu et al. (2018). In CBP, alveolar or interstitial emphysema occurs as a result of airflow obstruction and air penetration into deeply damaged lung parenchyma (Flock, 2004; López and Martinson, 2017). These serious alterations of the respiratory system lead to a severe lack of oxygen for the cow's metabolism, particularly in hot summer weather, thus resulting in hyperventilation, and mouth breathing. Lastly, CBP sonographic diagnosis is difficult to differentiate from other chronic lung diseases (Table 1), particularly when lesions are multiple and extensive. The consolidation phenomenon itself can be a factor of diagnostic uncertainty, as it may be due to infection, inflammation, granuloma formation, or neoplasia (Ali et al., 2014).

In cattle, primary pleural disorders are rare, and pleuropneumonia often results from subsequent bronchopneumonia (Braun et al 1997 ; Robcis et al., 2023). Clinically, the Case 3 presented with dyspnea, but no pleural friction rub on auscultation. However, ultrasound had shown a fragmented pleural line with subpleural consolidations, air bronchograms and CTAs (Figure 10), as seen by Kreuter and Mathis (2014) in pleurisy. But, a marked irregularity of the pleural line, with a thickened, fragmented, and/or blurred appearance, may also suggest an aggressive or advanced

stage of fibrotic disease (Rea et al., 2021). Also, pulmonary emphysema was diagnosed based on clinical examination as increased resonance of lung field upon percussion with silent lung sign in ausculttion; as well, the mixed dyspnea confirmed it. By sonography, pulmonary emphysema was characterized by numerous B-lines and A-lines (Figure 11), closely situated echo bands starting at the surface of the lung and running perpendicular to the pleura, which is in accordance with Manolescu et al. (2018) and Khalphallah et al. (2022) reports. Lung sliding, the movement between the visceral and parietal pleura during spontaneous respiration (Chen and Zhang, 2015), was present in this case, which helped to confirm the diagnosis of emphysema. The absence of this sign would have suggested the possibility of pneumothorax (Husain et al., 2012), in particular with the ping resonance detected on percussion of the ventral parts of the thorax.

Pulmonary tuberculosis may be revealed on ultrasound by consolidation, subpleural nodules, pleural thickening, fibrosis, and miliary lesions (Cocco et al., 2022). In their chronic phase, TB granulomas are necrotic and mineralized; they are irregularly shaped, multicentric, with prominent caseous necrosis and a thick capsule (Hunter et al., 2023). In our case, ultrasound showed thickening of pleural line (Figure 12). In addition, systemic tuberculosis infection may involve abscesses and chronic sinus formation, which are often secondary to pulmonary, pleural, or mediastinal tuberculous abscesses; they appear on the ultrasound image as hypoechoic areas with varying degrees of internal heterogeneity (Rea et al., 2021). In the present case, the abscess appears rather heterogeneous and hypoechoic in the center, surrounded by an anechogenic band; it was subpleural, which permitted its observation (Figure 13). When the pleura is in good condition and an abscess is located deep in the lung, it cannot be detected by ultrasound (Flock, 2004). As well, in pulmonary TB, the most common parenchymal findings on LUS are subpleural nodules and pulmonary consolidations (Rea et al., 2021), as shown in Figure 14. In addition to pleural and pulmonary abnormalities, LUS also revealed liver lesions. In endemic regions, TB diagnosis often relies on the detection of extra-pulmonary lesions through ultrasound (Hunter et al., 2016). In addition, the presence of fever and lymph node reactions are further signs in favor of the diagnosis of disease. Tuberculosis is, therefore, a neglected disease in our country, and screening is not automatic. This implies great vigilance on the clinician's part in examining cattle suspected of having tuberculosis, given the risk of contamination through direct contact or inhalation (Monde et al., 2023; Toribio et al., 2023).

In terms of differential diagnosis (Table 1), primary thoracic neoplasms should be considered, despite their rarity in adult cattle, when respiratory clinical signs and cachexia are concomitant (Robcis et al., 2023). Thus, adenocarcinoma is the most frequently reported lung neoplasm in cattle (Neto et al., 2019). LUS constitutes an excellent approach to the differential diagnosis of infectious lung diseases, as it can highlight specific lesions (Giannelli et al., 2022).

Table 1 Sonographic signs of the diagnosed respiratory conditions, with respective differential diagnoses, compared with bibliographic reports

Suspected disease and sonographic findings	Bibliographic reports	Conditions involving a differential diagnosis and sonographic signs	
Acute interstitial pneumonia : - Thickened pleura - Subplaural consolidation - Fluid alveolograms - Numerous CTAs (white lung)	- Coalescent B-lines and white lung - Irregular pleural line and reduced pleural sliding - Consolidations, subpleural nodules, or micronodules (Soldati and Demi, 2017) - Focal, patchy, or diffuse fan-shaped vertical comet-tail artifacts (interstitial syndrome) (Soldati et al., 2019) - Superficial fluid alveolograms (Tharwat and Oikawa, 2011).	Cardiogenic pulmonary edema	- No consolidations, no pleural nodules or pleural irregularities (Soldati and Demi, 2017) - Uniform distribution of comet-tail artifacts - Normal lung sliding - Homogenous pleural effusion (Grune et al., 2020)
		Pulmonary hemorrhages	- Diffuse B-line artifacts (Woolums, 2015) - Pleural effusion - Fibrin deposition sign (Liu et al., 2023b)
		Emphysema	- Diffuse comet-taila rtifacts (Buczinski et al., 2014; El-Zahar et al., 2021) - Multiple A-lines due to the increase in the amount of subpleural air (Manolescu et al., 2018)
		Inhalation pneumonia	-Separate, anechoic subpleural zone - Multiple hyperechoic patches (abscess/pyothorax) (Scott, 2012)
		Embolic pneumonia	- Consolidation - Hyperechoic nodules (Kim et al., 2023)

Bronchopneumonia and pleuropneumonia - Pleura effusion - Fragmented pleura - Cranioventralbilateral consolidation - Alveolograms (hyperechoic) - Bronchograms (hypoechoic) - Hepatization - Multiple B-lines (emphysema sign).	- Cranioventral bilateral consolidation (López and Martinson, 2017; Bayoumi et al., 2022) - Hypoechoic heterogeneous echotexture (Berman et al., 2019) - Lens-sized hyperechoic spots with B-lines artifacts (Nazerian et al., 2015) - Air bronchograms and hepatization (Copetti et al., 2008) - Abscess (Panciera and Confer, 2010)	Fibrinous pleurisy / fibrinous bronchopneumonia (shipping fever)	- Interruptedvisceral pleura - Pleural effusion with accumulation of fibrin: hyperechoic fibrin matrix and anechoic background (Scott and Sargison, 2012; Hussein et al., 2018) - Pleural and lung abscesses: uniform hypoechoic to anechoic area containing many hyperechoic spots representing gas echoes within the abscess (Scott and Sargison, 2012) - Absence of lung sliding with pleural adhesions (Husain et al., 2012)
		Millarytuberculosis	- Consolidation, subpleural nodules, fibrosis (Cocco et al., 2022)
		Lung fibrosis	- Numerous B-lines (Manolescu et al., 2018) - Consolidation with subpleural hypoechoic nodules (Yan et al., 2021)
		Chronic Interstitial Pneumonia	- Cranioventral consolidation (Doster, 2010) - Comparable features to chronic bronchopneumonia (Carvallo and Stevenson, 2022; Haydock et al., 2022)
		Pneumothorax	- Numerous B-lines - Absence of lung sliding (Husain et al., 2012; Chen and Zhang, 2015)

Tuberculosis : - Thickened pleura. - Consolidation. - Subpleural micronodules. - Subpleuralabscess.	- Pleural thickening, consolidation, subpleural nodules, fibrosis (Cocco et al., 2022). - Abscesses and chronic sinus with varying degrees of internal heterogeneity (Rea et al., 2021). - Associated extra-pulmonary lesions (Hunter et al., 2016).	Lung fibrosis	- Thickened and irregularly fragmented pleural line (Manolescu et al., 2018; Yan et al., 2021) - Consolidation with subpleural hypoechoic nodules (Yan et al., 2021) - Numerous B-lines (bronchiectasis, fibrotic alveolar syndrome) (Manolescu et al., 2018; Yan et al., 2021) - Eventual absence of lung sliding (Husain et al., 2012)
		Subacute and chronic forms of AIP	- Immature granulation tissue - Mature fibrosis (Haydock et al., 2022)
		Primarypulmona-ryneoplasms	- Lung metastasis appearing as large hypoechoic area bordered by a hyperechoic line (Scott and Sargison, 2012) - Heterogeneous parynchyma with hypoechoic areas of variable size - Homogeneous nodules, uncapsulated and hypoechoic - No B-line artifacts (Neto et al., 2019)

In summary, all the cases monitored in this clinical study presented with anorexia, poor body score, and respiratory conditions. Only one case had a fever, and this was a case of tuberculosis. In non-infectious or chronic lung diseases, body temperature generally remains within the normal range. These clinical and sonographic examinations indicate that auscultation is an essential tool in physical examination for the diagnosis of respiratory diseases. Percussion is another mean of examining the lung field that holds equal importance. Percussion offers many advantages, as it can confirm the results of auscultation. In addition to revealing chest pain, percussion provides precise indications of fluid or air infiltration in the lung parenchyma and thorax, as well as changes in the lung parynchyma. In this study, LUS had confirmed some of the physical examination findings and gave us a clear indication of the extent of the lung lesions, which enabled us to make accurate diagnoses and prognoses. Finally, this clinical study shows that examination of the respiratory tract in cattle using a portable ultrasound scanner is a practical and effective method that can be integrated into the routine of the bovine clinic.

In clinical practice, both percussion and auscultation have certain limitations when it comes to identifying the type of lesion, stage and extent of respiratory condition. The limitations of LUS are related to the non-perception of lesions under a healthy pleura on the one hand and to B-lines, which can indicate several types of pathological phenomena, on the other. Furthermore, LUS is a method that depends on the operator's experience, and results may therefore be biased.

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CONFLICT OF INTEREST

The author declares that he has no conflict of interest.

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FIZIKALNI PREGLED I SONOGRAFIJA U DIJAGNOSTICIRANJU TEŠKIH RESPIRATORNIH STANJA KOD STOKE: PRIKAZ SLUČAJA

SAŽETAK

Auskultacija i perkusija predstavljaju komplementarne i osnovne metode kod veterinarskog fizikalnog pregleda respiratornog sistema, ali nisu učinkovite u procjeni prirode i opsega plućne patologije. U našem kliničkom istraživanju smo prikazali četiri slučaja teške bovine respiratorne bolesti. Obavljen je opći klinički pregled počevši od inspekcije, mjerenja tjelesne temperature i pregleda mukoznih membrana. Ključne metode fizikalnog pregleda su bile auskultacija i perkusija. Nakon toga je izvršen ultrazvučni pregled cijelog plućnog polja s obje strane grudnog koša. Rezultati su pokazali da je sonografija pluća (LUS) zajedno sa fizikalnim pregledom omogućila postavljanje dosta precizne dijagnoze i prognoze kod odraslih krava sa plućnim oboljenjima. U diskusiji smo pružili pregled određenih koncepata sonografije pluća i njihov odnos prema običnom kliničkom pregledu.

Ključne riječi: Auskultacija, perkusija, plućna bolest, sonografija pluća, stoka

PROFESSIONAL PAPER

ULOGA I PRIMJENA 3R PRINCIPA U DOBROBITI ŽIVOTINJA TOKOM EKSPERIMENTALNIH ISTRAŽIVANJA

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SAŽETAK

Korištenje životinja u eksperimentima datira od najranijih historijskih razdoblja, a otkriće anestetika omogućilo je ublažavanje njihove patnje. Pojam dobrobit životinja podrazumijeva osiguravanje odgovarajućih uvjeta za smještaj, njegu, ishranu, zdravlje i ponašanje životinja, uz sprečavanje nanošenja nepotrebne boli, patnje i straha te zaštitu od bolesti i povreda. Cilj rada je predstaviti značaj primjene 3R principa, kao i njihove benefite koji doprinose dobrobiti životinja tokom eksperimentalnih studija. Bioetičari, Russell i Burch su sredinom 20. vijeka razvili 3R princip (zamjena, redukcija, poboljšanje), koji je postao temelj etički prihvatljivih istraživanja. S vremenom je nastao proširenu skup principa poznat kao 11R ili 12R, koji uključuju dodatne faktore koji predstavljaju sveobuhvatan pristup istraživanjima, usmjerenim na osiguravanje reproduktivnosti rezultata, relevantnosti istraživanja i prenosivosti podataka, uz poštivanje etičkih standarda. Neosporno je da je korištenje eksperimentalnih životinja danas, nažalost, još uvijek neizbježno, stoga primjena svih dostupnih mehanizama u cilju zaštite njihove dobrobiti pruža, između ostalog, i mogućnost razvoja pozitivnog dijaloga u smjeru šireg društvenog prihvaćanja još uvijek nužne primjene eksperimentalnih životinja, posebno u biomedicinskim istraživanjima.

Ključne riječi: 3R principi, dobrobit, eksperimentalne životinje

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UVOD

Posmatrano kroz historijski kontekst, istraživanja na životinjama kao eksperimentalnim modelima potječu još iz vremena Aristotela (384.-322. g. p.n.e.), Erasistratusa (304.-250. g. p.n.e.), Galena (129.-209. g. p.n.e.) pa sve do danas. Osnivač savremene eksperimentalne fiziologije Claude Bernard (1813.-1878.), jedan je od naučnika koji je zagovarao istraživanja na životinjama, smatrajući ih opravdanim, a djelovanje određenih komponenti na životinjski i ljudski organizam vrlo sličnim (Hajar, 2011; Franco, 2013). Životinje su korištene u cilju razumijevanja građe i funkcije organizma kako životinjskog, tako i ljudskog te su se na njima ispitivale učinkovitosti određenih preparata i zahvata kako bi se rezultati, odnosno iskustva, mogli primijeniti na ljudima u svrhu liječenja (Hajar, 2011; Gregurić Gračner i sar, 2019). Upotreba laboratorijskih životinja postaje s vremenom veoma značajna u biomedicinskim istraživanjima.

Posljednjih desetak godina, korištenje laboratorijskih životinja u naučnim istraživanjima jedno je od najčešće problematiziranih etičkih pitanja, kako u naučnim krugovima, tako i u javnosti. U današnje vrijeme, dosta se raspravlja o zabrani korištenja životinja kao modela u eksperimentima, iako su takva istraživanja neminovno doprinijela razvoju medicine (Franco, 2013; McLeod i Hartley, 2018).

U radovima na temu istraživanja i provođenja eksperimenata na životinjama uvijek se problematizira dobrobit. Ne postoji jedinstvena definicija dobrobiti koja bi bila primjenjiva na sve životinjske vrste. Dobrobit životinja je kompleksno pitanje koje ima svoju naučnu, etičku, ekonomsku, kulturnu, socijalnu, religijsku i političku dimenziju. S druge strane, pojam „dobrobit životinja“ se još definira kao stanje fizičkog i psihičkog zdravlja, koje omogućava uspješno prilagođavanje okolini. Za životinje jedan od najznačajnijih spoljašnjih (ekto) faktora je čovjek (Brown, 2013). Za laboratorijske životinje, dobrobit se definira kroz analizu njihovih bihevioralnih odgovora na eksperimentalne uvjete, gdje se pozitivna i negativna iskustva modeliraju i posmatraju unutar kontrolisanih laboratorijskih okvira, s ciljem razumijevanja prirode ponašanja u ovim specifičnim okolnostima (Baumans, 2005; Gregurić Gračner i sar, 2019).

3R principi: zamjena, smanjenje i poboljšanje (engl. *replacement, reduction* and *refinement*) međunarodno je priznat pristup smanjenju upotrebe životinja u istraživanjima u okviru kojeg se, kad god je to moguće, zahtijeva primjenu alternativnih metoda i/ili unapređenje metoda, kako bi se smanjila nelagoda životinja korištenih u eksperimentima.

Cilj rada je predstaviti značaj primjene 3R principa, kao i njihove benefite koji doprinose dobrobiti životinja tokom eksperimentalnih studija.

Početak korištenja 3R principa

Korištenje životinja u eksperimentima datira iz najranijih historijskih perioda, a otkrićem anestetika stvorili su se uvjeti za ublažavanje njihove patnje. Prvi zakon koji je ograničio okrutnost prema životinjama u naučnim istraživanjima je usvojen 1876. godine u Ujedinjenom Kraljevstvu. Bioetičari, William Russell i Rex Burch su sredinom 20. vijeka objavili revolucionarno djelo u kojem su utemeljili pristup prema humanijem korištenju laboratorijskih životinja, što je rezultiralo formiranjem tzv. 3R principa (zamjena, redukcija i poboljšanje), koji je postao ključni okvir za etička opravdana istraživanja (Gregurić Gračner i sar, 2019; Katica i Delibegović, 2019).

Razvojem društva i nauke, koncept je globaliziran i danas se koristi i širi skup principa poznat kao 11R ili 12R, koji uključuje dodatne faktore poput pouzdanosti (engl. *reliability*), relevantnosti (engl. *relevance*), ponovljivosti (eng. *reproducibility*), oporavka (engl. *rehabilitation*), odgovornosti (engl. *responsibility*), poštovanja (engl. *respect*), izbjegavanja nepotrebnih ponavljanja (engl. *redundancy avoidance*) i zakonitost postupaka (engl. *regulation*). Unutar Evropske unije, navedena načela su formalizirana Direktivom 2010/63/EU, koja je omogućila usklađivanje naučnih praksi s etičkim standardima. Baselska deklaracija iz 2010.

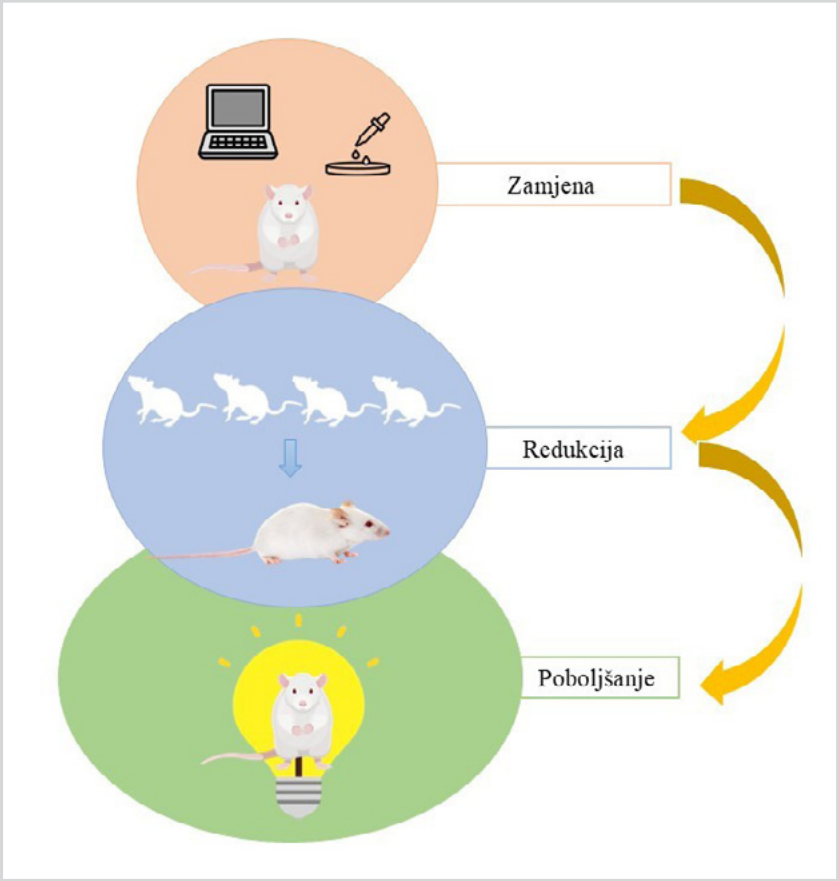
godine je dodatno ojačala napore u promovisanju ovih principa unutar naučne zajednice, sa ciljem uspostavljanja konstruktivnog dijaloga između istraživača i šire javnosti (Gregurić i sar, 2019).

Prvi akt na svijetu koji se odnosio na postupke sa laboratorijskim životinjama je donesen 1878. godine u Britanskom parlamentu pod nazivom „Akt o oktrutnosti prema životinjama“, kojim se zabranjuje neadekvatan tretman i okrutnost prema životinjama, kao što je to slučaj pri obavljanju bolnih eksperimenata, disekcije živih, svjesnih životinja i vivisekcije. Također, ovim aktom je naloženo posjedovanje relevantne licence za izvođenje eksperimenata od strane ministra unutrašnjih poslova. Uvjeti koji su bili neophodni za posjedovanje licence su bili iznimno strogi i kao takvi su se zadržali do danas (Uvarov, 1985).

Drugi akt, 1986. godine je regulirao korištenje laboratorijskih životinja i izvorno bio usklađen sa Direktivom EU 86/609/EEC koja je 2010. godine nadopunjena i zamijenjena Direktivom 2010/63/EU (Anonymous, 1986; Anonymous, 2010). U Sjedinjenim Američkim Državama akt sličan britanskom je donesen 1966. godine, pod nazivom „Akt o dobrobiti laboratorijskih životinja“, ali ovim aktom nisu bile obuhvaćene ptice, stahori i miševi (Gregurić Gračner i sar, 2019).

3R principi u eksperimentima na životinjama bazirani su na tri koncepta: zamjena, smanjenje i poboljšanje. Tokom faze planiranja i izvođenja *in vivo* eksperimenata, istraživač bi trebao slijediti ove principe, nastojeći zamijeniti, gdje je moguće, životinjski model alternativnim modelom, smanjiti što je više moguće broj životinja u eksperimentu te unaprijediti eksperimentalne uvjete (Slika 1), (Russel i Burch, 1959).

Nakon primjene osnovnih 3R principa u *in vivo* eksperimentima, važno je proširiti etički okvir kroz dodatne smjernice koje osiguravaju potpuno naučnu i društvenu odgovornost, pri čemu se aktuelizira

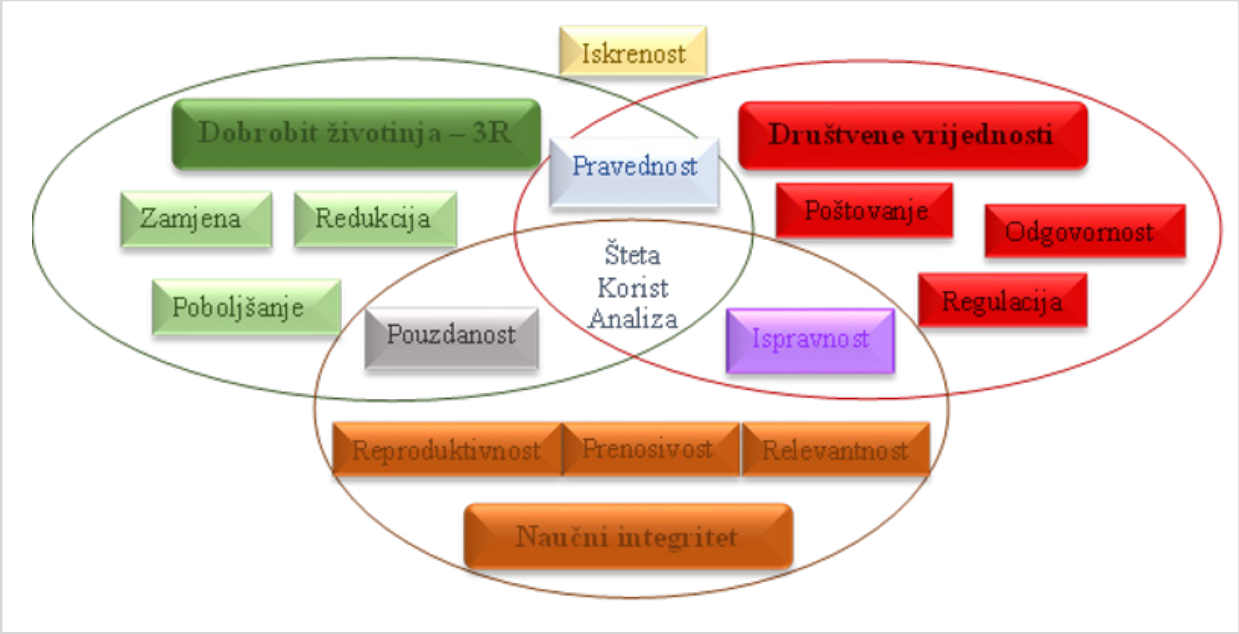


Slika 1

3R principi eksperimenata na životinjama

Figure 1

3R Principles of Animal Experimentation



Slika 2 Modificirani shematski prikaz 12R principa (Brink i Lewis, 2023)

Figure 2 Modified scheme of 12R Principles (Brink and Lewis, 2023)

12R princip, koji obuhvata mnogo više ključnih elemenata esencijalnih za korektno provođenje i realizaciju naučno-istraživačkog rada. Ovi principi predstavljaju sveobuhvatan pristup istraživanjima, usmjerenim na osiguravanje reproduktivnosti rezultata, relevantnosti istraživanja i prenosivosti podataka, uz poštivanje etičkih standarda (Slika 2) (Brink i Lewis, 2023).

Naučni integritet uključuje primjenu principa reproduktivnosti (engl. *reproducibility*), relevantnosti (engl. *relevance*) i prenosivosti (engl. *transferability*) u cilju dobivanja adekvatnih rezultata istraživanja. Istraživački dizajn, adekvatne veličine uzorka i precizne statističke analize su ključni za postizanje pouzdanih rezultata, dok transparentno izvještavanje i distribucija podataka povećavaju reproduktivnost, smanjujući nepotrebnu potrošnju resursa. Dobrobit životinja fokusirana je na već spomenute 3R principe – zamjena, redukcija i poboljšanje, dok se društvene vrijednosti odnose na odgovornost istraživača prema zajednici, poštivanje zakona, te svijest o kulturnim i etičkim aspektima eksperimenata, uključujući moralnu i zakonsku obavezu zaštite životinja i društvene odgovornosti u naučnom procesu. Preklapanjem ovih domena stvaraju se dodatni principi: ispravnost (engl. *righteousness* - etičko postupanje prema životinjama i društvu), pouzdanost (engl. *reliability* - generiranje tačnih podataka kroz integraciju naučnog integriteta i dobrobit životinja) i odgovornost (engl. *reckoning* - odgovorno planiranje i izvođenje studija uz poštivanje etičkih i zakonskim smjernica). 12R principi pružaju balans između naučnih potreba, etičkih obaveza i društvene odgovornosti, omogućavajući održiv i odgovoran pristup istraživanju na životinjama (Brink i Lewis, 2023).

Dobrobit životinja

Pojam ″dobrobit životinja″ se sve češće koristi, ali njegovo značenje varira zavisno od konteksta i tumačenja. Dobrobit životinja podrazumijeva osiguravanje odgovarajućih uvjeta za smještaj, njegu, ishranu, zdravlje i ponašanje životinja, uz sprečavanje nanošenja nepotrebne boli, patnje i straha, te zaštitu od bolesti i povreda

(Sl. glasnik BiH, 25/09). Veterinari i farmeri su se fokusirali na fizičko stanje i okoliš (smještaj) životinja, smatrajući da je zdravlje znak njihove dobrobiti, dok su naučnici koristili fiziološke parametre (nivoi hormona stresa i srčanog ritma) kako bi procijenili koliko se životinje uspješno prilagođavaju na njihovo okruženje. Međutim, ovaj pristup je ograničen jer ne obuhvata mentalno stanje životinja. Savremeni pristupi dobrobiti uključuju i emocionalna stanje životinja, budući da većina smatra da životinje osjećaju strah i frustraciju. Postoje teorije koje sugeriraju da je dobrobit životinja praktično direktno povezana sa njihovim osjećanjima. Najprihvaćenija definicija danas uključuje kombinaciju fizičkog zdravlja, emocionalnog stanja i mogućnosti zadovoljavanja prirodnih potreba, jer su svi ovi aspekti međusobno povezani i utječu na cjelokupnu dobrobit životinje (Hewson, 2003). Postoji pet (5) osnovnih prava koja čine temelje dobrobiti životinja, a svaki od njih je usmjeren na osiguranje zdravlja i blagostanja životinja:

Prva je sloboda od gladi i žeđi, što podrazumijeva osiguravanje pristupa adekvatnoj hrani i vodi, kako bi se zadovoljile osnovne nutritivne potrebe životinje.

Druga je sloboda od nelagode, koja se odnosi na pružanje odgovarajućih uvjeta života (sklonište, prostrano okruženje).

Treća je sloboda od boli, povreda i bolesti, što uključuje veterinarsku njegu kako bi se spriječila i liječila svaka patnja, povreda ili bolest.

Četvrta, sloboda izražavanja normalnog ponašanja, koja podrazumijeva omogućavanje prostora i samim time prirodno ponašanje i interakciju sa drugim životinjama.

Peta, sloboda od straha i stresa, koja se odnosi na zaštitu životinja od uznemirujućih situacija koje mogu izazvati strah, stres ili anksioznost, čime se osigurava mentalno blagostanje (Mellor, 2016).

Navedenih pet (5) sloboda može se nazvati i pet (5) prava svih životinja, što bi moglo biti poveznica i zajednički stav, između dva gotovo sukobljena tabora:

1. Dobrobit životinja,
2. Životinjska prava.

U Bosni i Hercegovini postoji nekoliko pravnih akata koji definiraju postupanje sa životinjama, njihovu dobrobit, kao i korištenje eksperimentalnih životinja u istraživanjima. Krovni zakon u ovoj oblasti je Zakon o zaštiti i dobrobiti životinja iz 2009. godine (Sl. glasnik BiH, 25/09). GLAVA X – Zaštita životinja za eksperimente i druga naučna istraživanja član 31-34, kojom se uređuje zaštita životinja od mučenja, zanemarivanja i neprimjerenog postupanja, a nadležna tijela za provedbu zakona su Ministarstvo vanjske trgovine i ekonomskih odnosa i Ured za veterinarstvo BiH. Uz ovaj zakon postoje i brojni pravilnici koji detaljnije uređuju specifične oblasti, npr. Pravilnik o načinu vođenja evidencije o eksperimentalnim životinjama (Sl. glasnik BiH, 19/10). Ovi propisi pružaju pravni okvir za osiguravanje dobrobiti životinja.

Zamjena (engl. *replacement*)

Većina naučnih i medicinskih istraživanja uključuje eksperimentalno korištenje životinja, koje itekako predstavlja važan aspekt u određenim oblastima, iako bi u idealnom svijetu trebale postojati alternative (Robinson, 2005). Iako još uvijek ne postoje sredstva za zamjenu svih oblika upotrebe životinja bez usporavanja naučnog napretka, zamjena je posebno relevantna za fundamentalna i primijenjena biomedicinska istraživanja, regulatorno testiranje i upotrebu životinja u edukaciji i obuci (Russel i Burch, 1959).

Koncept principa je baziran na mogućnosti zamjene životinjskog modela alternativnim (Russel i Burch, 1959). Zamjenske metode se uobičajeno predstavljaju kao adekvatna alternativa korištenju modela (eksperimenata) sa životinjama te nude niz prednosti, omogućavajući brži napredak, i u nekim slučajevima pružajući naučne uvide koji „nisu bili mogući korištenjem životinjskih modela“ (Rusell i Burch, 1959). Rusell i Burch su opisali niz alternativnih metoda, kao što su biljke, mikroorganizmi, biohemijski sistemi i neživi fizički modeli, te su napravili razliku

između relativnih i apsolutnih tehnika zamjene, koji su kasnije evoluirali u djelomičnu (relativnu) i potpunu zamjenu (apsolutnu) (Russel, 1957).

Djelimična zamjena podrazumijeva upotrebu druge vrste organizama sa relativno manje složenim nervnim sistemom, a za koje se smatra da nisu sposobne za doživljavanje patnje (NC3RS, n.d.), primjer su beskičmenjaci i nezreli oblici kičmenjaka, kao i primarne ćelije (i tkiva) uzeti od životinja koje su ubijene isključivo u tu svrhu.

Kod potpune zamjene, Buchanan-Smith je 2005. godine izjavio da „3R i dalje predstavljaju najbolji pristup alternativama“ i definirao koncept zamjene kao „skup procedura koje u potpunosti eliminiraju upotrebu nekih vrsta životinja“.

Zbog kontinuiranog istraživanja u različitim naučnim oblastima postoje brojne alternativne metode i korištenje zamjenskih modela čiji je prikaz dat u produžetku (Tabela 1).

Tabela 1 Alternativne zamjene za životinjske modele

Table 1 Alternative replacements for animal models

Zamjena	Opis	Referenca
Fizičko-hemijska svojstva	Korištenje pH i kapaciteta pufera za predviđanje potencijalnih ozbiljnih iritacija oka ili korozije; testovi reaktivnosti peptida za screening hemikalije koje potencijalno izazivaju senzibilizaciju kože; korištenje računarskih i matematičkih modela koji povezuju molekularnu strukturu sa specifičnim biološkim aktivnostima.	Lalko i sar., 2012; OECD, 2017
Neosjetilni organizmi	Korištenje bakterija, glista i voćnih mušica kao alternativnih organizama za testiranje bioloških efekata, uključujući *Caenorhabditis elegans* za testiranje toksičnosti i istraživanje procesa ćelijske smrti i razvoja nervog sistema, *Drosophila melanogaster* za razumijevanje određenih aspekata složenih poremećaja, poput Parkinsove bolesti.	Ames sar., 1973; Robinson, 2005; Leung i sar., 2008; Perrimon i sar., 2016
Nezreli oblici osjetilnih vrsta	Korištenje nezrelih oblika osjetilnih vrsta koje nisu sposobne osjetiti bol, patnju ili stres, poput riblje larve za procjenu akvatične toksičnosti.	Lilicrap i sar., 2016
Jednoćelijski organizmi	Korištenje jednoćelijskog kvasca za proučavanje poremećaja ćelijske diobe kod ćelija raka.	Robinson, 2005
Ex vivo i *in vivo* sistemi	Korištenje sistema, životinjskog ili ljudskog porijekla, na nivou organa, presjeka tkiva, kulture/suspenzije ćelija ili subćelijske komponente. Ovi sistemi mogu predstavljati apsolutne ili relativne zamjene. Napredni bioinžinjerski sistemi poput organoida, 3D skela i mikrofluidnih sistema za simulaciju ljudske fiziologije.	Marx i sar., 2016; Vulto i Joore, 2021; Taebnia i sar., 2023
Prikupljanje životinjskog materijala	Prikupljanje izmeta, dlake, sline i urina od životinja iz kojeg se može izdvojiti DNK, kao alternativa invazivnim metodama hvatanja i uzorkovanja.	Bischof i sar., 2020
Istraživanja na ljudima	Uz odgovarajuće etičke mjere zaštite (prikupljanje podataka tokom volonterskih studija, kliničkih ispitivanja, epidemioloških studija i sl.)	Smith i Richmond, 2024

Redukcija (engl. *reduction*)

Princip redukcije brojnosti životinja, kao dio 3R principa je izuzetno važan, a odnosi se na smanjenje broja eksperimentalnih jedinica korištenih u dizajnom eskperimenta kreiranom protokolu, a da pri tome redukcija nema utjecaja na kvalitet rezultata koji moraju ostati relevantni i vjerodostojni (Robinson, 2005; Smith i Richmond, 2024). Međutim, bitno je razmisliti o načinu smanjenja eksperimentalnih životinja, jer ovaj princip ne smije u startu utjecati na statističke pokazatelje. Ponekad se javlja dilema između smanjenja broja životinja i poboljšanja uvjeta (engl. *refinement*), npr. kako bi reducirali broj ženki u proizvodnji genetski modificiranih miševa uobičajena praksa je davanje hormona u cilju povećanja proizvodnje jajnih stanica ili tzv. superovulacija. Ali, superovulacija zahtijeva da svaka životinja primi intraperitonealnu injekciju u abdomen, što može izazvati bol, a konsekventno i stres. Zbog toga se redukcija ponekad smatra neadekvatnim ili nepodesnim principom (Robinson, 2005).

Redukcija se može postići korištenjem rezultata iz prethodnih provedenih sličnih studija ili pilot-studija, usvajanjem najučinkovitijeg eksperimentalnog dizajna i veličine uzorka prilagođene cilju, te metodologija i iskustavima koje koriste različite laboratorije ili istraživačke institucije. Postoje tri načina za realizaciju principa redukcije (Tabela 2) (de Boo i Hendriksen, 2005).

Tabela 2 Tri načina realizacije redukcije (de Boo i Hendriksen, 2005)

Table 2 Three types of reduction (de Boo i Hendriksen, 2005)

Načini redukcije	Opis
Intra-eksperimentalna	Smanjenje broja životinja unutar svakog pojedinačnog eksperimenta ili protokola. Poboljšanje statističkog dizajna, izvođenje pilot-studija i retrospektivna analiza podataka omogućavaju optimizaciju minimalnog broja životinja.
Supra-eksperimentalna	Širenje koncepta redukcije između istraživača kroz organizovane kurseve i sastanke o eksperimentalnim dizajnima i statističkim metodama kako bi se smanjila upotreba životinja u istraživačkim eksperimentima.
Ekstra-eksperimentalna	Redukcija na međunarodnom nivou kroz usklađivanje pravila o eksperimentisanju i razvoj novih strategija za istraživanje i testiranje, čime se smanjuje broj životinja potrebnih za naučne svrhe.

Korištenje prevelikog broja eksperimentalnih jedinica, osim što je neetično, dovodi do rasipanja resursa u finansijskom i vremenskom smislu. Istovremeno, korištenje nedovoljnog broja životinja može utjecati na rezultate statističke obrade podataka, što ponovo predstavlja rasipanje resursa (de Boo i Hendriksen, 2005; Verderio i sar., 2023).

Poboljšanje (engl. *refinement)*

Poboljšanje, kao dio 3R principa, ne podrazumijeva samo brigu o dobrobiti eksperimentalnih životinja, već i poboljšanje kvaliteta njihovog života, tokom svih procedura kojima su izložene u zatočeništvu (Russel i Burch, 1959). Buchanan-Smith (2005) je predložio novu definiciju poboljšanja, koja glasi: „bilo koji pristup koji izbjegava ili minimizira stvarnu ili potencijalnu bol, stres i druge nepovoljne efekte koje životinje doživljavaju u bilo kojem trenutku svog života i koji poboljšava njihovo

blagostanje“. Poboljšanje nije samo smanjenje broja nepovoljnih efekata ili broja korištenih životinja, već smanjenje ukupne kumulativne boli, patnje, stresa i trajne štete koja može biti nanesena životinjama koje se uzgajaju, drže i koriste u naučno-istraživačke svrhe (Smith i Richmond, 2024). Stres se najčešće manifestuje kroz poremećaje u ponašanju, kao što je očigledni bijeg, preference - povlačenje i izbjegavanje pristupa ili pak suprotno kroz pojavu agresivnosti. Pokazatelj poremećaja mogu biti promjene u kretanju, vokalizaciji, promjeni srčanog ritma, disfunkciji simpatičkog nervnog sistema i sl. (Meyer, 2015). U novoj Evropskoj direktivi 2010/63, kao faktor poboljšanja navodi se obogaćivanje i unapređenje faktora okoline (mikroambijenta), te je dokazan način da se životinjama omogući veća kontrola nad okruženjem i podstakne ispoljavanje ponašanja svojstvenih ekologiji i etologiji korištenih vrsta (Tabela 3) (Olsson i Dahlborn, 2002; Verderio i sar., 2023). Bitno je napomenuti da različite životinje imaju specifične modele ponašanja, npr. miševi vole praviti gnijezda i držati drvene grančice, također, stahori koji su nekada radi lakšeg održavanja držani na rešetkastim podovima, danas se drže u kavezima sa čvrstim podovima koji sadrže mjesta za skrivanje, naime u naučnim eksperimentima su vršili pritisak tijelom na vrata da bi dobili pristup kavezima sa čvrstim podom ili dodatnim prostorom. Samim time, nevjerovatno je da stahori podignu 83% svoje tjelesne težine kako bi dobili pristup takvom kavezu, što upućuje koliko su im takvi resursi važni (Robinson, 2005). Obezbjeđivanje olakšanja bola, iako spada u jedan od bitnijih elemenata poboljšanja, također je teško identificirati i zahtjevno za prepoznati kod određenih vrsta eksperimentalnih životinja. Glodari su u prirodnim uvjetima najčešće plijen, te patnju i bol često instiktivno prikrivaju. Naučnici na Univerzitetu u Newcastle-u su posmatrali i snimali stahore nakon provedenih operativnih zahvata kako bi identificirali specifično ponašanje boli, komparirajući ih s onima koji nisu prošli operativni zahvat.

Korištenjem ovakvog principa, identificirali su nekoliko prepoznatljivih znakova, kao što su pokreti istezanja slični mačjem istezanju leđa. Takvi znakovi kod stahora su nestajali nakon aplikacije analgetika (Robinson, 2005).

Tabela 3 Vrste poboljšanja (Verderio i sar., 2023)

Table 3 Types of improvement (Verderio et al., 2023)

Vrsta poboljšanja	Opis
Higijena	Okruženje treba biti slobodno od insekata i parazita, te lako za čišćenje i dezinfekciju. Osigurati prostor za odmor koji je opremljen prostirkom ili drugim mekanim, suhim i čistim materijalom. Površina po kojoj se životinje kreću trebaju biti suhe i čiste, kako ne bi došlo do klizanja i mogućih povreda nogu.
Okruženje	Temperature u prostorijama za smještaj trebaju biti prilagođene vrsti, starosti životinja i njihovoj gustoći naseljenosti, kako bi se izbjegle situacije toplotnog ili hladnog stresa. Potrebna je ventilacija prostora, redovno mjerenje kvaliteta zraka, kao i prisustvo kisika i nivoa ugljen-dioksida. Previsoka vlažnost može pogodovati razvoju amonijaka u prostorijama i kavezima, a preniska vlažnost uzrokuje patološka stanja kod glodara, kao što je ″ringtail″ i gubitak toplote.
Smještaj	Životinja treba biti u mogućnosti da organizira prostor prema svojim etološkim potrebama, zbog toga potrebna je opremljenost sa materijalom za izgradnju gnijezda, adekvatna prostirka, prečke i platforme. Pri neadekvatnim uvjetima smještaja javlja se ugroženost dobrobiti životinje, što posljedično može dovesti do psihofizičke nelagode, npr. razvoj atipičnog ponašanja, stereotipija ili prekomjerna agresija.

Vrsta poboljšanja	Opis
Senzorne stimulacije	Ukoliko eksperimentalni protokol ne dozvoljava interakciju sa pripadnicima iste vrste, neophodno je omogućiti indirektni socijalni kontakt (vizuelni, slušni i olfaktorni). Na ovaj način smanjuje se stres tokom eksperimentalnih postupaka i osigurava bolja interakcije između životinja i osoblja/istraživača.
Socijalizacija	Životinjama je potrebna socijalna interakcija sa pripadnicima iste vrste, usljed čega ih je potrebno držati u parovima ili grupama koje su stabilnije i harmoničnije, iako s druge strane to može uključivati negativne i stresne aspekte poput agresije dominantnih jedinki nad podređenim.

ZAKLJUČAK

3R načela, koja se svakodnevno i bez iznimke spominju kada se naglasak želi staviti na napore naučne zajednice koji se ulažu u osiguranje dobrobiti životinja u eksperimentu, razvojem nauke, tehnologije, ali i evolucijom svijesti kako naučnika, tako i šire društvene zajednice, svakodnevno se unapređuju, tako da danas već možemo govoriti i o 12R načelima. Međutim, upravo se naziv "3R načelo" koristi uvijek kada se u raspravi žele istaknuti aktivnosti u vezi s osiguranjem dobrobiti životinja u eksperimentu, gotovo kao priznanje naučnicima koji su postavili temelje nove primijenjene naučne discipline koja je trebala poboljšati postupke u radu s laboratorijskim životinjama, a ujedno i pridonijeti kvaliteti naučnog rada u kojem se te životinje koriste. Neosporno je da je korištenje eksperimentalnih životinja danas, nažalost, još uvijek neizbježno, stoga primjena svih dostupnih mehanizama u cilju zaštite njihove dobrobiti pruža, između ostalog i mogućnost razvoja pozitivnog dijaloga u smjeru šireg društvenog prihvaćanja još uvijek nužne primjene eksperimentalnih životinja, posebno u biomedicinskim istraživanjima.

Potreba za ispitivanjem na životinjama se kontinuirano povećava s razvojem humane i veterinarske medicine, te farmaceutske industrije pa je neophodno razvijati testove koji mogu pratiti i potrebe industrije i osiguranje od hazarda. Alternativni testovi često omogućuju preliminarno, brže i jeftinije ispitivanje u odnosu na *in vivo* testove koji su dugotrajniji i skuplji. Korištenje životinja u testiranjima se smanjuje u svim naučnim poljima zbog razvoja alternativnih metoda i veće svijesti istraživača i naučnika, ali i poštivanja pravila (poput 3R principa), legislative i zakona.

Apsolutna zamjena svih testova na životinjama alternativnim testovima je nemoguća zbog složenosti organskih sistema koji učestvuju u odgovoru na unešeni inzult, zbog potrebe za tačnim i pouzdanim rezultatima i zbog nemogućnosti primjene nekih alternativnih metoda. Ispitivanje na životinjama se smatra etički opravdanim ukoliko je procijenjena potencijalna korist rezultata istraživanja za ljude i životinje veća od procijenjene štete koja im se nanosi. Zakonska regulativa koja ograničava korištenje životinja za naučna istraživanja ima ulogu u regulaciji, ali i poticanju razvoja alternativnih metoda. Tako je na primjer zabrana testiranja kozmetičkih proizvoda na životinjama u Evropskoj uniji iz 2013. godine imala za rezultat nagli porast razvoja alternativnih testova za ispitivanje dermalne toksičnosti.

Postojeće zakonodavstvo je, zbog vrlo velike zabrinutosti javnosti o opravdanosti korištenja životinja u naučne svrhe, usmjereno na potpunu zamjenu životinja u eksperimentima drugim metodama koje ne uključuju žive životinje, kad to postane naučno izvodivo. Međutim, korištenje živih životinja i dalje je neophodno radi zaštite zdravlja ljudi, životinja i zaštite okoliša. U tom smislu primjenom aktuelnih propisa potiče se i omogućava razvoj alternativnih pristupa i primjene načela 3R, te nastoji osigurati visoka razina zaštite životinja koji se još uvijek moraju koristiti u eksperimentima.

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ROLE AND IMPLEMENTATION OF 3R PRINCIPLES IN ANIMAL WALFARE IN EXPERIMENTAL RESEARCH

ABSTRACT

Use of animals in experimentation dates back to the ancient era, and the discovery of anesthetics have enabled alleviation of their suffering. Animal welfare implies the creation of appropriate conditions of housing, caring, nutrition and animal behavior in order to prevent unnecesarry pain, suffering and fear, and also protection from diseases and injuries. Aim of our research is to evaluate the importance of the application of 3R principles and their benefits to animal welfare in experimentation. In the mid-20th century, bioethicists Russel and Burch developed a 3R principle (replacement, reduction, refinement) to have become a foundation for the ethically acceptable research. Over time, the principle have expanded into a set of principles known as 11R or 12R to include some extra factors referring to a comprehensive approach to research with the aim to improve reproducibility of results, relevance of research and transfer of data, all abiding by ethical standards. Unfortunately, indisputable is the necessity to still use the experimental animals, however, the implemetation of all mechanisms at hand to protect animal welfare is supportive of strenghtening of an affirmative dialogue toward a wider societal acceptance of experimentation on animals, which is still indespensable in biomedical research

Keywords: 3R principles, experimental animals, welfare

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