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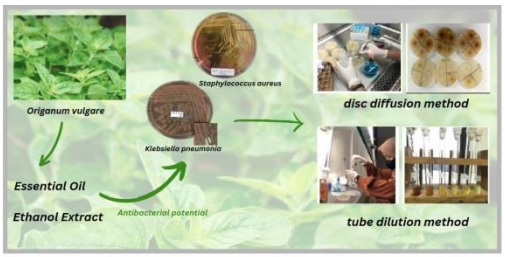
Research Paper

Potential Antibacterial Effects of Ethanol Extract and Essential Oil of Origanum vulgare on *Klebsiella pneumonia* and *Staphylococcus aureus*

Amri IA, Ramadani NF, Hamidah F, Dameanti FNAEP, and Adrenalin SL.

World Vet. J. 13(4): 486-491, 2023; pii:S232245682300051-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj51



Amri IA, Ramadani NF, Hamidah F, Dameanti FNAEP, and Adrenalin SL (2023). **Potential Antibacterial Effects of Ethanol Extract and Essential Oil of *Origanum vulgare* on *Klebsiella pneumonia* and *Staphylococcus aureus*.** *World Vet. J.*, 13 (4): 486-491. DOI: https://dx.doi.org/10.54203/scil.2023.wvj51

ABSTRACT: *Klebsiella pneumonia* (*K. pneumonia*) and *Staphylococcus aureus* (*S. aureus*) are pathogenic bacteria causing various infectious diseases in humans and animals. Currently, herbal ingredients are widely used as antibacterial agents to combat bacterial infections due to their lower side effects, compared to chemical drugs. One such plant with medicinal promise as an antibacterial agent is the oregano plant (*Oregano vulgare*). It contains substances, such as tannin, flavonoids, carvacrol, thymol, and saponin. Therefore, the current study was conducted to regularly compare the *in vitro* antibacterial potential of ethanol extract essential oil oregano (*Oregano vulgare*) on *K. pneumonia* and *S. aureus*. In this research, the diffusion method using discs was employed to observe the inhibition zones, while the dilution tube method was utilized to determine the minimum inhibitory concentration (MIC) of the ethanol extract and essential oil of oregano against the test bacteria. The bacterial treatment group received the test material at concentrations of 100%, 50%, 25%, and 12.5%. The obtained data were analyzed descriptively in terms of zone inhibition and MIC values. According to the disc diffusion test, the essential oil of *oregano* demonstrated greater efficacy as an antibacterial agent against *K. pneumoniae* at a concentration of 100%, resulting in an average inhibition zone of 18 mm. Conversely, for *S. aureus*, a concentration of 1.5% of the essential oil exhibited higher effectiveness, yielding an average inhibition zone of 30 mm. Based on the MIC values, the essential oil was more effective as an antibacterial for *K. pneumonia* at a concentration of 0.2% (2 mg/mL), while for *S. aureus* it was more effective at a concentration of 0.19% (1.9 mg/mL).
Keywords: Antibacterial, Ethanol Extract, Essential Oil, *Oregano vulgare*

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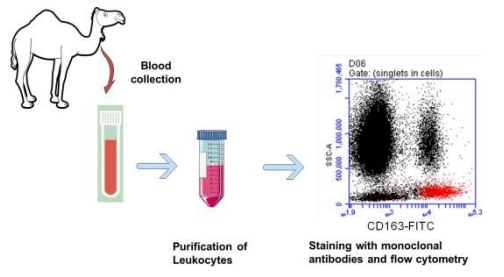
Research Paper

Interaction of Specific Monoclonal Antibodies with Leukocyte Antigens in Camels

Alalai MA, Alkuwayti MA, Alrabiah NA, and Hussen J.

World Vet. J. 13(4): 492-500, 2023; pii:S232245682300052-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj52



Alalai MA, Alkuwayti MA, Alrabiah NA, and Hussen J (2023). **Interaction of Specific Monoclonal Antibodies with Leukocyte Antigens in Camels.** *World Vet. J.*, 13 (4): 492-500. DOI: https://dx.doi.org/10.54203/scil.2023.wvj52

ABSTRACT: The dromedary camel as a livestock species significantly impacts the economy of arid and semi-arid regions worldwide. The identification of cross-reactive antibodies against pivotal immune cell markers acts as a valuable method to investigate the immune system of camels. The aim of the present study was to identify new monoclonal antibodies that react with camel leukocyte subsets using flow cytometry and multicolor immunofluorescence. The expression patterns of the tested antibodies indicated cross-reactivity of the anti-bovine CD9 monoclonal antibody clones LT86A and HI9a with different binding potential. Although all leukocyte subpopulations stained positively with the CD9 antibodies, monocytes showed the highest CD9 abundance, compared to lymphocytes and granulocytes. No cross-reactivity was identified for the tested monoclonal antibodies against equine CD8a (clone: ETC142BA1), mouse CD3 (clone: CD3-12), human CD3 (clone: T3/2/16A9), human CD206 (clone: MMR), and bovine granulocytes (clone: CH138A). The present study revealed that only camel monocytes showed positive staining with the anti-ovine CD5 mAb (clone ST1), which is in contrast to the human and murine systems. The present findings indicated low homogeneity between camels and other species in the antigenic structure of leukocyte antigens, highlighting the need to develop camel-specific mAbs against the main immune cell markers.
Keywords: Antibodies, Camel, Cell marker, Flow cytometry, Immunity

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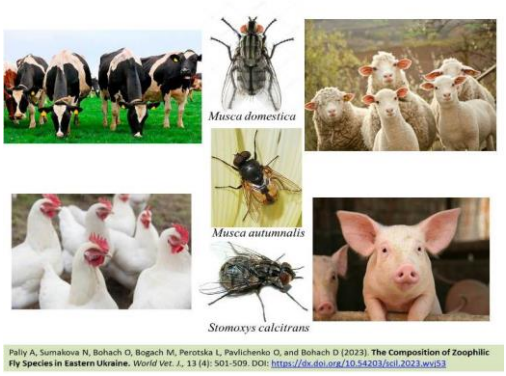
Research Paper

The Composition of Zoophilic Fly Species in Eastern Ukraine

Paliy A, Sumakova N, Bohach O, Bogach M, Perotska L, Pavlichenko O, and Bohach D.

World Vet. J. 13(4): 501-509, 2023; pii:S232245682300053-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj53



ABSTRACT: Zoophilic Diptera plays a leading role in the epizootic foci formation of many infectious and parasitic diseases and directly affects the quality of livestock products. The current study aimed to analyze the number and species composition of parasitic Diptera in industrial, farm, and homestead agrobiocenoses of large and small cattle, pig, and poultry farms in Eastern Ukraine. The research involved entomological collection during the peak activity daylight hours in early May, July, and early September 2021-2022 per farm. A total of 360 entomological collections were made, and 4310 zoophilous flies were examined. In livestock farms of five districts of the Kharkiv region of Ukraine, 28 species of zoophilic flies were registered, among which *Musca domestica*, *Muscina stabulans*, *Stomoxys calcitrans*, *Lucilia sericata*, *Protophormia terraenovae*, and *Drosophila* species were dominant species. The analysis revealed that cattle biocenoses hosted 27 fly species, pigs had 8 species, and poultry and small cattle each had 7 species. The study indicated an increase in the population of *Musca autumnalis*, the main species in the pastures, near livestock premises during the summer. *Stomoxys calcitrans* was also recorded in livestock agrobiocenoses. The species *Musca domestica, Musca autumnalis,* and *Stomoxys calcitrans* account for 78.8% to 88.3% of the entire complex of zoophilous flies. The two species of *Ortellia caesarion* (shiny dung beetle) and *Ortellia cornicina* (green dung beetle), known for their role as manure mineralizers and deemed non-threatening to animals, were completely absent during the research period. The findings indicated the species of *Eristalis tenax* in agrobiocenoses in 2021. Therefore, it can be concluded that zoophilic flies are physical irritants to animals and potential carriers of many infectious diseases, especially diseases caused by unicellular organisms.
Keywords: Biotopes, *Musca autumnalis, Musca domestica*, *Stomoxys calcitrans*, Zoophilic flies

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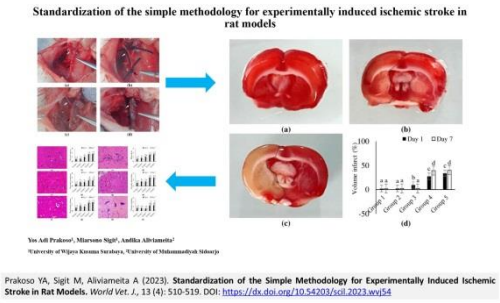
Research Paper

Standardization of the Simple Methodology for Experimentally Induced Ischemic Stroke in Rat Models

Prakoso YA, Sigit M, Aliviameita A.

World Vet. J. 13(4): 510-519, 2023; pii:S232245682300054-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj54



ABSTRACT: Stroke is a globally significant and devastating disease that requires prompt treatment. Animal models are commonly used to investigate stroke therapy, often through experimentally induced ischemic stroke (EIIS). However, challenges arise in implementing EIIS in animal models. The current study aimed to present a simple EIIS methodology for animal models. A total of 60 male Sprague-Dawley rats were randomly divided into five groups, namely Group 1 (sham-operated), Groups 2 to 5 (EIIS groups) with different duration of common carotid artery (CCA) ligation, including 1, 2, 4, and 8 hours, respectively. The ligation was performed on the CCA and its branches. Before the experiment, the rats were anesthetized, and the incision area was shaved and disinfected. The sagittal ventral midline was incised, with neck muscles retracted to expose the right CCA. The occlusion was performed on three sides of a carotid artery (common, external, and internal) using a simple interrupted suture. The occlusion of blood flow using ligation was performed at different times depending on the groups. After that, the CCA ligations were re-perfused by cutting the suture knot. The brain and blood were collected on days 1 and 7 after reperfusion. The results indicated that 4 and 8 hours of CCA ligation significantly impacted the general condition and neuro-deficit score. Moreover, 4 and 8 hours of CCA ligation could induce ischemic stroke by its capacity to cause infarction within the brain parenchyma and increase the platelet-to-white blood cell ratio, C-reactive protein, and De Ritis ratio. In contrast, 1 and 2 hours of CCA ligation did not significantly affect the observed parameters. It can be concluded that the EIIS using 4 and 8 hours of CCA ligation can be applied to induce ischemic stroke in rat models with consistent impacts on general conditions, neuro-deficit, hematology, and serology.
Keywords: Common carotid artery, Ischemic stroke, Ligation, Rat model, Standardization

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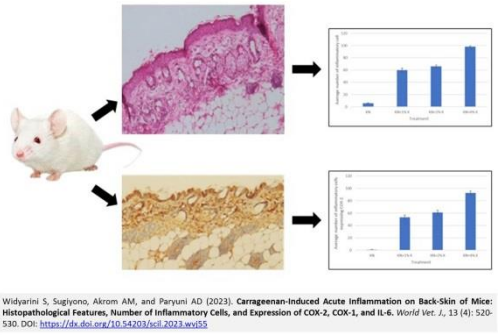
Research Paper

Carrageenan-Induced Acute Inflammation on Back-Skin of Mice: Histopathological Features, Number of Inflammatory Cells, and Expression of COX-2, COX-1, and IL-6

Widyarini S, Sugiyono, Akrom AM, and Paryuni AD.

World Vet. J. 13(4): 520-530, 2023; pii:S232245682300055-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj55



ABSTRACT: Carrageenan is a sulfated polysaccharide obtained from red seaweed (Rhodophyceae) and can trigger inflammatory activation in both humans and laboratory animals. This study aimed to investigate the expression of cyclooxygenase-2 (COX-2), cyclooxygenase-1 (COX-1), and interleukin-6 (IL-6) and the number of inflammatory cells (neutrophil) involved in a carrageenan-induced acute inflammatory model in the back skin of mice. Paraffin blocks from the back skin of female Swiss mice aged 8 weeks were used in this study. The back-skins of 4 groups of 5 mice in each group were subcutaneously injected with 1%, 2%, and 4% carrageenan powder in 0.9% buffer saline and 0.9% buffer saline as control. Skin samples on paraffin blocks were taken 6 hours after carrageenan injection. Furthermore, paraffin blocks were stained with hematoxylin-eosin (HE) to count the number of inflammatory cells. Immunohistochemistry staining using anti-COX-2, COX-1, and IL-6 antibodies was performed to determine the role of inflammatory mediators. The results showed that the number of inflammatory cells (neutrophils) increased significantly following an increase in carrageenan concentrations. The COX-2, COX-1, and IL-6 expressed by inflammatory cells increased significantly at carrageenan concentrations of 1% to 4%. Histopathological features supported the results obtained from the calculation of the number of inflammatory cells and the expression of COX-2, COX-1, and IL-6. The inflammatory markers consisting of COX-2, COX-1, and IL-6 were expressed on the back skin of mice at 6 hours post-injection with 1% to 4% carrageenan. It can be concluded that carrageenan can be used for an acute inflammatory model of the back skin of a mouse. This inflammation model is intended to facilitate the evaluation or measurement of therapeutic and inflammatory responses when test substances are administered topically or transdermal.
Keywords: Carrageenan, Cyclooxygenase-2, Cyclooxygenase-1, Interleukin-6, Inflammatory cell, Skin inflammation

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Research Paper

Observational Study on Reproductive Behavior in Semi-Wild Sambar Deer (*Rusa unicolor*) for Wildlife Conservation and Assisted Reproductive Management

Murad A, Ithnin H, Putra TR, Ngau C, and Fitri W-N.

World Vet. J. 13(4): 531-538, 2023; pii:S232245682300056-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj56

ABSTRACT: Understanding the reproductive behaviors of different wildlife species is essential to unravel their reproductive strategies, ecological adaptations, and conservation requirements. This study delved into the reproductive biology of the sambar deer (*Rusa unicolor*), with a focus on promoting assisted reproductive technology for wildlife conservation and investigating the reproductive behaviors of male and female sambar deer. The study was conducted at Pusat Konservasi Hidupan Liar (PKHL) Sungkai, Perak, Malaysia. The observation focused on one male and two female sambar deer. Direct observations of the deer were conducted for 14 days in September 2022. The direct observations were performed in the morning (Session 1= 8-10 am), afternoon (Session 2 = 10-12 pm), and evening (Session 3= 3-5 pm), using the instantaneous sampling method. A total of 75 behavior instances were recorded, in which male deer exhibited the most reproductive behavior at 58 instances (77.3% of the total reproductive behavior). Successful mating was observed on day 6, elucidating a crepuscular preference in the male animal in exhibiting reproductive behavior. The female's reproductive behavior lasted for a short period, from 24 hours for Female 2 and 72 hours for Female 1. In conclusion, there was a distinct behavior between the male and female deer during the rutting season. Understanding the reproductive behavior to estimate the length of estrus can be useful as a non-invasive tool to detect heat and can be considered to improve breeding management and implement assisted reproductive technology.
Keywords: Breeding, Conservation, *Ex-situ,* Release program, Wildlife

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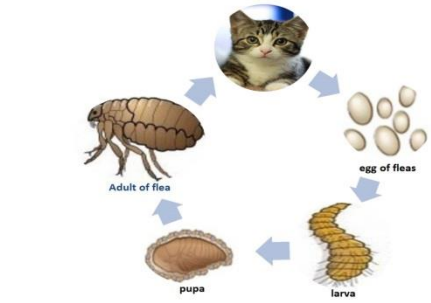
Research Paper

Efficiency Evaluation of Silica Nanoparticles as a Pesticide against *Ctenocephalides felis*

Aziz Anah S and Aziz Anah S.

World Vet. J. 13(4): 539-542, 2023; pii:S232245682300057-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj57

ABSTRACT: The increasing resistance of arthropods to many insecticides has encouraged researchers to search for new alternatives to combat harmful insects. The present study aimed to evaluate the effectiveness of silica nanoparticles (NPs) on *Ctenocephalides felis* (*C. felis*), a prevalent species among cats and a known vector for diseases. The killing efficacy of SiO2-NPs against *C. felis* was tested at three different concentrations (50, 100, and 150 mg/ml) over three different time intervals (10, 20, and 40 minutes), alongside positive and negative control groups (distilled water and cypermethrin). The results of the current study indicated that all concentrations had a fleacidal effect, with SiO2-NPs demonstrating increased efficacy with higher concentrations and longer exposure periods. The concentration of 150 mg/mL of SiO2-NPs led to the highest effect at 96% upon exposure for 40 minutes. The results of the current study revealed significant differences between the control groups and all the groups treated with Sio2-NP concentrations. It can be concluded that Sio2-NPs are a practical approach to flea control although it is necessary to search for environmentally friendly pesticides. The current results indicate that SiO2-NPs have anti-parasitic effects against *C. felis*.



Aziz Anah S and Aziz Anah S (2023). **Efficiency Evaluation of Silica Nanoparticles as a Pesticide against *Ctenocephalides felis*.** *World Vet. J.*, 13 (4): 539-542. DOI: https://dx.doi.org/10.54203/scil.2023.wvj57

Keywords: Cat fleas, Iraq, Nanoparticles, Pesticide, Silica

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Hematological Profile and Aminotransferase Activity in Kintamani Bali Puppies Injected with High Doses of Ivermectin

Sudimartini LM, Mufa RMD, and Merdana IM.

World Vet. J. 13(4): 543-550, 2023; pii:S232245682300058-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj58

ABSTRACT: Ivermectin toxicity is known to cause harmful side effects or even death in dogs intolerant to the medication. Intolerant dogs have a mutation in the MDR-1 (Multi-Drug Resistance) gene, so they lack the P-glycoprotein gene that removes drugs from the brain. Therefore, this study aimed to determine ivermectin toxicity in Kintamani Bali puppies by examining physiological responses based on hematological profiles and aminotransferase activity after a high-dose injection. A laboratory observational approach was used, and the samples were 25 healthy female Kintamani puppies based on a veterinary examination, aged 3-6 months, weighing 6.32 ± 1.18 kg, randomly divided equally into five treatment groups. The treatments included a placebo (1ml Aqua Pro Injection) as a control, as well as a single dose of ivermectin injection sequentially 200, 400, 800, and 1600 µg/kg subcutaneously. Blood samples were collected before treatment and after 7 and 14 days post-treatment. The hematologic parameters observed included levels of hemoglobin, erythrocytes, hematocrit, total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, as well as blood biochemistry, namely aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. Observation results after 4 hours of administration of ivermectin at doses of 800 and 1600 µg/kg of puppies showed changes in behavior, restlessness, depression, tremors, mydriasis, hypersalivation, anorexia, and polydipsia. Meanwhile, the results of hematological examination on the seventh day after ivermectin treatment showed a trend of erythropenia, leukocytosis, a decrease in hemoglobin levels, and an increase in aminotransferase enzyme activity. This condition continued until day 14, but the physiological parameter values showed that the puppy's condition gradually improved compared to the seventh day after treatment. There were significant differences in the blood profile, AST, and ALT of Kintamani puppies injected with ivermectin at doses of 800 and 1,600 ug/kg compared to controls on days 7 and 14 after and before treatment. It was concluded that high-dose ivermectin injections in Kintamani Bali puppies caused toxicity with clinical signs of erythropenia, decreased hemoglobin, leukocytosis, and increased aminotransferase activity.

Sudimartini LM, Mufa RMD, and Merdana IM (2023). **Hematological Profile and Aminotransferase Activity in Kintamani Bali Puppies Injected with High Doses of Ivermectin.** *World Vet. J.*, 13 (4): 543-550. DOI: https://dx.doi.org/10.54203/scil.2023.wvj58

Keywords: Aminotransferase, Blood profile, Ivermectin, Kintamani dogs, Toxicity

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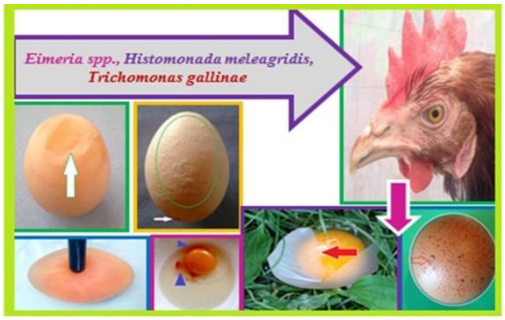
Research Paper

Effects of *Histomonas*, *Trichomonas*, and *Eimeria* Co-infection on Productivity and Macro-morphological Indicators of Eggs in Laying Hens

Liulin P, Bogach M, Lyakhovich L, Petrenko A, and Kostyuk I.

World Vet. J. 13(4): 551-560, 2023; pii:S232245682300059-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj59



Liulin P, Bogach M, Lyakhovich L, Petrenko A, and Kostyuk I (2023). **Effects of *Histomonas*, *Trichomonas*, and *Eimeria* Co-infection on Productivity and Macro-morphological Indicators of Eggs in Laying Hens.** *World Vet. J.*, 13 (4): 551-560. DOI: https://dx.doi.org/10.54203/scil.2023.wvj59

ABSTRACT: The study of macro-morphological changes is important for recognizing disturbances in egg formation that cause pathologies, especially co-infection. The current study aimed to evaluate the level of egg productivity and macro-morphological parameters of eggs in domestic chickens of the Rhode Island breed with co-infection of *Histomonas*, *Trichomonas*, and *Eimeria*. Clinical and parasitological, coproscopic, morphometric research, and statistical analysis methods were used for this research. Pathogens of *Histomonas* and *Trichomonas* were detected by microscopy of smears of fresh feces, and *Eimeria* oocysts were identified by flotation according to the Fullenborn method. During 30 days of research, there was a significant decrease in egg production (52%), a decrease in egg weight by 16.8%, and a decrease in the shell thickness by 30.43% during spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection in laying hens. The eggshell indicated noticeable macro-morphological changes, including deformations and defects resulting from insufficient calcification. These changes manifest as combined damage to the shell, characterized by small cracks, roughness, bumpy or spilled thickenings, and complete or partial depigmentation. When evaluating the internal content of eggs in 12% of their samples, there were bloody spots, relatively smaller and lighter yolks, thinning of the protein part. Thus, the specified macro-morphological changes and egg defects were the result of the negative impact of co-infection on the processes of egg formation, which indicates the systemic nature of the lesion and the morphofunctional insufficiency of the egg-forming organs.
Keywords: Comorbidity, Egg defect, Egg production, Eimeriosis, Histomonosis, Laying hen, Trichomoniasis

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Research Paper

Effects of Adding Glutathione to AndroMed Diluent on Intact Plasma and Acrosome Membranes, and Progressive Motility of Cattle Spermatozoa During Freezing Processes

Bebas W, Gorda IW, Agustina KK, and Merdana IM.

World Vet. J. 13(4): 561-570, 2023; pii:S232245682300060-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj60

Bebas W, Gorda IW, Agustina KK, and Merdana IM (2023). **Effects of Adding Glutathione to AndroMed Diluent on Intact Plasma and Acrosome Membranes, and Progressive Motility of Cattle Spermatozoa During Freezing Processes.** *World Vet. J.*, 13 (4): 561-570. DOI: https://dx.doi.org/10.54203/scil.2023.wvj60

ABSTRACT: Adding endogenous antioxidants to the diluent is significantly associated with semen quality during the freezing process. This study aimed to investigate the effects of adding glutathione to AndroMed diluent on the preservation of crucial sperm attributes, namely, intact plasma membrane (IPM), intact acrosome membrane (IAM), and progressive motility of Bali cattle spermatozoa. A completely randomized design was used, and spermatozoa samples were obtained from a Bali cattle and divided into two diluent treatment groups (36 diluent samples in each group with six replications), namely pure AndroMed as the control and a group with the addition of glutathione (1 mmol) to AndroMed. Each treatment was replicated six times and evaluated at three freezing stages, including post-dilution, post-equilibration, and post-thawing, for crucial sperm properties. The results indicated that fresh Bali cattle spermatozoa had progressive motility, IAM, and IPM of 75%, 89%, and 88%, respectively. During the freezing process, there was a significant decrease in semen quality, including progressive motility, IAM, and IPM of spermatozoa after dilution to post-equilibration and post-equilibration to post-thawing in both treatment groups. Meanwhile, the addition of 1 mmol of glutathione to AndroMed diluent had a significant difference in increasing progressive motility, IAM, and IPM of Bali cattle spermatozoa at each stage of semen freezing, including post-dilution, post-equilibration, and post thawing when compared with controls. Based on the results, it can be concluded that adding 1 mmol of glutathione to the AndroMed diluent enhanced the quality and integrity of Bali cattle semen, including progressive motility, IAM, and IPM during the freezing process.
Keywords: Bali cattle, Freezing Process, Glutathione, Progressive Motility

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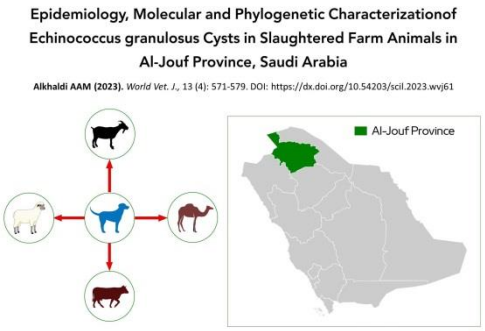
Research Paper

Epidemiology, Molecular, and Phylogenetic Characterization of Echinococcus granulosus Cysts in Slaughtered Farm Animals in Al-Jouf Province, Saudi Arabia

Alkhaldi AAM

World Vet. J. 13(4): 571-579, 2023; pii:S232245682300061-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj61



ABSTRACT: Echinococcosis, or hydatidosis, is a serious veterinary disease and public health issue worldwide, particularly in rural areas in which dogs have frequent contact with local herbivores. This study assessed the frequency of hydatidosis found among farm animals slaughtered in Al-Jouf Province in northern Saudi Arabia in 2021. A total of 156754 sheep, 36337 goats, 8590 camels, and 986 cattle were inspected for hydatidosis infection by comprehensive evaluation involving meticulous visual inspection and manual exploration of the internal organs through palpation. The cysts were subjected to molecular and phylogenetic analysis. The overall prevalence rates of hydatid cysts were 0.43%, 0.19%, 0.54%, and 0.51% in the inspected sheep, goats, camels, and cattle, respectively. The highest disease prevalence rates among sheep (27.8%) and goats (30.9%) occurred in the spring, and the highest prevalence rates among camels (41.3%) and cattle (80%) were in the summer. Regarding the prevalence of the disease in four slaughterhouses in the Al-Jouf Province, the highest prevalence in sheep, goats, and camels was in the Tabarjal slaughterhouse (1.43%, 0.81%, and 1.08%, respectively), although the Al-Qurayat slaughterhouse had the highest prevalence rate in cattle (1.98%). Complete molecular analysis indicated that cytochrome c oxidase subunit 1 (*cox1*) sequences from cyst isolates belonged to *Echinococcus granulosus* (*E. granulosus*). Moreover, there was high homology (98-100%) with associated Genbank sequences of *E. granulosus* isolated from sheep in the Kingdom of Saudi Arabia (KSA). Sheep and camels were a significant source of hydatidosis transmission to dogs and helped to maintain disease incidence in the Al-Jouf Province. Thus, significant efforts should focus on preventing cyst transmission from abattoirs and infected stray dogs.
Keywords: *Echinococcus granulosus*, Epidemiology, Molecular characterization, Farm animals

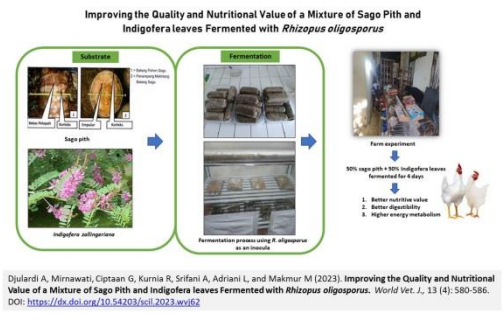
[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Improving the Quality and Nutritional Value of a Mixture of Sago Pith and Indigofera leaves Fermented with *Rhizopus oligosporus*

Djulardi A, Mirnawati, Ciptaan G, Kurnia R, Srifani A, Adriani L, and Makmur M.

World Vet. J. 13(4): 580-586, 2023; pii:S232245682300062-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj62



ABSTRACT: The nutritional value of sago pith is limited due to its low protein content, making it less suitable for poultry feed. To increase the benefit values of the sago pith, it is necessary to process it through fermentation. The current study aimed to determine the effects of substrate composition and fermentation time of fermented sago (*Metroxylon sagu*) pith (SP) and *Indigofera* (*Indigofera zollingeriana*) leaves (IL) mixture using *Rhizopus oligosporus* as an inoculum on crude protein, crude fat and crude fiber content of fermented SP and IL, nitrogen retention, crude fiber digestibility, and energy metabolism in broiler chickens. The study was performed on 30 broiler chickens, average weighing ± 1.5 kg at 6 weeks of age, along with SP, IL, and *R. oligosporus*. This experiment was conducted using a randomized design in a 3×3 factorial with three replications. Substrate composition, or factor A, was made up of A1 (80% SP + 20% IL), A2 (60% SP + 40% IL), and A3 (50% SP + 50% IL). Fermentation time as Factor B entailed B1 (2 days), B2 (3 days), and B3 (4 days). The findings demonstrated a significant interaction between the time of fermentation and the composition of the substrate in relation to crude protein content, nitrogen retention, crude fat, crude fiber digestibility, and energy metabolism. It can be concluded that the composition of substrate 50% SP and 50% IL with 3 days of fermentation yielded the best result, with crude protein at 25.45%, nitrogen retention at 59.72%, crude fat at 0.020%, crude fiber at 6.40%, crude fiber digestibility at 57.34%, and metabolic energy at 2658.44 kcal/kg.
Keywords: Broiler chickens, Crude protein, *Indigofera*, Sago pith, *Rhizopus oligosporus*

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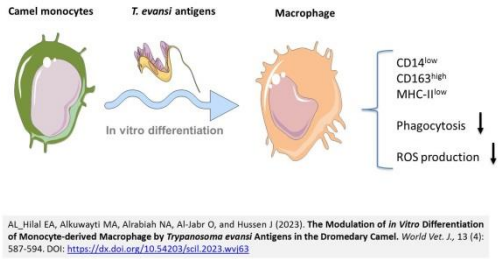
Research Paper

The Modulation of in Vitro Differentiation of Monocyte-derived Macrophage by *Trypanosoma evansi* Antigens in the Dromedary Camel

AL_Hilal EA, Alkuwayti MA, Alrabiah NA, Al-Jabr O, and Hussen J.

World Vet. J. 13(4): 587-594, 2023; pii:S232245682300063-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj63



AL_Hilal EA, Alkuwayti MA, Alrabiah NA, Al-Jabr O, and Hussen J (2023). **The Modulation of *in Vitro* Differentiation of Monocyte-derived Macrophage by *Trypanosoma evansi* Antigens in the Dromedary Camel.** *World Vet. J.*, 13 (4): 587-594. DOI: https://dx.doi.org/10.54203/scil.2023.wvj63

ABSTRACT: Studies on the camel immune response to *Trypanosoma (T.) evansi,* the causative agent of Surra, are very limited. In the present study, flow cytometry was employed to investigate the modulatory effects of different *T. evansi* antigens on the *in vitro* differentiation of camel blood monocytes into macrophages. For this, *in vitro*, separated camel monocytes were differentiated into monocyte-derived macrophages (MDM) in the presence or absence (control) of formalin-fixed (inactivated) *T. evansi* whole parasite (*T. evansi* group) or the purified Ro Tat 1.2 antigen (Ro Tat 1.2 group). The analysis of the antimicrobial functions of MDM (phagocytosis and reactive oxygen species (ROS) production) revealed reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens. In addition, a lack of ROS-response was observed in camel MDM generated in the presence of *T. evansi* antigens after stimulation with PMA. These results indicated a compromising effect of *T. evansi* on the innate defense mechanisms in camels. Phenotypic analysis revealed the upregulation of major histocompatibility complex (MHC) class II molecules together with the lower abundance of the scavenger receptor for haptoglobin–hemoglobin complexes (CD163) on MDM generated in the presence of whole *T. evansi* parasites, indicating a polarizing effect of *T. evansi* on the differentiation of camel monocytes into an M1 phenotype. However, the reduced antimicrobial functions of these cells argue against their pro-inflammatory nature. Although both MDM generated in the presence of whole *T. evansi* antigens or their purified Ro Tat 1.2 proteins indicated similar expression levels of CD14 and MHCII molecules, the different abundance of the cell surface molecules CD172a, CD163, CD45, and CD44 indicated different phenotypes of the two MDMs. The results of the present study revealed compromising effects of *T. evansi* antigens on camel macrophages differentiated *in vitro* from blood monocytes. Whether these effects contribute to the *in vivo* pathogenesis of *T. evansi* in camels remains to be determined in future studies.
Keywords: Camel, Flow cytometry, Immunity, Macrophage, Monocyte, *Trypanosoma evansi*

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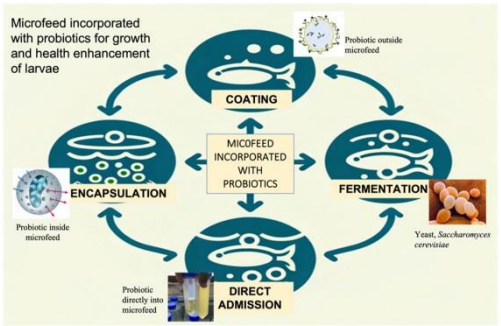
Review

Microfeed Incorporated with Probiotic for Aquaculture: A Review

Yaslikan NM, Yaminudin J, Rasdi NW, and Karim M.

World Vet. J. 13(4): 595-605, 2023; pii:S232245682300064-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj64



Yaslikan NM, Yaminudin J, Rasdi NW, and Karim M (2023). **Microfeed Incorporated with Probiotic for Aquaculture :A Review.** *World Vet. J.*, 13(4): 595-605. DOI: https://dx.doi.org/10.54203/scil.2023.wvj64

ABSTRACT: Ensuring the availability of high-quality larvae in sufficient quantities remains a significant bottleneck for the grow-out phase of aquaculture. Over the past century, various alternative dietary solutions for larval stages have been explored, encompassing bacteria, microalgal pastes, yeasts, and various inert microparticles, though with inconsistent outcomes. This review aimed to discuss the innovative integration of probiotics into microfeeds, highlighting encapsulation, coating, and fermentation techniques to propel aquaculture productivity. Microfeeds, which are often nutrient-rich and easily assimilated in powdered or liquid form, play a crucial role in larval fish nutrition. These can be classified into microencapsulated, dry, liquid, and live feeds. The choice of microfeed is pivotal, ensuring appeal, digestibility, and water stability tailored to each larval stage. As probiotics gain popularity in aquaculture for their potential to enhance growth, bolster disease resistance, and improve water quality, their administration methods have diversified. The probiotics can be administered through direct immersion and bath treatments to biofloc systems and feed additives. The results indicated that microfeed incorporated with probiotics showed a positive result impact on the aquaculture industry.
Keywords: Alternative diets, Aquaculture, Microfeed, Probiotics

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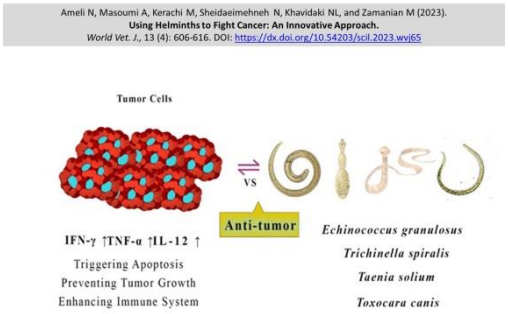
Review

Using Helminths to Fight Cancer: An Innovative Approach

Ameli N, Masoumi A, Kerachi M, Sheidaeimehneh N, Khavidaki NL, and Zamanian M.

World Vet. J. 13(4): 606-616, 2023; pii:S232245682300065-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj65



ABSTRACT: As an alternative treatment in cancer therapy, there has been a growing interest in using helminths, such as *Trichinella spiralis* (*T. spiralis*), *Echinococcus granulosus* (*E. granulosus*), *Toxocara canis* (*T. canis*), and *Taenia solium* (*T. solium*). This study aimed to investigate the antigens and mechanisms that contribute to the anticancer properties of helminths, providing insights into how helminths may be used as a new and innovative treatment modality for cancer. The current review analyzed preclinical and clinical studies published between 2000 and 2023. The present study sought to obtain information on helminths, such as *E. granulosus*, *T. spiralis*, *T. canis*, and *T. solium*, to treat cancers of the breast, pancreas, melanoma, and leukemia by exploring databases, such as PubMed, Google Scholar, and Scopus. Studies focusing on helminth therapy against particular cancer types for *in vitro* and animal models were included. Several studies have shown the possibility of inhibiting breast, colon, melanoma, and leukemia tumor growth, inducing apoptosis, and modulating the tumor microenvironment with *E. granulosus*, *T. spiralis*, *T. canis*, and *T. solium* based on *in vitro* and animal models studies. Some studies have indicated that helminth therapy can improve survival rates, reduce tumor growth, and stimulate the immune system in cancer patients. A potential improvement in treatment outcomes can be used for combination therapies, such as antigen selection, immune profiling, and individualized approaches based on helminth therapy. Helminth therapy is an additional option for cancer treatment, emphasizing *T. spiralis*, *E. granulosus*, *T. canis*, and *T. solium*. These helminth antigens could modulate immune responses and directly cause cytotoxicity in cancer cells.
Keywords: Cancer, *Echinococcus granulosus*, *Taenia solium, Toxocara canis, Trichinella spiralis*

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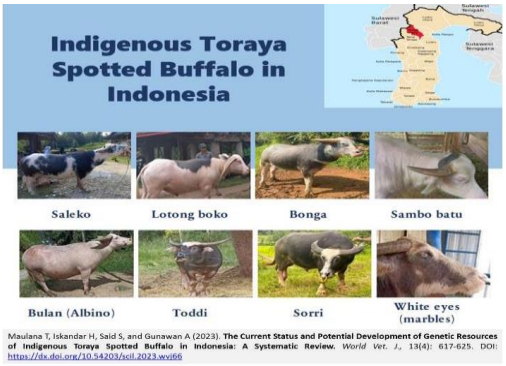
Review

The Current Status and Potential Development of Genetic Resources of Indigenous Toraya Spotted Buffalo in Indonesia: A Systematic Review

Maulana T, Iskandar H, Said S, and Gunawan A.

World Vet. J. 13(4): 617-625, 2023; pii:S232245682300066-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj66



ABSTRACT: Buffaloes are integral to the Asiatic market as they are crucial for agricultural work and transportation and provide a significant source of dairy and meat, contributing to various industries, local economies, and cultural practices across the region. Indonesia is a mega biodiversity country abundant in livestock genetic resources, including indigenous, local, and introduced breeds that play a crucial role in agriculture and the livestock industry. These genetic resources offer the potential for selective breeding and improving the quality of livestock populations through well-designed breeding programs, ensuring sustainable livestock production for the future. The current study was performed using the "Publish or Perish" software, and the data obtained was analyzed using the CADIMA web tool. The Toraya buffalo population is the largest in the Tana Toraja and North Toraja regencies and is widely distributed within South Sulawesi Province, Indonesia. The population of Toraya buffalo in this region reached 43674 heads. Toraya buffaloes exhibit diverse body color characteristics, such as black, gray, white, and mixed. Moreover, Toraya buffaloes possess distinct quantitative traits that set them apart from other swamp buffalo breeds. Although there are limited studies on Toraya buffaloes, the potential for broader and more comprehensive studies offers opportunities to uncover new information on the characteristics, genetics, reproduction, health, and management of Toraya buffaloes. The development of Toraya buffalo farms also holds significant economic promise, as it can lead to increased agricultural productivity and improved livelihoods for local communities by enhancing the quality by implementing well-planned breeding programs and leveraging reproductive technology, and genetics-based selection, growth, and productivity can be produced. To increase the population and productivity of Toraya buffaloes, a well-structured breeding program integrating reproductive technology and selection based on quantitative and molecular genetics is essential. The development potential of Toraya buffalo is vast, not only due to its high cultural value but also its superior quantitative traits compared to common swamp buffalo, positioning it as a potential national meat provider. The present review article aimed to discuss the characteristics and development potential of Toraya buffaloes, along with the implementation of reproductive biotechnology and molecular genetics to enhance the population, productivity, and quality of Toraya buffaloes in Indonesia.
Keywords: Genetic resource, Spotted buffalo, Swamp buffalo, Toraya buffalo

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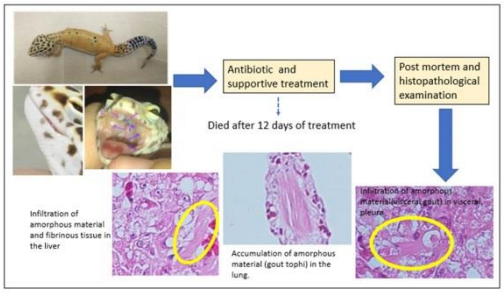
Case Report

Pathologic Case of Gout Tophi Deposition with a Concurrent Systemic Bacterial Infection in a Leopard Gecko (*Eublepharis macularius*)

Mazlan M, Asrul Syafiq M, Zakaria MA, Samad LA, and Che-Amat A.

World Vet. J. 13(4): 626-629, 2023; pii:S232245682300067-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj67



Mazlan M, Asrul Syafiq M, Zakaria MA, Samad LA, and Che-Amat A (2023). **A Pathologic Case of Gout Tophi Deposition with a Concurrent Systemic Bacterial Infection in a Leopard Gecko (*Eublepharis macularius*).** *World Vet. J.*, 13 (4): 626-629. DOI: https://dx.doi.org/10.54203/scil.2023.wvj67

ABSTRACT: Gout is caused by excessive uric acid in the blood deposited in tissues (visceral gout) or joints (articular gout), leading to severe inflammation and pain. A female leopard gecko was presented to the University Veterinary Hospital, University of Putra, Malaysia, with a history of swelling at the left caudal mandible, inappetence, and weight loss. An oral examination indicated a swollen mouth with scabs on the upper right mandible and multiple whitish deposits inside the mouth. The preliminary diagnosis was mouth rot, and the treatment included metronidazole and a multivitamin supplement. The leopard gecko died 12 days after treatment since there was no improvement. Post-mortem examination revealed that the liver was slightly enlarged with generalized moderate congestion and the presence of whitish deposits, as well as noticeable whitish deposits on the pleural surface of the lungs. Histopathological examination of the lungs revealed a granuloma with an inflammatory reaction predominantly by abundant mononuclear cells and fibrin deposition. An irregular collection of amorphous materials in the visceral pleura suggested gout tophi. The liver was infiltrated with amorphous material and fibrinous tissue, and it had mild congestion, indicating visceral gout and bacterial infection. Klebsiella pneumoniae and Proteus mirabilis were isolated from the lungs and liver samples, respectively. In conclusion, gout tophi is common in reptiles, but visceral involvement is rare, and early detection is critical to avoid secondary bacterial infection, as demonstrated in this case.
Keywords: Amorphous material, Bacterial infection, Gout tophi, Histopathology, Leopard gecko

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Case Report

Hematological and Biochemical Parameters of Macropod Progressive Periodontal Disease in Wild Western Gray Kangaroos

Zhelavskyi M, Kernychnyi S, and Betlinska T.

World Vet. J. 13(4): 630-635, 2023; pii:S232245682300068-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj68

Zhelavskyi M, Kernychnyi S, and Betlinska T (2023).
Hematological and Biochemical Parameters of Macropod Progressive Periodontal Disease in Wild Western Gray Kangaroos.
World Vet. J., 13 (4): 630-635. DOI: https://dx.doi.org/10.54203/scil.2023.wvj68

ABSTRACT: Macropod progressive periodontal disease (MPPD), known as Lumpy Jaw, poses a persistent and potentially fatal threat in Western gray kangaroos when they are kept in captivity. Such a condition leads to the development of osteomyelitis and sepsis in Western gray kangaroos (Macropus fuliginosus). This case study presented the inaugural examination of hematological and biochemical aspects of MPPD with a progression toward sepsis in a captive environment. The primary objective of this research was to pinpoint hematological and biochemical indicators associated with severe MPPD in a Western gray kangaroo held in captivity. The study employed various methods, including clinical, radiographical, hematological, and biochemical analyses, as well as microbiological study methods. The case was a 2.5-year-old male wild Western gray kangaroo with fever (39.7 °C), dehydration, dyspnea, tachycardia, and involuntary jaw clenching due to stress and agitation. The kangaroo had a history of lethargy, anorexia, swelling of the soft tissues of the lower jaw on the left side, and tenderness during palpation. A radiograph of the head revealed mandible proliferative lesions. The hematological and biochemical examinations indicated an increase in the total count of leucocytes, level of neutrophils, number of erythrocytes, hematocrit level, and lymphopenia. Increased activity of alkaline phosphatase, amylase, and creatinine elevated azotemia. There was a decrease in the content of albumin, glucose, and total bilirubin. The bacteria, consisting of Fusobacteriaceae spp., Porphyromonadaceae spp., and Bacteroidaceae spp., were found and identified in all samples. However, this comprehensive diagnosis of MPPD based on clinical signs, radiography, and especially hematological and biochemical parameters of the septic process can be helpful in diagnosis and treatment.
Keywords: Macropod Progressive Periodontal Disease, Macropus fuliginosus, Hematological and Biochemical parameters

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Case Report

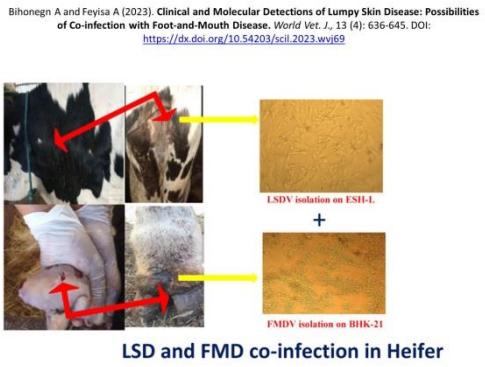
Clinical and Molecular Detections of Lumpy Skin Disease: Possibilities of Co-infection with Foot-and-Mouth Disease

Bihonegn A and Feyisa A.

World Vet. J. 13(4): 636-645, 2023; pii:S232245682300069-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj69

ABSTRACT: Lumpy skin disease (LSD) and foot and mouth disease (FMD) are notable viral diseases of cattle. This report aimed to highlight the possibilities of an uncommon case of LSD and FMD co-infection. The report also presents the clinical and molecular detection of LSD virus in six crossbred calves and LSD and FMD virus co-infection in a heifer at small-scale dairy farms located in northern Ethiopia. Nasal swabs and tissue samples were collected following aseptic techniques from the six calves suspected of having LSD and a tissue sample from one heifer suspected of having LSD-FMD co-infection and submitted to the laboratory for cell culture and real-time polymerase chain reaction (PCR) tests. Different-sized, firm, painful skin nodules with necrotic centers were seen on different parts of the calves' body. Swelling of the prescapular and prefemoral lymph nodes, conjunctivitis, and corneal cloudiness were also observed. Uniquely, one heifer was seen with erosive lesions in the oral cavity and tongue, salivation, lameness, and skin nodules. Intracytoplasmic inclusion bodies, a distinctive feature of LSD virus, and the formation of syncytia, a characteristic of FMD virus, were observed in the cell lines. The heifer was diagnosed with a rare co-infection of LSDV and FMDV based on clinical signs, cell culture, and real-time PCR test results. The other six calves were diagnosed with the LSD virus. Treatment with broad-spectrum antibiotics, local wound cleansing, and anti-inflammatory drugs was initiated. Unfortunately, the heifer with LSD-FMD co-infection died while under treatment, and just three calves with LSD were recovered. It can be concluded that vaccination of animals against both diseases and promotion of bio-security protocols in farms is more helpful than treatment, and early case reporting is also warranted to avoid losses related to the diseases.
Keywords: Calves, Co-infection, Foot and mouth disease, Heifer, Lumpy skin disease



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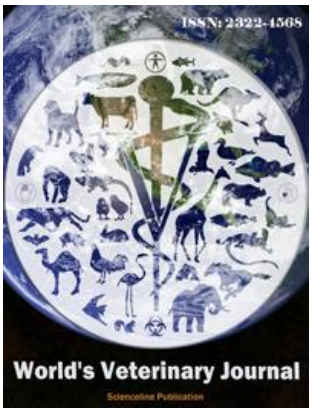

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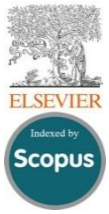

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Potential Antibacterial Effects of Ethanol Extract and Essential Oil of *Origanum vulgare* on *Klebsiella pneumonia* and *Staphylococcus aureus*

Indah Amalia Amri[1*], Nurul Fitri Ramadani[2], Farah Hamidah[2], Fidi Nur Aini Eka Puji Dameanti[1], and Sruti Listra Adrenalin[1]

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ABSTRACT

*Klebsiella pneumoni*a (*K. pneumonia*) and *Staphylococcus aureus* (*S. aureus*) are pathogenic bacteria causing various infectious diseases in humans and animals. Currently, herbal ingredients are widely used as antibacterial agents to combat bacterial infections due to their lower side effects, compared to chemical drugs. One such plant with medicinal promise as an antibacterial agent is the oregano plant (*Oregano vulgare*). It contains substances, such as tannin, flavonoids, carvacrol, thymol, and saponin. Therefore, the current study was conducted to regularly compare the *in vitro* antibacterial potential of ethanol extract essential oil oregano (*Oregano vulgare*) on *K. pneumonia* and *S. aureus*. In this research, the diffusion method using discs was employed to observe the inhibition zones, while the dilution tube method was utilized to determine the minimum inhibitory concentration (MIC) of the ethanol extract and essential oil of oregano against the test bacteria. The bacterial treatment group received the test material at concentrations of 100%, 50%, 25%, and 12.5%. The obtained data were analyzed descriptively in terms of zone inhibition and MIC values. According to the disc diffusion test, the essential oil of *oregano* demonstrated greater efficacy as an antibacterial agent against *K. pneumoniae* at a concentration of 100%, resulting in an average inhibition zone of 18 mm. Conversely, for *S. aureus*, a concentration of 1.5% of the essential oil exhibited higher effectiveness, yielding an average inhibition zone of 30 mm. Based on the MIC values, the essential oil was more effective as an antibacterial for *K. pneumonia* at a concentration of 0.2% (2 mg/mL), while for *S. aureus* it was more effective at a concentration of 0.19% (1.9 mg/mL).

Keywords: Antibacterial, Ethanol Extract, Essential Oil, *Oregano vulgare*

INTRODUCTION

Natural ingredients are increasingly used as an alternative medicine against bacterial infection. Natural ingredients have low side effects and are easy to obtain compared to chemical drugs. Oregano plant (*Oregano vulgare*) is one of the natural ingredients commonly used to process food and is also used as medicine (Milagres De Almeida et al., 2023). Recently, the oregano plant has gained significant recognition as an herbal medicine, supported by robust scientific evidence. Oregano plant is very influential against Gram-positive and Gram-negative bacteria (Zhong et al., 2023). The most common compounds in the oregano plant are carvacrol, thymol, tannins, phenols, and flavonoids (Chevallier, 2016). Carvacrol and thymol disrupt the phospholipid layer to inhibit protein synthesis, which can damage bacterial cell membranes. This leads to an increase in cell membrane permeability. Carvacrol can inhibit the movement of the flagellum due to a decrease in ATP production (Walczak et al., 2021). The mechanism of phenol compounds will react with cell membrane phospholipid compounds. These processes lead to alterations in fatty acid compositions, phospholipid levels, and disruptions in the bacterial cell walls, resulting in cellular damage. Tannins function by attaching to proteins located on the surface of bacterial cells, preventing them from allowing water to enter. Flavonoids operate similarly, with the potential to induce cell or bacterial cell wall leakage (Chevallier, 2016).

Klebsiella (K.) pneumonia is a short rod-shaped Gram-negative bacterium with no spores and a thick and non-motile capsule with a size of 0.5-1.5 µ. *Klebsiella pneumonia* is a significantly opportunistic and gram-negative bacteria

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that causes infections in the respiratory tract, circulatory system, urinary system, and wounds in individuals with underlying disease (Ranjbarian et al., 2023). *Staphylococcus (S.) aureus* is a Gram-positive bacteria (clustered like grapes) and easily grows on various deep bacterial media aerobic states. *Staphylococcus aureus* is a prevalent pathogen in human infections, often contributing to various diseases, including skin and soft tissue infection, infectious arthritis, and septicemia (Zhao et al., 2022). Therefore, the current study aimed to investigate and compare the antibacterial potential of essential oil (EO) and extract ethanol (EE) of *Oregano vulgare* against *K. pneumonia* and *S. aureus*.

MATERIALS AND METHODS

Ethical approval

The current study was conducted according to the guidelines of the University Brawijaya, Puncak Dieng Eksklusif Malang, East Java, Indonesia.

Extract preparation

Oregano vulgare was obtained from the local farmers in Malang, Indonesia. The leaves were dried in an open and shady place for 7 days. Then, the dried *Oregano vulgare* leaves were powdered to obtain 500 grams. *Oregano vulgare* powder was soaked for 3 days with a maceration procedure using 3 L of 96% methanol at room temperature for 24 hours and stirred once daily for 3 days. The extract was evaporated using a rotary evaporator (Sarmira et al., 2021).

The EO was extracted through the hydro-distillation process using a Clevenger's type apparatus, as described by Clevenger in 1928. The distillations were conducted by subjecting aerial plant parts to boiling for 3 hours. The resulting EO was then transferred to airtight, sealed vials and subsequently stored at a temperature of 4°C until it was to be utilized for subsequent purposes.

Bacterial culture and preparation

The bacteria used are *K. pneumonia* and *S. aureus*. These isolates were obtained from the Laboratory of Microbiology, Faculty of Veterinary Medicine, University Brawijaya, Malang, Indonesia. The *K. pneumonia* was reidentified using Mac Conkey Agar *(*MCA) media. The confirmation tests were carried out in the form of biochemical tests and Gram staining with a solution of crystal violet, acetone alcohol, Lugol and safranin with the results of bacteria in the form of Gram-negative bacilli (Riedel et al., 2019). At the same time, *S. aureus* reidentification tests were carried out in the form of mannitol fermentation tests, gram staining, catalase tests, and coagulase tests. The confirmed bacteria were then propagated using the Nutrient Broth (NB) media on *K. pneumonia* and *S. aureus* and incubated at 37°C for 24 hours.

Antibacterial testing tube dilution

In tube dilution, 13 reactions were provided for EE and EO. Each tube was filled with 0.5 ml of Mueller hilton broth (MHB) and 0.5 ml of the test material. It was then put into the second test tube, and 1 ml of bacterial suspension was added so that each test tube would be filled with 2 ml and incubated at 37^{O}C for 24 hours. The turbidity was observed, and the MIC value was determined on the media. Tubes tested for MIC were subculture on MHA media and incubated at 37^{O}C for 18-24 hours. The growth of bacteria from incubation was observed with the presence or absence of colony growth (Aiemsaard et al., 2023).

Antibacterial testing disc diffusion

The disc method was carried out by inoculating a bacterial suspension on MHA media as much as 0.1 ml using a sterile cotton swab and left to dry. Disc paper soaked in EE and EO common oregano for 15 minutes. The soaking time of disc paper could affect the absorption of the active ingredients contained in the extract into the paper disc because a longer soaking time results in increased absorption of active materials. For the agar disk diffusion method, the sterile filter paper disk was saturated with 100%, 50%, 25%, and 12.5%. The soaked paper discs were aseptically placed on the surface of the MHA media and incubated at 37°C for 24 hours. The bacterial growth activity was determined by measuring the diameter of the zone of inhibition (Nordin et al., 2019). Dimethyl sulfoxide (DMSO) 5% was used as a negative control because it is a solvent with no antibacterial properties (Adiwibawa Prasetya et al., 2019).

Data analysis

Research data on the antibacterial potential of EE and EO of *Oregano vulgare* was analyzed descriptively by looking at the Minimum Inhibitory Concentration (MIC) and inhibition zone results. The obtained data were analyzed using SPSS software (version 29 IBM) with one-way ANOVA and a follow-up Tukey when the p-value was significant ($p < 0.05$).

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RESULTS AND DISCUSSION

Antibacterial activity test

The disc diffusion method was used in this study, where EE and EO of *Oregano vulgare* were tested for their antibacterial activity against *K. pneumonia*. The inhibition diameter of the bacterial activity was measured as a result of the disc diffusion method. According to Sarmira et al. (2021), the strength of the antibacterial activity can be determined based on the diameter of the inhibition. Therefore, very strong activity is for a diameter of inhibition > 20 mm, strong activity has an inhibition zone diameter of 10-20 mm, moderate activity corresponds to an inhibition zone diameter of 5-10 mm, and inhibition zone diameter < 5 shows a weak activity.

The results obtained after measuring the average diameter of the clear zone formed against the test bacteria *K. pneumonia* and *S. aureus* with EE and EO of *Oregano vulgare* are shown in Tables 1 and 2.

Table 1. The antibacterial activity of ethanol extract of *Oregano vulgare*

Bacteria	Concentration (%)	Inhibition Zone (mm)
Klebsiella pneumonia	DMSO 5%	0 ± 0^{a}
	100%	6.4 ± 0.5^{c}
	50%	4.7 ± 0.5^{b}
	25%	3.74 ± 0.5^{b}
	12.5%	0.0 ± 0^{a}
Staphylococcus aureus	DMSO 5%	0 ± 0^{a}
	100%	14.3 ± 0.9^{d}
	50%	12.3 ± 0.5^{c}
	25%	9 ± 0.5^{b}
	12.5%	7.3 ± 0.5^{b}

Values represent mean ± SD. ^{abcd}Means with different superscripts in a column differ significantly among different concentrations ($p < 0.05$)

Table 2. The antibacterial activity of the essential oil of *Oregano vulgare*

Bacteria	Concentration	Inhibition Zone (mm)
Klebsiella pneumonia	DMSO 5%	0 ± 0^{a}
	100%	18 ± 0^{c}
	50%	16.3 ± 0.9^{c}
	25%	13 ± 0.8^{b}
	12.5%	12 ± 1.4^{b}
Staphylococcus aureus	DMSO 5%	0 ± 0^{a}
	100%	30.3 ± 0.5^{d}
	50%	28.3 ± 0.5^{d}
	25%	18.6 ± 0.9^{c}
	12.5%	14 ± 1.4^{b}

Values represent mean ± SD. ^{abcd}Means with different superscripts in a column differ significantly among different concentrations ($p < 0.05$)

The study indicated that EE and EO of *Oregano vulgare* had antibacterial activity against *K. pneumonia* and *S. aureus* (Tables 1 and 2). The average value of the diameter of the bacterial inhibition zone *K. pneumonia* with ethanol extract test material *Oregano vulgare* at a concentration of 100% is 6.4 mm, which is with a medium inhibition, and *S. aureus* concentration of 100% was 14.33 mm with strong inhibition. Table 2 indicates that *Oregano vulgare* EO to *K. pneumonia* at a concentration of 100% was 18 mm, which is a strong inhibition. Notably, a concentration of 100% *Oregano vulgare* EO demonstrated a very strong inhibition against *S. aureus*, evidenced by an inhibition zone diameter of 30.33 mm. *Oregano vulgare* EO indicated a more powerful antibacterial effect against bacteria than EE, which was likely to inhibit Gram-positive bacteria more than Gram-negative bacteria. The *Oregano vulgare* EE antibacterial activity of 100% against *K. pneumonia* and *S. aureus* showed a medium and strong inhibition (6.4 ± 0. 5 and 14.3 ± 0.9 mm, respectively; Table 1). Moreover, the antibacterial activity of EO of 100% *Oregano vulgare* against *K. pneumonia* and *S. aureus* showed a strong and very strong inhibition (18 ± 0 and 30.3 ± 0.5 mm, respectively; Table 2). It was found that EO had more powerful antibacterial effects than the EE of *Oregano vulgare*, especially in this research using *K. pneumonia* (Gram-negative) and *S. aureus* (Gram-positive bacteria). The antibacterial activity against gram-positive *S. aureus* was stronger than that against gram-negative *K. pneumonia*. The reason could be the fact that Gram-negative bacteria are usually more resistant to antibacterial compounds since their cell walls are more complicated (Nurhayati et al., 2020).

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Essential oils exhibit potent antibacterial properties due to their capacity to enhance membrane permeability and induce cytoplasmic membrane depolarization. These actions effectively inhibit the production of microbial toxins and the formation of biofilms while also reducing fimbriae production (Băicuș et al., 2022). Carvacrol and thymol, the main components of oregano EO, are antibacterial and antioxidant (Coccimiglio et al., 2016).

The EE of oregano leaves contains several secondary metabolite compounds, such as phenolics, tannins, flavonoids, saponins, triterpenoids, and steroids (Cortés-Chitala et al., 2021). Ethanol extract of oregano leaves contains secondary metabolite compounds that have various mechanisms of action, such as antibacterial. The mechanism of action of phenolic compounds involves the denaturation of proteins, leading to the disruption of their tertiary structure and the subsequent loss of their original properties. Phenolic compounds can denature protein; thus, the main structure of the protein will be damaged (Coccimiglio et al., 2016). The cell wall of bacteria can be easily damaged by the antibacterial properties of *Oregano vulgare*, namely carvacrol and thymol (Can Baser, 2008). However, the mechanisms by which thymol and carvacrol cause cell death in bacteria have not been thoroughly investigated. Koparal and Zeytinoglu (2003) demonstrated that carvacrol is a very potent inhibitor of cell growth in A549 cell line, as evidenced by the concentration-dependent decreases in cell number, degeneration of cell morphology, and a decrease in total protein amount (Koparal and Zeytinoglu, 2003). Based on Hsu et al. (2011), thymol induces cell death in human osteosarcoma and astrocytes and may involve apoptosis via mitochondrial pathways.

Minimum inhibition concentration

The MIC is defined as the smallest concentration of an antimicrobial agent that prevents the visible growth of test microorganisms in a tube. The first with no visible growth after the incubation period is taken as a MIC. Minimum inhibition concentration (MBC) is a minimal concentration of antimicrobials that kills inoculum and can be determined by the MIC assay (Ngadino et al., 2018). The tube dilution method was used to determine MIC for the antibacterial potential of *Oregano vulgare* EE and EO against *K. pneumonia* and *S. aureus*.

Table 3. Determination of minimum inhibition concentration

Bacteria	*Oregano vulgare*	Concentration of *Oregano vulgare* (%)												
		100	**50**	**25**	**13**	**6.3**	**3.1**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**0.05**	**0.02**
Klebsiella pneumonia	Ethanol extract	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	Essential oil	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)
Staphylococcus aureus	Ethanol extract	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	Essential oil	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)

(+): Growing; (-): Not growing

Tube dilution test results using EE and EO against bacteria *K. pneumonia* turbidity at each concentration can be observed after incubation (Table 3). In the ethanol extract that had been given a bacterial culture of 1 ml, the observed MIC results were at a concentration of 50% or the equivalent of 500,000 µg/mL (500 mg/ml). The observed MIC results were at a concentration of 0.2% or equivalent to 2,000 µg/mL (2 mg/ml) found on *S. aureus* tube dilution test using ethanol extract. The smallest concentration was found in the tube where there was no growth of bacterial colonies, as indicated by the clarity of the tube. In EO, the lowest MIC value at 12.5% was equivalent to 125 mg/mL, and in EO, at a concentration of 0.19% or 1.9 mg/mL.

Essential oil *Oregano vulgare* has a greater antibacterial potential than ethanol extract *Oregano vulgare*, as measured by the MIC values. The literature explains that a drug substance or test material can be classified as antibacterial if it has bacteriostatic and bactericidal properties. Bacteriostatic refers to a drug's ability to inhibit bacterial growth at a specific concentration. In contrast, bactericidal refers to the capacity of a drug or test substance to kill bacteria at a particular concentration. In this study, bacteriostatic activity was indicated by the variation in turbidity levels and the reduction in the number of bacteria growth in response to the administration of the extract at varying concentrations. In contrast, bactericidal activity was indicated by the presence or absence of bacterial growth on the medium supplemented by *Oregano vulgaris* extract in various concentrations.

CONCLUSION

This study revealed that *Oregano vulgare* EE and EO is a potential alternative natural antibacterial agent against *Klebsiella. pneumonia* and *S. aureus*. Compared to ethanol extract, EO has a greater antibacterial potential, and both are more effective against gram-positive bacteria.

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DECLARATIONS

Authors' Contributions

Indah Amalia Amri, Nurul Fitri Ramadani, Farah Hamidah, Fidi Nur Aini EPD, and Sruti Listra Adrenalin contributed manuscript writing, data analysis, and study design. All authors contributed review data from this research and approved the final draft of the manuscript.

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Ethical consideration

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material. For inquiries, please contact the corresponding author/s.

Conflict of interests

The authors have not declared any conflict of interest.

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Interaction of Specific Monoclonal Antibodies with Leukocyte Antigens in Camels

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ABSTRACT

The dromedary camel as a livestock species significantly impacts the economy of arid and semi-arid regions worldwide. The identification of cross-reactive antibodies against pivotal immune cell markers acts as a valuable method to investigate the immune system of camels. The aim of the present study was to identify new monoclonal antibodies that react with camel leukocyte subsets using flow cytometry and multicolor immunofluorescence. The expression patterns of the tested antibodies indicated cross-reactivity of the anti-bovine CD9 monoclonal antibody clones LT86A and Hl9a with different binding potential. Although all leukocyte subpopulations stained positively with the CD9 antibodies, monocytes showed the highest CD9 abundance, compared to lymphocytes and granulocytes. No cross-reactivity was identified for the tested monoclonal antibodies against equine CD8a (clone: ETC142BA1), mouse CD3 (clone: CD3-12), human CD3 (clone: T3/2/16A9), human CD206 (clone: MMR), and bovine granulocytes (clone: CH138A). The present study revealed that only camel monocytes showed positive staining with the anti-ovine CD5 mAb (clone ST1), which is in contrast to the human and murine systems. The present findings indicated low homogeneity between camels and other species in the antigenic structure of leukocyte antigens, highlighting the need to develop camel-specific mAbs against the main immune cell markers.

Keywords: Antibodies, Camel, Cell marker, Flow cytometry, Immunity

INTRODUCTION

Monoclonal antibodies (mAbs) play a crucial role in the process of identifying immune cells and monitoring their distribution and mobilization in distinct tissues such as the lungs, the ovary, and the lymph node (Gunnes et al., 2003; Maecker et al., 2012; Hussen et al., 2022; Rivers et al., 2022). In order to avoid the laborious procedure of generating species-specific mAbs, researchers frequently employ cross-reactive mAbs (Farady et al., 2009; Irani et al., 2016; Grandoni et al., 2020; Grandoni et al., 2023; Hussen et al., 2023a; Hussen et al., 2023b). Over the past few years, several studies have been performed to test the cross-reactivity of mAbs against leukocyte antigens sourced from humans or other veterinary animals with their corresponding camel antigens (Hussen et al., 2017; Hussen and Schuberth, 2020; Hussen et al., 2022). These studies have successfully identified cross-reactive mAbs that target significant cell marker antigens. The list of mAbs and the target cluster of differentiation antigen molecules have been recently reviewed (Hussen and Schuberth, 2020). The identification of such mAbs has been employed in several important studies on camel immune cells (Hussen et al., 2020a; Hussen et al., 2020b; Hussen et al., 2021; Hussen and Al-Sukruwah, 2022; Hussen et al., 2022; Hussen et al., 2023a; Hussen et al., 2023b).

Flow cytometry has been demonstrated to be a valuable technique for identifying and characterizing immune cells in body fluids or cell suspensions prepared from tissue homogenates. The relevancy of this technique has been examined in the fields of infection immunity, immune response to vaccination, immunopathology, and tumor research and diagnosis through the determination of the manner in which a population of cells is distributed within tissue homogenates (Koshiol and Lin, 2012; Lyons et al., 2017; Boonyaratanakornkit and Taylor, 2019).

The cell marker CD9 is one of the tetraspanin family with a key role in the essential cellular functions of many immune cells and endothelial cells. This mainly includes intracellular signaling, cell stimulation and proliferation, cell viability, and cell adhesion and migration (Veenbergen and van Spriel, 2011; Rocha-Perugini et al., 2014). CD9 exhibits a broad distribution among cells and tissues and was initially recognized as a marker for lymphohematopoiesis (Rocha-Perugini et al., 2014). Subsequently, it became associated with numerous cellular processes such as motility, proliferation, differentiation, fusion, and adhesion (Wright et al., 2004; Tohami et al., 2004; Pugholm et al., 2016).

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The identification of T cells is usually possible using antibodies to the pan-T cell marker CD3 in combination with the T cell co-receptors CD4 and CD8. Although camel helper T cells can be identified using anti-bovine CD4 antibody, there are no antibodies to camel CD3 or CD8. In addition, the set of available mAbs against camel T cell activation markers is very limited. The transmembrane receptor CD5 is a 67 kDa type 1 T cell surface protein with key regulatory roles in T cell activation and development (Burgueno-Bucio et al., 2019). In humans and mice, CD5 is expressed in thymocytes, mature T cells, and a subset of B cells known as B1a (Li et al., 2019). Although the role of CD5 in lymphocytes has been subjected to extensive investigation, its function in other populations of immune cells remains largely unexplored. In T cells, the primary function of CD5 is to regulate signaling through the T cell receptor (TCR). This is in addition to their role during the development of thymocytes (Axtell et al., 2006). Furthermore, CD5 showed an inhibitory effect on peripheral T cells through interference with the immunological synapse formation (Raman, 2002). Therefore, increased CD5 expression in T cells is associated with a suppressed response to stimulation by antigens (Hawiger et al., 2004). On the other hand, a high level of CD5 expression in T cells contributes to the induction of tolerance and the generation of regulatory T cells (Treg) (Ordonez-Rueda et al., 2009).

The main target of the present study was to test the reactivity of camel leukocyte subsets with some commercially available monoclonal antibodies for leukocyte antigens from other species using flow cytometry and multicolor immunofluorescence. The identification of reactive monoclonal antibodies against new cell surface antigens will pave the way for in-depth exploration of the camel immune system.

MATERIALS AND METHODS

Ethical approval

Animals' ethical approval was obtained from the Ethics Committee of King Faisal University, Saudi Arabia, with an approval number (KFU-REC-2021- DEC -EA000326).

Blood samples

Blood samples (10 mL blood) were collected from five male dromedary camels of the Al-Majahim breed (between 8 and 13 years with an average weight of 468 ± 46 kg). The animals were selected from the camels admitted to Al-Omran Slaughterhouse in Al-Ahsa Region in Saudi Arabia. All animals were apparently healthy based on the clinical examination (performed by a veterinarian) for any symptoms such as mastitis, metritis, diarrhea, and respiratory diseases. The samples were collected from the jugular vein into blood collection tubes with the anticoagulation agent EDTA (Becton Dickinson, Heidelberg, Germany) and transported to the laboratory within one hour.

Purification of camel leukocytes

Peripheral blood leukocytes were separated as previously described (Hussen, 2021). Briefly, 5 mL camel blood was diluted 1:2 in cold phaosphate buffered saline (PBS) and centrifuged for 20 minutes at 3000 rpm and 10 ºC. After removing the supernatant plasma, the remaining pellet of leukocytes and red blood cells was suspended and incubated for 20 seconds with 10 mL aqua dist. Followed by the addition of 10 mL of 2-times concentrated PBS. This lysis step was repeated 2-3 times (centrifugation at 1550 rpm, 1100 rpm, and 100 ×g for 10 min at 10 ºC) until complete removal of RBCs. Finally, the cell pellet was suspended in PBS and adjusted to 5×10^6 cell/mL. Cell viability (always more than 95%) was evaluated by flow cytometry after adding propidium iodide (2 µg/mL) to the cells.

Membrane immunofluorescence and flow cytometry

Separated blood leukocytes were labeled with monoclonal antibodies (mAbs) to selected leukocyte antigens and analyzed by flow cytometry (Eger et al., 2015; Hussen et al., 2013). Separated cells (5×10^5 cell/well) were incubated in the wells of a 96-well plate with mAbs to cluster of differentiation CD9, CD5, CD3, CD206, CD46, CD163, and the granulocyte marker CH138A (Table 1). After incubation at 4°C for 15 minutes, cold PBS was added to the cells (150 µL per well) to wash out unbound antibodies. After that, cells were stained with secondary fluorochromes-labeled anti-mouse antibodies (Invitrogen) or with isotype controls (Becton Dickinson Biosciences). After washing, cells were analyzed on flow cytometry (Becton Dickinson Accuri C6 flow cytometer San Jose, California, USA). At least 100000 cells were measured and analyzed with C-Flow.

Statistical analysis

Data analysis was performed using the flow cytometric software C-Flow (Becton, Dickinson; Accuri C6 Software 1.0.264.21 BD, USA). Means and standard error of the mean (SEM) were calculated using the column statistic function of the Prism software (GraphPad version 5; California, USA). Differences between means were tested with a t-test (for repeated measures), with p value of less than 0.05 considered significant.

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Table 1. List of monoclonal antibodies involved in the study to test their reactivity with camel antigens

Antigen	Labeling	Antibody clone	Target species	Source
CD9	-	LT86A	Bovine	Kingfisher, USA
CD9	PE	Hl9a (V P018)	Bovine	BioLegend, UK
CD8a	-	ETC142BA1	Equine	Kingfisher, USA
CD46	FITC	MEM-258	Bovine	Biorad, UK
CD3	FITC	CD3-12	Mouse	Abcam, USA
CD3	-	T3/2/16A9	Human	Mybiosource, USA
CD206	PE	MMR	Human	BioLegend, USA
Granulocyte	-	CH138A	Bovine	Kingfisher, USA
CD5	-	ST1	Ovine	Kingfisher, USA

CD: Cluster of differentiation, PE: Phycoerythrin, FITC: Fluorescein isothiocyanate.

RESULTS AND DISCUSSION

Monoclonal antibodies to cell surface antigens are valuable tools for characterizing immune cells and monitoring changes in immune cell phenotype during infection or vaccination (Maecker et al., 2012). Although some subsets of camel immune cells can be identified using cross-reactive antibodies (Hussen et al., 2017), comprehensive immunophenotyping of camel immune cells requires the identification of other monoclonal antibodies to extend the toolbox for the characterization of important cell populations and subpopulations.

The cell marker CD9 is one of the tetraspanin family with a key role in the essential cellular functions of many immune cells and endothelial cells (Kinashi, 2005; Veenbergen and van Spriel, 2011; Rocha-Perugini et al., 2014). In the present study, two clones of antibodies (Hl9a and LT86A) against bovine CD9 showed reactivity with camel leukocytes (Figure 1A-B). Although both clones showed the same expression pattern with significantly higher ($p < 0.05$) expression on camel monocytes compared to lymphocytes and granulocytes, the staining intensity with the Hl9a clone was significantly (6 times) higher than the LT86A clone ($p < 0.05$). A possible explanation for this difference is the magnification of the fluorescence signal due to the use of an indirect membrane immunofluorescence test to detect the unlabeled Hl9a mAb, while the LT86A mAb was directly labeled with phycoerythrin (PE, Figure 1A-D). The current results confirm the previous reports about the wide distribution of CD9 on human leukocyte subsets, including B cells, helper CD4+ T cells, cytotoxic CD8+ T cells, natural killer cells, and myeloid cells (Wright et al., 2004; Tohami et al., 2004; Pugholm et al., 2016) and indicates similar expression pattern of CD9 on camel and human leukocytes. Given its key role in several functions of different immune cells, the identification of two monoclonal antibodies with cross-reactivity against camel CD9 will enable conducting functional studies to uncover its role in the camel immune system.

The characterization of T cell subpopulations is usually achieved by the combined staining with mAbs to the pan-T cell marker CD3 and the T cell co-receptors CD4 and CD4 to identify helper and cytotoxic T cells, respectively. In the present study, the anti-mouse CD3 mAbs clone CD3-12 and the anti-human CD3 mAb clone (T3/2/16A9) did not show any reactivity with camel lymphocytes, indicating no cross-reactivity with the corresponding camel CD3 antigen (Figure 2). Similarly, an anti-equine CD8alpha mAb (clone: ETC142BA1) did not label any cell population within camel lymphocytes (Figure 2).

Currently, the identification of camel myeloid cell subsets depends on using mAbs to the pan-myeloid marker CD172a, the monocyte markers CD14 and CD163 in combination with the major histocompatibility complex (MHC) molecules II. Furthermore, camel leukocytes did not show reactivity to monoclonal antibodies to the bovine pan-granulocyte marker (clone: CH138A), the human macrophage marker CD206 (clone: MMR, Figure 2), and the bovine CD46 antigen (MEM-258).

The transmembrane receptor CD5 is a 67 kDa type 1 T cell surface protein with key regulatory roles in T cell activation and development (Raman, 2002). In humans and mice, CD5 expression has been considered limited to cells of the lymphoid lineage, mainly on thymocytes, T, and B lymphocytes (Li et al., 2019). Recently, CD5 was detected in dendritic cells from human blood (Wood and Freudenthal, 1992; Li et al., 2019; He et al., 2023) and monocyte-derived dendritic cells and macrophages (Takahashi et al., 1998; Helft et al., 2015). In the present study, camel lymphocytes showed no reactivity with the anti-ovine CD5 mAb (clone: ST1). On the other hand, the antibody showed positive staining for camel monocytes that were identified by their CD163 expression. This is in contrast to the negative staining of monocytes to the isotype control antibodies (Figure 3A-E). Whether the expression of CD5 on camel monocytes represents a camel-specific expression pattern of CD5 or whether the antibody nonspecifically recognizes an epitope on camel monocytes could not be answered based on the data of the current study. To clarify this, blocking FC receptors on camel monocytes and comparative staining of camel and human leukocytes with the CD5 antibody are required.

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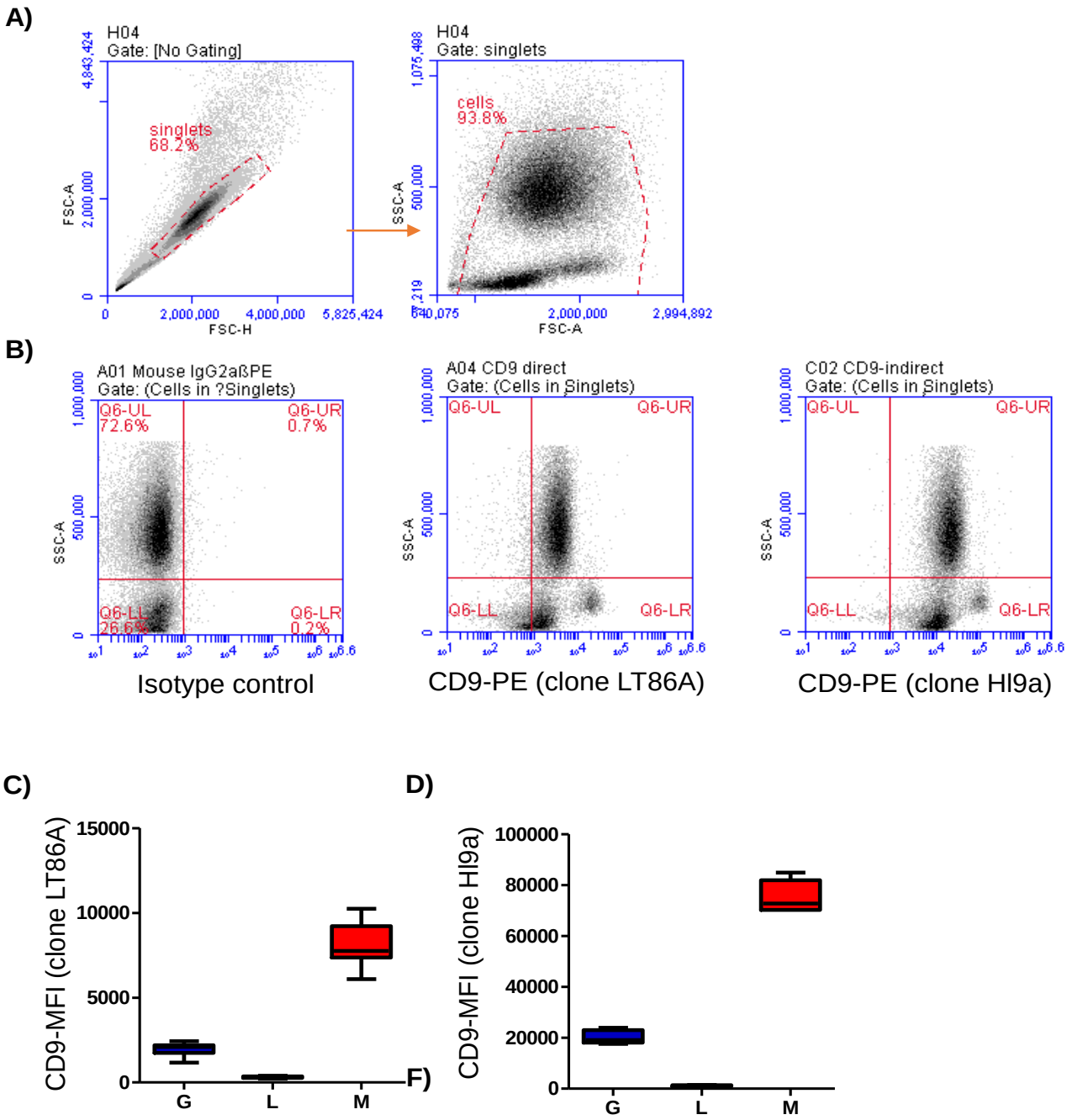

Figure 1. Reaction of camel immune cells toward mAbs against CD9 antigen. Cell duplicates were excluded from the analysis based on forward scatter (FSC)-H/FSC-A signals, and leukocytes were gated in a side scatter (SSC)-A/FSC-A dot plot to exclude cell debris (**A**). Staining of leukocytes with isotype control antibody or with CD9-specific antibodies of the clone LT86A or Hl9a (**B**). Mean fluorescence intensity (MFI) values were presented for granulocytes, lymphocytes, and monocytes (n= 5 animals) were presented for the clone LT86A (**C**) or Hl9a (**D**). The letters G, L, and M indicate granulocytes, lymphocytes, and monocytes, respectively.

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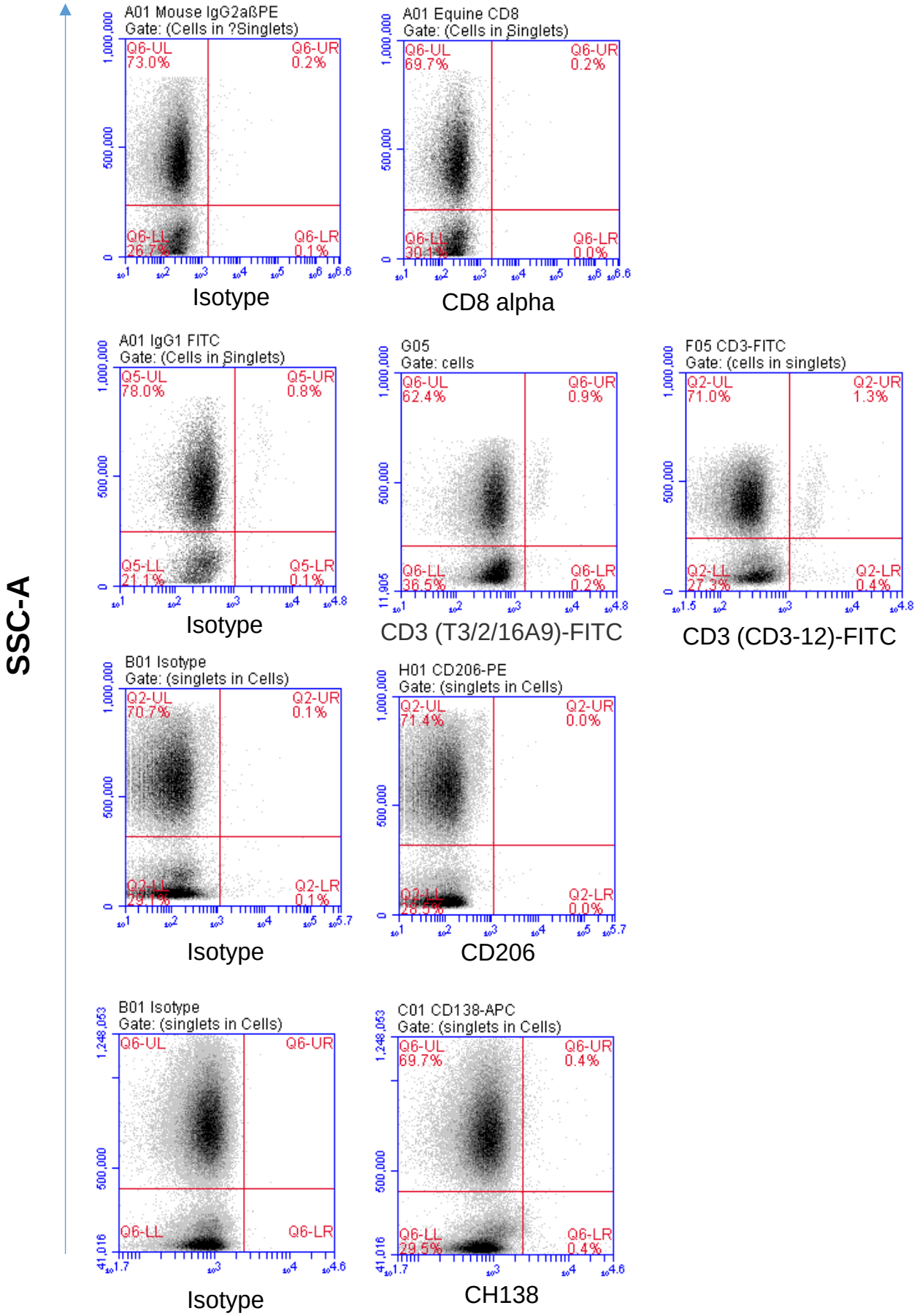

Figure 2. Staining of camel leukocytes with monoclonal antibodies to selected leukocyte antigens from other species or with isotype antibody controls. Camel leukocytes were labeled with indicated antibodies and analyzed on the accuri cytometer. Representative side scatter against staining (fluorescence intensity) dot plots of five separate experiments.

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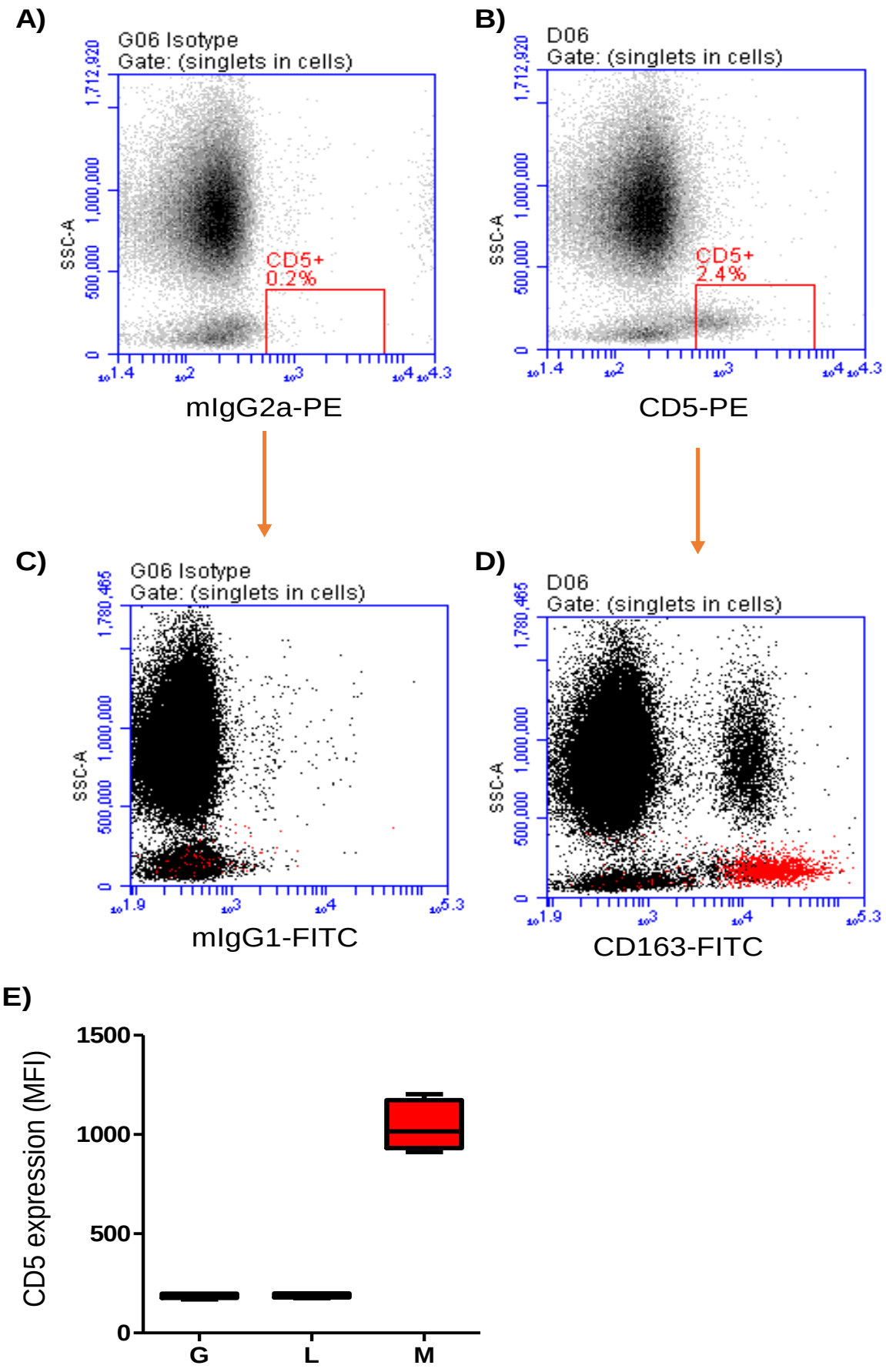

Figure 3. Reactivity of camel leukocytes with isotype control (**A**) or specific antibodies against ovine CD5 (**B**). A gate was set on CD5+ cells (red gate), and positive cells were marked in red in a separate dot plot of mouse IgG1 isotype control (**C**) or CD163 (**D**), indicating the co-expression of CD163 and CD5 on monocytes. **E**: Mean fluorescence intensity values of CD5 expression were presented for camel leukocyte subsets. The letters G, L, and M indicate granulocytes, lymphocytes, and monocytes, respectively.

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CONCLUSION

The present study tested the reactivity of camel leukocyte subsets with some commercially available monoclonal antibodies for leukocyte antigens from other species. The expression patterns of the tested antibodies indicate cross-reactivity of the anti-bovine CD9 monoclonal antibody clones LT86A and Hl9a with different binding potential. In contrast, no cross-reactivity was identified for the anti-equine CD8a mAb (ETC142BA1), anti-mouse CD3 mAb (CD3-12), anti-human CD3 mAb (T3/2/16A9), anti-human CD206 mAb (MMR), anti-bovine granulocytes mAb (CH138A). Only camel monocytes showed positive staining with the anti-ovine CD5 mAb (clone ST1), in contrast to the human and murine systems. The present study indicated low homogeneity between camels and other species in the antigenic structure of leukocyte antigens. It strengthened the need to develop camel-specific mAbs against the main immune cell markers. Further studies may focus on using the identified monoclonal antibodies for phenotypic and functional studies on the camel immune system.

DECLARATIONS

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

Mohammed Ameer Alalai did sample collection and manuscript revision; Mayyadah Abdullah Alkuwayti did supervision and manuscript preparation; Noof Abdulrahman Alrabiah did manuscript preparation and revision. Jamal Hussen did the analysis, funding acquisition, and writing of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

There are no competing interests to disclose.

Ethical consideration

Animals ethical approval was obtained from the Ethics Committee of King Faisal University, Saudi Arabia, with an approval number (KFU-REC-2021- DEC -EA000326). Ethical issues, such as data fabrication, double publication, and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal.

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The Composition of Zoophilic Fly Species in Eastern Ukraine

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ABSTRACT

Zoophilic Diptera plays a leading role in the epizootic foci formation of many infectious and parasitic diseases and directly affects the quality of livestock products. The current study aimed to analyze the number and species composition of parasitic Diptera in industrial, farm, and homestead agrobiocenoses of large and small cattle, pig, and poultry farms in Eastern Ukraine. The research involved entomological collection during the peak activity daylight hours in early May, July, and early September 2021-2022 per farm. A total of 360 entomological collections were made, and 4310 zoophilous flies were examined. In livestock farms of five districts of the Kharkiv region of Ukraine, 28 species of zoophilic flies were registered, among which *Musca domestica*, *Muscina stabulans*, *Stomoxys calcitrans*, *Lucilia sericata*, *Protophormia terraenovae*, and *Drosophila* species were dominant species. The analysis revealed that cattle biocenoses hosted 27 fly species, pigs had 8 species, and poultry and small cattle each had 7 species. The study indicated an increase in the population of *Musca autumnalis*, the main species in the pastures, near livestock premises during the summer. *Stomoxys calcitrans* was also recorded in livestock agrobiocenoses. The species *Musca domestica*, *Musca autumnalis*, and *Stomoxys calcitrans* account for 78.8% to 88.3% of the entire complex of zoophilous flies. The two species of *Ortellia caesarion* (shiny dung beetle) and *Ortellia cornicina* (green dung beetle), known for their role as manure mineralizers and deemed non-threatening to animals, were completely absent during the research period. The findings indicated the species of *Eristalis tenax* in agrobiocenoses in 2021. Therefore, it can be concluded that zoophilic flies are physical irritants to animals and potential carriers of many infectious diseases, especially diseases caused by unicellular organisms.

Keywords: Biotopes, *Musca autumnalis*, *Musca domestica*, *Stomoxys calcitrans*, Zoophilic flies

INTRODUCTION

Animal health and production are critical factors in the agricultural industry, and the health of livestock directly affects its productivity and, in turn, farmers' income (Nanka et al., 2018; Hernandez-Patlan et al., 2023). Ensuring stable epizootic well-being is also an integral part of the livestock industry. Despite the successes achieved in the fight against animal diseases of various etiologies, the issue of controlling ectoparasites, studying their biodiversity, and developing new means of eradication remain relevant today (Belluco et al., 2023). Stable flies, *Stomoxys calcitrans* (Linnaeus, 1758), are significant threats to humans and animals worldwide (Rochon et al., 2021). Studies have indicated that in individual animal herds infested with flies, the average annual loss of productivity per animal was 139 kg of milk for dairy cows, 6.26 of body weight for preweaning calves, and 9 kg of body weight for fattening cattle (Taylor et al., 2012; Narladkar, 2018).

Research on the biodiversity of flies (Diptera) was conducted in different ruminant farming systems in Poland (Nosal et al., 2019). Moreover, studies in farms in Romania have investigated the role of the intermediate host *Musca autumnalis* (*M. autumnalis*) in the spread of equine thelasiosis (Cotuțiu et al., 2022).

Flies are permanent residents of livestock premises, farm territories, and pastures. Large accumulations of cattle manure in feedlots, wet grain feed, and unprotected silage lead to an increase in stable fly population within the livestock industry (Cook et al., 2018). Hematophages, which are direct animal pests, are particularly important for veterinary medicine (El Ashmawy et al., 2021). When parasitizing directly on animals, flies cause discomfort, anxiety, and irritability, negatively affecting their productivity (Machtinger et al., 2021). In addition, these insects can serve as carriers of many pathogens (Khamesipour et al., 2018). Houseflies can acquire and transmit various enteric bacterial pathogens, such as *Salmonella* and *Campylobacter* (Thomson et al., 2021). *Musca domestica* (*M. domestica*) plays a significant role in the dissemination of bacteria resistant to antimicrobial drugs (Bertelloni et al., 2023). Houseflies carry

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clonal lines of multidrug-resistant bacteria identical to those found in animal feces. (Zurek and Ghosh, 2014). Moreover, 26 types of pathogens causative agents of bovine mastitis were isolated from flies (Gioia et al., 2022).

The housefly *M. domestica* (Linnaeus, 1758) is known for its ability to carry exogenous forms of ascarids, esophagostoma, and hookworm on its body (Förster et al., 2009; Otu-Bassey et al., 2022), and *M. autumnalis* is an intermediate host of *Parafilaria bovicola* (Hund et al., 2021). The *M. domestica* may act as as a potential mechanical vector or reservoir in the epidemiology of bovine leukemia virus (Panei et al., 2019) and contagious ovine ecthyma (Raele et al., 2021).

To combat zoophilic parasites in animal husbandry, many insecticides and repellents have been developed and proposed, which differ in their method of application and the active substances they contain. However, their widespread and uncontrolled use leads to the emergence of resistance generations of insects, creating uncontrolled environmental risks in limited areas (Espinoza et al., 2021). Today, litigation against livestock producers associated with pest filth flies has become more frequent and has a high profile (Machtinger and Burgess, 2020). Thus, the timely and scientifically based control of parasitic dipterans in livestock biocenoses is of great sanitary, epizootological, epidemiological, and social importance. Successful implementation of antiparasitic measures is possible only with a preliminary study of the species composition and number of zoophilous dipterans. This study aimed to study the species composition of zoophilic flies in various livestock biocenoses of Eastern Ukraine.

MATERIALS AND METHODS

Ethical approval

The current study followed the current legislation of Ukraine (Article 26 of the Law of Ukraine 5456-VI of 16.10.2012 "On the Protection of Animals from Cruel Treatment)" and international biotic norms (materials of the IV European Convention on the Protection of Vertebrate Animals, which are for experimental and other purposes, Strasbourg, 1985) (Simmonds, 2018). The research program was reviewed and approved by the bioethics commission of the Institute of Animal Husbandry of the National Academy of Sciences (Kharkiv, Ukraine) in the current order.

Sampling time and study design

The collection of insects was carried out on the premises of industrial, farm, and homestead farms on walking platforms in summer camps, places of mass grazing of animals in five districts of the Kharkiv region (Bogoduhivskyi, Krasnogradskyi, Lozivskyi, Kharkivskyi, and Izyumskyi) in Ukraine (Figure 1).

Entomological collection was carried out in each household in three time periods of early May, July, and early September 2021-2022 during daylight hours during mass fly activity. In each district, one industrial, farm, and home farm was surveyed, where different types of animals were kept. A total of 360 entomological collections were held.

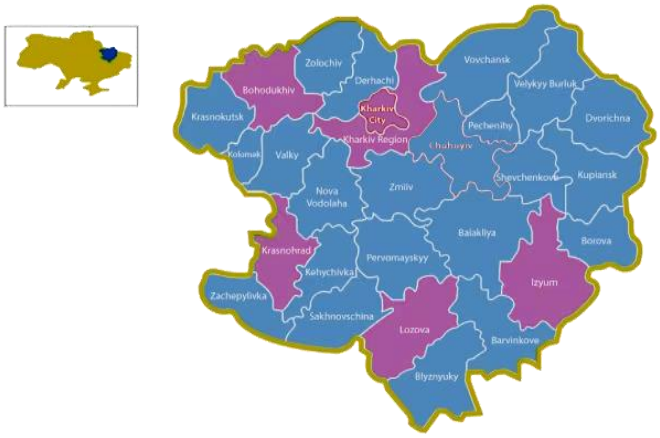

Figure 1. The studied regions in the Kharkiv, Ukraine

Entomological analysis

According to the generally accepted methods used in ecological and entomological studies, a survey of livestock agrobiocenoses was carried out for the presence of dipterans (McCravy, 2018; Marchioro et al., 2020). Diptera field collections were conducted according to generally accepted methods used in entomology (Lamarre et al., 2018). Insects were collected from various sources, including the skin surface of animals both indoors and on pastures, as well as from windows and other structures of livestock premises. During the active flight of insects, the collection was carried out with the help of an entomological trap by the method of mowing (the selection of standard samples of 50 sweeps is foreseen, which made it possible to calculate the average indicators of the qualitative and quantitative composition of insects with different biotopes). The activity and dynamics of insect attacks on animals were recorded with a digital camera (GoPro HERO12 Black Creator Edition, CHDFB-121-EU, China), and subsequently, the number of insect adults was counted on a computer monitor. The 4310 collected insects were fixed in 70% ethanol and delivered to the laboratory for further identification and quantification. Collected and caught insects were identified using the identifier atlas (Sorokina and Pont, 2010; Gregor et al., 2016; Lendzele et al., 2019).

Data analysis

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The dominance of Diptera (D) was estimated according to the following formula.

Dominance = (number of one species/total number of species) × 100%

RESULTS AND DISCUSSION

Regarding the diversity of the species of parasitic insects in the agricultural biotopes of animals in five districts of the Kharkiv region in Ukraine, the obtained results indicated the presence of 28 species of zoophilic flies. A different composition of zoophilic parasites could depend on the species of animals and the season (Table 1). According to the research results, 28 species of flies were identified in 2021, of which 21 belong to the Muscidae family. Among these, the highest diversity of flies was observed in cattle biocenoses, particularly the Ukrainian black and spotted breeds (27 species), followed by large white pig breeds (8 species). In contrast, poultry (Rhode Island white breed) and small cattle (Ascanian blackhead breed) exhibited the lowest diversity, with 7 species each.

In the subsequent insect collections in 2022, 10 species of zoophilous flies were found in the biocenoses of small cattle, while 11 species were found in the biocenoses of poultry. During 2021-2022 insect collections, *Lucilla sericata* (*L. sericata*) was found in all the investigated livestock farms despite its small number. Species, such as *M. domestica, Stomoxys salcitrans, Protophormia terraenovae, Muscina stabulans, L. sericata, Drosophila* spp., were found in all livestock premises regardless of the type of animals kept there. Notably, *Ortellia caesarion* was absent in the 2021-2022 insect collections. Therefore, the main species from the Muscidae family under modern climatic conditions were *M. domestica, M. autumnalis, Stomoxys calcitrans*, and *Muscina stabulans*. According to the results of entomological collections, an analysis of the frequency of distribution of zoophilous dipterans among cattle was carried out, depending on their species and the place of parasitism (Table 2).

Table 1. Species composition and abundance of the main species of zoophilous flies in agrobiocenoses of the Kharkiv region during 2021-2022

Kind of insects	2021				2022			
	Cattle	Pigs	Small cattle	Poultry	Cattle	Pigs	Small cattle	Poultry
Calliphora vicina (Robineau-Desvoidy, 1830)	+	–	+	–	+	–	+	+
Drosophila spp.	+	+	–	+	+	+	+	+
Eristalis tenax (Linnaeus, 1758)	–	+	–	–	–	+	–	–
Fannia canicularis (Linnaeus, 1761)	+	+	–	+	+	+	–	+
Fannia scalaris (Fabricius, 1794)	+	+	–	+	+	+	–	+
Haematobia atripalpis (Bezzi,1895)	+	–	–	–	+	–	–	–
Haematobia stimulans (Meigen, 1824)	+	–	–	–	+	–	–	–
Hydrotaea dentipes (Fabricius, 1805)	+	–	–	–	+	–	–	–
Lucilia caesar (Linnaeus, 1758)	+	–	–	–	+	–	–	+
Lucilia sericata (Meigen, 1826)	+	+	–	+	+	+	+	+
Lyperosia irritans (Linnaeus, 1758)	+	–	+	–	+	–	+	–
Lyperosia titilans (Bezzi)	+	–	–	–	+	–	–	–
Mesembrina meridiana (Linnaeus, 1758)	+	–	–	–	+	–	–	–
Morellia hortorum (Fallén, 1817)	+	–	–	–	+	–	–	–
Morellia simplex (Loew, 1857)	+	–	–	–	+	–	–	–
Musca amita (Linnaeus, 1771)	+	–	–	–	+	–	–	–
Musca autumnalis (De Geer, 1776)	+	–	+	–	+	+	+	+
Musca domestica (Linnaeus, 1758)	+	+	+	+	+	+	+	+
Musca larvipara (Linnaeus, 1758)	+	–	–	–	+	–	–	–
Musca osiris (Wiedemann, 1830)	+	–	–	–	+	–	–	–
Musca tempestiva (Fallén, 1817)	+	–	–	–	+	–	–	–
Musca vitripennis (Meigen, 1826)	+	–	–	–	+	–	–	–
Muscina assimilis (Fallen, 1823)	+	–	–	–	+	–	–	–
Muscina stabulans (Fallén, 1817)	+	+	–	+	+	+	+	+
Ortellia cornicina (Fabricius, 1805)	+	–	–	–	+	–	–	–
Protophormia terraenovae (Robineau-Desvoidy, 1830)	+	+	+	–	+	+	+	+
Stomoxys calcitrans (Linnaeus, 1758)	+	+	+	+	+	+	+	+
Wohlfahrtia magnifica (Schiner, 1862)	+	–	+	–	+	–	+	–

+: The presence of a species of Diptera, –: The species is missing

Table 2. Species composition and abundance of the main species of zoophilic flies in industrial cattle agrobiocenoses of the Kharkiv region during 2021-2022

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Type of flies	Number	Percentage	Biotopes					
			Pastures		Summer camp		Livestock premises	
			Number	Percentage	Number	Percentage	Number	Percentage
Calliphora vicina (Robineau-Desvoidy, 1830)	1	0.02	–	–	–	–	1	0.08
Drosophila species	40	0.92	–	–	16	0.7	24	1.9
Fannia canicularis (Linnaeus, 1761)	1	0.02	1	0.15	–	–	–	–
Fannia scalaris (Fabricius, 1794)	38	0.9	–	–	5	0.2	33	2.7
Haematobia atripalpis (Bezzi, 1895)	1	0.02	1	0.15	–	–	–	–
Haematobia stimulans (Meigen, 1824)	2	0.05	1	0.15	1	0.04	–	–
Hydrotaea dentipes (Fabricius, 1805)	1	0.02	1	0.15	–	–	–	–
Lucilia caesar (Linnaeus, 1758)	1	0.02	–	–	–	–	1	0.08
Lucilia sericata (Meigen, 1826)	1	0.02	–	–	–	–	1	0.08
Lyperosia irritans (Linnaeus, 1758)	161	3.7	72	10.5	66	2.8	23	1.8
Lyperosia titilans (Bezzi)	50	1.2	27	3.9	23	0.9	–	–
Mesembrina meridiana (Linnaeus, 1758)	1	0.02	1	0.15	–	–	–	–
Morellia hortorum (Fallén, 1817)	48	1.1	29	4.2	18	0.8	1	0.08
Morellia simplex (Loew, 1857)	1	0.02	1	0.15	–	–	–	–
Musca amita (Linnaeus, 1771)	1	0.02	1	0.15	–	–	–	–
Musca autumnalis (De Geer, 1776)	813	18.9	326	47.7	482	20.2	5	0.4
Musca domestica (Linnaeus, 1758)	1624	37.7	16	2.34	792	33.2	816	65.9
Musca larvipara (Linnaeus, 1758)	115	2.6	68	9.9	46	1.9	1	0.08
Musca osiris (Wiedemann, 1830)	1	0.02	1	0.15	–	–	–	–
Musca tempestiva (Fallén, 1817)	1	0.02	1	0.15	–	–	–	–
Musca vitripennis (Meigen, 1826)	1	0.02	1	0.15	–	–	–	–
Muscina assimilis (Fallen, 1823)	1	0.02	1	0.15	–	–	–	–
Muscina stabulans (Fallén, 1817)	22	0.51	–	–	10	0.4	12	0.9
Ortellia caesarion (Meigen)	5	0.11	5	0.7	–	–	–	–
Ortellia cornicina (Fabricius, 1805)	7	0.16	7	1.0	–	–	–	–
Protophormia terraenovae (Robineau-Desvoidy, 1830)	1	0.02	1	0.15	–	–	–	–
Stomoxys calcitrans (Linnaeus, 1758)	1368	31.7	120	17.5	928	38.8	320	25.8
Wohlfahrtia magnifica (Schiner, 1862)	1	0.02	1	0.15	–	–	–	–
Total	4310	100	683	100	2389	100	1238	100

It has been proven that the species composition of zoophilic flies is represented by different families. The Muscidae family had the most numerous in the general structure of insects. The occurrence index of the house fly (*M. domestica*) was 37.7%, and this species occupied the main livestock biotopes and was dominant (65.9%) indoors. It was established that in the case of violations of sanitary conditions in animal husbandry premises, the number of house fly adults that attack one animal at the same time ranged from 200 to 300 in specimens.

The number of *Stomoxys calcitrans* was within 31.7%, and it was also observed in all the main livestock biotopes. This species of fly-on animals were registered in 60-90 specimens. *Musca autumnalis* was also recorded in the main biotopes, and its abundance was 18.9%. At the same time, it was the dominant parasitic species on pastures, accounting for 47.7%, and its number per animal ranged from 40 to 335 individuals. These three main species of flies accounted for 88.3% of the entire complex of zoophilic parasites. It should also be noted that the species *M. larvipara* (2.6%), *L. irritans* (3.7%), *M. hortorum* (1.1%), *L. titillans* (1.2%), *Muscina stabulans* (0.5%), and *F. scalaris* (0.9%) occupied a certain place in parasitocenosis and formed the main species composition of zoophilic flies. The populations of other species of flies were small and did not have notable effects on farm animals in the general biocenosis. Along with industrial biocenoses of cattle, parasitism of zoophilous dipterans was noted in other maintenance centers, namely, farm and homestead farms (Table 3).

The species composition of zoophilous flies in specialized farms in 2021 included 9 species, and in homesteads, there were 10 species. The three main species of flies accounted for 83.7% and 64.3%, respectively, of the entire complex of zoophilic parasites. In 2022, three species (*M. domestica, Stomoxys calcitrans,* and *M. autumnalis*) accounted for 78.9% and 63.2%, respectively. Such species, including *Muscina stabulans, Fannia canicularis, Fannia scalaris,* and *Protophormia terraenovae,* were 2-4 times more abundant in homestead farms than specialized arms. This is because these types of flies are more synanthropic, and their number depends on the sanitary condition of the territories. The data does not include 11 species of flies caught in one specimen, and the species of *L. sericata* was present in one specimen in collections from both specialized farms and homestead farms. The species and numerical composition of mass species of zoophilic flies in pig farms are presented in Table 4.

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Table 3. Species composition and abundance of the main species of zoophilic flies in specialized and homestead agrobiocenoses of cattle farms in Kharkiv region during 2021-2022

Type of flies	2021				2022			
	Specialized farms		Homestead farms		Specialized farms		Homestead farms	
	Imago. number	Percentage	Imago. number	Percentage	Imago. number	Percentage	Imago. number	Percentage
Musca domestica	184	57.5	123	41.4	206	59.4	205	49.8
Stomoxys calcitrans	56	17.5	47	15.8	48	13.8	40	9.8
Musca autumnalis	28	8.7	21	7.0	20	5.7	15	3.6
Muscina stabulans	13	4.0	24	8.0	12	3.5	41	9.9
Fannia canicularis	–	–	19	6.4	4	1.2	4	0.9
Fannia scalaris	6	1.8	28	9.4	8	2.3	18	5.1
Protophornia terraenovae	4	1.2	17	5.7	5	1.4	25	6.0
Drosophila spp.	18	5.6	18	6.0	25	7.2	36	8.6
Liperosia irritans	11	3.4	–	–	14	4.0	8	1.9
Lucilia sericata	1	0.3	1	0.3	5	1.4	19	4.6
Total	321	100	298	100	347	100	411	100

Table 4. Species and numerical composition of species of zoophilous flies in pig farms of the Kharkiv region during 2021-2022

Type of flies	2021				2022			
	Industrial farms		Homestead farms		Industrial farms		Homestead farms	
	Imago. number	Percentage	Imago. number	Percentage	imago. number	Percentage	imago. number	Percentage
Musca domestica	241	57.9	217	46.5	203	51.5	192	46.9
Muscina stabulans	24	5.7	72	15.4	21	5.3	64	15.6
Stomoxys calcitrans	87	20.9	65	13.9	96	24.3	51	12.5
Fannia scalaris	–	–	34	7.4	–	–	21	5.1
Fannia canicularis	7	1.7	37	7.9	3	0.8	11	2.7
Protophormia teraenovae	–	–	18	3.8	3	0.8	23	5.6
Drosophila spp.	33	7.9	23	4.9	31	7.9	38	9.3
Eristalis tenax	24	5.7	–	–	25	6.3	–	–
Musca autumnalis	–	–	–	–	1	0.2	1	0.2
Lucilia sericata	11	3.4	1	0.2	11	2.7	8	1.9
Total	416	100	467	100	394	100	409	100

Table 5. Species and numerical composition of mass species of zoophilic flies in poultry farms of the Kharkiv region during 2021-2022

Type of flies	2021				2022			
	Industrial farms (cage maintenance)		Homestead farms		Industrial farms (cage maintenance)		Homestead farms	
	Imago. number	Percentage	Imago. number	Percentage	Imago. number	Percentage	Imago. number	Percentage
Musca domestica	312	93.1	332	81.3	239	59.5	222	50.5
Muscina stabulans	1	0.3	22	5.3	14	3.5	48	10.9
Musca autumnalis	–	–	–	–	2	0.5	18	4.1
Stomoxys calcitrans	–	–	–	–	6	1.5	21	4.8
Fannia scalaris	–	–	4	0.9	15	3.7	11	2.5
Fannia canicularis	1	0.3	7	1.7	3	0.7	9	2.0
Protophormia teraenovae	14	4.1	18	4.4	63	15.6	53	12.1
Calliphora vicina	–	–	–	–	25	6.2	18	4.1
Lucilia sericata	6	2.0	15	3.6	11	2.7	8	1.8
Lucilia caesar	–	–	–	–	4	0.9	4	0.9
Drosophila species	1	0.3	10	2.4	22	5.4	27	9.3
Total	335	100	408	100	404	100	439	100

In industrial pig farms in 2021, the composition of mass zoophilous flies was seven, and eight were in homesteads. The dominant species were *M. domestica* and *Stomoxys calcitrans*, which accounted for 78.8% of the total flies in industrial farms and 60.4% in homestead farms. The number of flies in the synanthropic complex was 21.2% and 39.6% in industrial and homestead farms, respectively. In the 2022 collection, nine species of flies were recorded on farms, and the total numbers of *M. domestica* and *Stomoxys calcitrans* were 75.8% and 59.4%, respectively. The number of flies of the synanthropic complex increased to 24.2% in industrial farms and 40.6% in homestead farms, compared to the previous year. A notable number of *Eristalis tenax* (5.7%) was observed in industrial pig farms, and their larvae were found in large numbers in liquid manure. The species and numerical composition of mass species of zoophilous flies in poultry farms are presented in Table 5.

In 2021, 7 species of flies were recorded in poultry houses during entomological collection. In the same setting in 2022, the number of recorded species increased to 11. *Musca domestica* (93.1%) was the dominant species in poultry houses with caged birds in 2021, followed by *Protophormia teraenovae* (4.1%), *L. sericata* (2.0%), and other species collectively accounted for 0.9%. In the vicinity of poultry houses, the dominant species was *M. domestica*, with a

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dominance index of 81.3%, in 2021. The subdominant species were *Muscina stabulans* (5.3%), *Protophormia teraenovae* (4.4%) and *L. sericata* (3.6%). In 2022, *M. domestica* was the dominant species (59.5%) on industrial farms and 50.5% on homesteads. The subdominant species was *Protophormia teraenovae*, accounting for 15.6% of industrial farms and 12.1% of homestead farms.

DISCUSSION

Animal productivity is directly influenced by the technology of their keeping, genetic potential, and epizootological status of the farms. Additionally, the hygiene of livestock product production plays a crucial role, with the parasitic component posing a significant challenge (Aliiev et al., 2022; Pavlenko et al., 2023). Insects, due to their widespread distribution and ability to occupy various ecological niches, as well as the strict and sometimes forced coexistence of insects and humans, are of significant concern for public health (Belluco et al., 2023). House flies (*M. domestica*) are ubiquitous insects that live in close contact with humans and farm animals (Nayduch et al., 2023). Due to their behavior and life cycle, these insects can easily become infected with bacteria, becoming mechanical carriers of potentially pathogenic microorganisms (Bertelloni et al., 2023). The present study used modern methodical approaches for catching insects, which increased the accuracy of the findings.

Research conducted from 2000 to 2020 on the territory of Ukraine among agricultural biocenoses registered 27 species of dipterous insects, among which species of the Muscidae family dominated (74.1%). The largest number of parasitic Diptera was found in livestock premises for keeping cattle, and the least number of species were found in premises for keeping poultry (Paliy et al., 2021a). Considering pig farms, the largest number of zoophilic flies can be found in premises where sows with suckling piglets and animals are designated for fattening (Paliy et al., 2021b).

In a study performed by Domatskiy et al. (2021), more than 120 species of insects came into contact with animals, and of this number, 92 species were found in pastures, 57 in cowsheds, 48 in pig houses, and 27 in stables. Moreover, 30 most harmful species of flies (5 species of stable flies, 4 bloodsuckers, 16 licking, and 5 species of flies) caused myiasis in animals. In another study, Machtinger and Burgess (2020) found that the dominant species in pig production was the housefly *M. domestica* L. (Diptera: Muscidae), followed by the black landfill fly *Hydrotaea aenescens* (Wiedemann) (Diptera: Muscidae).

The abundance of *M. domestica* throughout the year was relatively high although some fluctuations were noted in winter. The reason is that if all sanitary standards for manure collection are observed in livestock premises, there are still hidden breeding places for flies (Paliy et al., 2020a).

The stable fly *Stomoxys calcitrans* is a major blood-sucking pest of livestock that is distributed almost worldwide. The fight against these flies was limited to strengthening sanitary measures and using insecticides to suppress larval stages (Cook et al., 2018). The species *Stomoxys calcitrans* displaces *Haematobia stimulans* and *Haematobia atripalpis* from its habitat (Olafson et al., 2021).

Musca domestica, M. autumnalis, M. larvipara, Stomoxys calcitrans, Haematobia stimulans, and *Haematobia atripalis* were identified as the main parasitic species among zoophilic flies in the northwestern region of Ukraine (Katiukha, 2020). Other researchers found that the greatest number of dipterans in livestock biocenoses was made up of insects from the family *Drosophilidae* (61.4%), and the dominant species of *Muscidae* were *M. domestica* (19.2%) and *Stomoxys calcitrans* (5.7%). At the same time, more than half (55.5%) of all flies were caught in the morning, the vast majority of them (71.8%) were near livestock premises, and 28.2% were in the middle of the premises (Nosal et al., 2019).

A total of 22 species of Diptera were found on pig farms in Estonia, of which 96.6% belonged to the order of true flies (Diptera insects) (Tummeleht et al., 2020). It has been reported that the number of zoophilic parasites directly depends on the species of animals, diet, season of the year, and the use of insecticides on the farm (El Ashmawy et al., 2021). It should be noted that parasitic dipterans could negatively affect the crop industry (Saurabh et al., 2021). Therefore, the spread of dipterans in animal husbandry is an urgent problem that requires the development of innovative means and methods for their control, taking into account modern ecological requirements (Saini et al., 2017; Lakew et al., 2021).

CONCLUSION

A total of 28 species of zoophilic flies were registered in livestock farms of five districts of the Kharkiv region of Ukraine, among which *M. domestica*, *Muscina stabulans*, *Stomoxys calcitrans*, *L. sericata*, *Protophormia terraenovae*, and *Drosophila* species dominated. In the biocenoses of cattle, the population of flies is represented by 27 species, in pigs by 8 species, and the lowest in the biocenoses of poultry (n = 7) and small cattle (n = 7).

DECLARATIONS

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Authors' contributions

Mykola Bogach and Anatoliy Paliy participated in the data collection, analysis, preparation, and revision of the manuscript. Natalia Sumakova, Ludmila Perotska, and Denis Bohach were involved in the collection of data and laboratory analysis, while Olena Pavlichenko and Olena Bohach formatted and edited the manuscript. All the authors have read and approved the final manuscripts.

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Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

Availability of data and materials

The authors confirm that all the data of this study are available by request from the authors.

Competing interests

The authors have not declared any conflict of interest.

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Standardization of the Simple Methodology for Experimentally Induced Ischemic Stroke in Rat Models

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ABSTRACT

Stroke is a globally significant and devastating disease that requires prompt treatment. Animal models are commonly used to investigate stroke therapy, often through experimentally induced ischemic stroke (EIIS). However, challenges arise in implementing EIIS in animal models. The current study aimed to present a simple EIIS methodology for animal models. A total of 60 male Sprague-Dawley rats were randomly divided into five groups, namely Group 1 (sham-operated), Groups 2 to 5 (EIIS groups) with different duration of common carotid artery (CCA) ligation, including 1, 2, 4, and 8 hours, respectively. The ligation was performed on the CCA and its branches. Before the experiment, the rats were anesthetized, and the incision area was shaved and disinfected. The sagittal ventral midline was incised, with neck muscles retracted to expose the right CCA. The occlusion was performed on three sides of a carotid artery (common, external, and internal) using a simple interrupted suture. The occlusion of blood flow using ligation was performed at different times depending on the groups. After that, the CCA ligations were re-perfused by cutting the suture knot. The brain and blood were collected on days 1 and 7 after reperfusion. The results indicated that 4 and 8 hours of CCA ligation significantly impacted the general condition and neuro-deficit score. Moreover, 4 and 8 hours of CCA ligation could induce ischemic stroke by its capacity to cause infarction within the brain parenchyma and increase the platelet-to-white blood cell ratio, C-reactive protein, and De Ritis ratio. In contrast, 1 and 2 hours of CCA ligation did not significantly affect the observed parameters. It can be concluded that the EIIS using 4 and 8 hours of CCA ligation can be applied to induce ischemic stroke in rat models with consistent impacts on general conditions, neuro-deficit, hematology, and serology.

Keywords: Common carotid artery, Ischemic stroke, Ligation, Rat model, Standardization

INTRODUCTION

Stroke is a prevalent degenerative disease worldwide caused by the disruption of the circulatory system. The most common type of stroke is ischemic stroke (Yi et al., 2020), characterized by thromboembolism within a cerebral artery (Chugh, 2019). Recognized as a catastrophic disease, stroke significantly impacts human life, posing substantial social and economic challenges. In Indonesia, ischemic stroke has emerged as a leading financial burden, with an annual cost of IDR 2.56 trillion (Venketasubramanian et al., 2022). Hence, the ischemic stroke study is one of the major research topics of the Indonesian national research program.

Experimentally induced ischemic stroke (EIIS) studies on animal models have some shortcomings concerning the methodology and duration of middle cerebral artery occlusion (MCAO). Additionally, the study of EIIS in animal models encounters issues related to research parameters (Bacigaluppi et al., 2010). Several frequent procedures to induce ischemic stroke include craniotomy and middle cerebral artery (MCA) occlusion by inserting a nylon suture (Bertrand et al., 2017). Due to the limited number of veterinary surgeons in animal models, those methods are brutal to be conducted in developing countries, including Indonesia. Further, the MCAO duration impairs the volume of the infarct area (Narayan et al., 2021). Too short occlusion time impacts the minimum infarction area and unclear neurological score. In contrast, too-long occlusion has significant mortalities (Singh et al., 2022).

There is evidence suggesting that artificial induction of ischemic stroke in animals may not consistently result in significant brain damage, as the brain exhibits a certain level of tolerance to ischemic insults (Gidday, 2006). Accordingly, this preliminary study aimed to provide brief information regarding the simple method of EIIS procedure in rat models that could be reliable to support the pre-clinical study of ischemic stroke. This study was supported by several biomarkers for ischemic stroke, including platelet-to-white blood cell ratio, neutrophil/lymphocyte ratio, C-reactive protein, and De Ritis ratio.

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MATERIALS AND METHODS

Ethical statement

This study has been approved by the ethics committee of the Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya. The approval number of this study was KKE-34/IX/2022, and it was stated as 2 September 2022. All the experimental procedures were performed in the Laboratory of Pharmacology, Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya, Indonesia, from September 2022 until January 2023.

Research design and sample collection

This study used 60 male Sprague-Dawley rats, six months old, weighing 254.25 ± 8.18 grams (Laboratory of Pharmacology, Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya). The rats were divided into five groups. Each group was maintained inside the acrylic cage (80 cm × 40 cm × 50 cm), with husk bedding, 12/12 hours light/dark, 25°C, and 60% humidity. The rats were provided water and feed access *ad libitum*.

Group 1 was sham-operated (skin neck incision without CCA ligation). Groups 2 to 5 were EIIS groups, including Group 2 with EIIS using 1 hour of CCA ligation, Group 3 with 2 hours of CCA ligation, Group 4 with 4 hours of CCA ligation, and Group 5 with 8 hours of CCA ligation. After the EIIS procedure, the rats were observed regarding their general condition and neurodeficit score on days 1 and 7 after reperfusion. The scoring system was conducted following a previous study with modifications (Bertrand et al., 2017), and it is embedded in Table 1.

Table 1. A scoring system of general condition and neurodeficit of experimentally induced ischemic stroke in rat models

Category	Parameter	Score			
		1	2	3	4
General condition	Hair condition	Worst	Moderate	Clean	-
	Ear position	Droopy	-	Raised	-
	Hearing	Unresponsive	-	Responsive	-
	Eye condition	Unresponsive	-	Responsive	-
	Posture	Crawling	Leaning	Normal	-
	Spontaneous activity	Unconscious	Low activity	Normal	-
Neurodeficit	Gait	Unconscious	Crawling	Walking to one side	Normal
	Climbing	Unconscious	Crawling	Walking to one side	Normal
	Compulsory circling	Unconscious	Falling to one side	Leaning	Normal
	Front limb symmetry	No grabbing at all	One side grabbing	Both grabbing but continually loose	Normal
	Whisker response	No response	Whisker movement only	Turn trunk	Normal
	Epileptic behavior	General tonic spasm	Transient general tonic spasm	Transient focal tonic spasm	Normal

Experimentally induced ischemic stroke procedure

The ligation of a common carotid artery (CCA) was conducted to induce EIIS in animal models. The ligation was performed on the CCA and its branches. Before the EIIS procedure, the rats were anesthetized using a combination of 50 mg/kg BW ketamine (Agrovet Market, Canada) and 4 mg/kg BW xylazine (Interchemie, Holland). The rats were placed on their back on the operating table. The incision area was shaved and disinfected using 70% alcohol. Further, the sagittal ventral midline incision was performed. The neck muscle and salivary glands were carefully retracted to expose the right CCA. The right CCA was carefully separated from the vagal nerve to prevent transient parasympathetic nerve dysfunction. After that, the CCA was observed to find its bifurcation and both the external carotid artery (ECA) and internal carotid artery (ICA, Figure 1a). The occlusion was performed on three sides of a carotid artery (CCA, ECA, and ICA) using a simple interrupted suture using a 2-0 braided silk suture (Gea Medical, Indonesia; Figure 1b). The occlusion of blood flow using ligation was performed at different times depending on the groups. Furthermore, the ligation was released by cutting the suture knot using a surgical scissor (Figure 1c-d). The reperfusion was performed for seven days.

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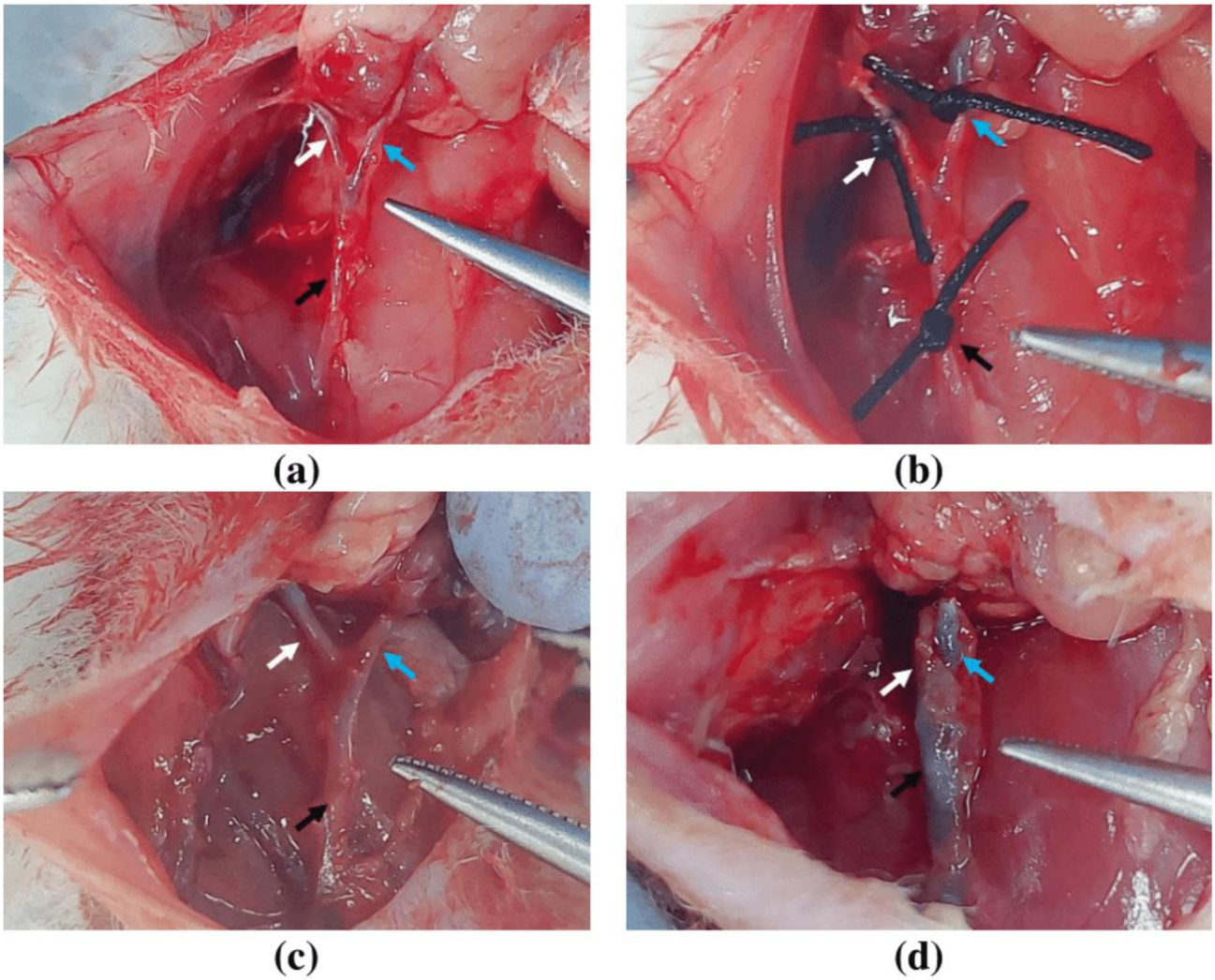

Figure 1. The ligation procedure of experimentally induced ischemic stroke in a Sprague-Dawley rat model. The common carotid artery has been visible (**a**), a simple interrupted suture was applied on the common carotid artery, external carotid artery, and internal carotid artery (**b**); the reperfusion of common carotid artery after occlusion (**c**); the common carotid artery condition after seven days of occlusion (**d**). Black arrow: Common carotid artery, White arrow: External carotid artery, Blue arrow: Internal carotid artery.

Laboratory test

The blood of rats was collected via the jugular vein (2 mL) on days 1 and 7 after the EIIS procedure. The blood was separated into two parts, one for hematology and the rest for serology tests. After that, the rats were euthanized using lethal doses of ketamine (150 mg/kg BW, Agrovet Market, Canada) combined with xylazine (10 mg/kg BW, Interchemie, Holland). The skull was opened to collect their brain. The blood and serum were tested against several biomarkers for ischemic strokes, such as platelet-to-white blood cell ratio (PWR, Amalia and Dalimonthe, 2020), neutrophil/lymphocytes ratio (NLR, Sharma et al., 2021), C-reactive protein (CRP, den Hertog et al., 2009), and De Ritis ratio (Gao et al., 2017).

Before brain cutting, the brain was incubated at -20°C for 15 minutes to prevent the brain from being crushed during sectioning. The brain sample was cut (2-3 mm) using a surgical blade and stained using 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, Indonesia) at 37°C for 10 minutes. The stained brain section was then analyzed regarding its ischemic area using ImageJ software. Further, the organ was fixed using 10% neutral buffer formalin for routine histopathology (H&E staining) and thionine staining (Ramírez et al., 2020).

Statistical analysis

The normal and homogenous data were analyzed using a parametric test, including ANOVA and post hoc tests using Bonferroni's test. In contrast, the abnormal and non-homogenous data were analyzed using a non-parametric test, including the Kruskal Wallis and Mann Whitney-U test. This study used a significance level of 0.05. The statistical analysis was performed using SPSS version 26.

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RESULTS AND DISCUSSION

The result indicated that EIIS in rats significantly impacts the score of the general condition until seven days after reperfusion ($p \leq 0.05$). Group 2, with 1 hour of CCA ligation, did not show differences compared to Group 1 ($p \geq 0.05$). This finding indicated that 1 hour of CCA ligation does not impact general conditions in rat models. Moreover, Group 3 showed a significant difference in general condition parameters compared to Group 1, especially in hair condition parameters ($p \leq 0.05$). The severe general condition score was shown by Groups 4 and 5 in all parameters ($p \leq 0.05$), except for ear position ($p \geq 0.05$) compared to the others. However, both groups 4 and 5 did not show any differences regarding the general condition ($p \geq 0.05$, Figure 2). The neurodeficit score of EIIS in rat models indicated similar statistical results to the general condition score. The neurodeficit score in Group 2 has no differences compared to Group 1. Group 3 showed significant differences in gait and whisker response compared to the control ($p \leq 0.05$) but not regarding the other parameters ($p \geq 0.05$). Furthermore, groups 4 and 5 showed severe neurodeficit scores compared to the other groups ($p \leq 0.05$, Figure 2).

Further, the general condition and neurodeficit measurement were confirmed by hematological and serological biomarkers for ischemic stroke. Several markers were PWR, NLR, CRP, and De Ritis ratio. This study obtained an elevated PWR and De Ritis ratio in Group 5 in 1 and 7 days after the EIIS procedure compared to the others ($p \leq 0.05$). However, the PWR and De Ritis ratio in Group 4 increased on day seven only. Groups 4 and 5 showed consistent increases in NLR and CRP in 1 and 7 days after the EIIS procedure compared to the other groups ($p \leq 0.05$, Table 2).

Table 2. Hematology and serology biomarkers of experimentally induced ischemic stroke in rat models

Parameter	Day	Group 1	Group 2	Group 3	Group 4	Group 5
PWR	1	96.17 ± 4.15^{a}	98.16 ± 1.63^{a}	111.30 ± 5.59^{b}	129.56 ± 1.55^{c}	130.19 ± 1.23^{c}
	7	99.66 ± 1.05^{a}	99.57 ± 4.69^{a}	128.23 ± 4.20^{b}	134.43 ± 4.33^{c}	131.86 ± 2.08^{c}
NLR	1	0.27 ± 0.00^{a}	0.25 ± 0.02^{a}	0.32 ± 0.03^{a}	0.42 ± 0.07^{b}	0.36 ± 0.01^{b}
	7	0.27 ± 0.00^{a}	0.31 ± 0.02^{a}	0.31 ± 0.02^{a}	0.38 ± 0.03^{b}	0.37 ± 0.02^{b}
CRP (mg/dL)	1	34.73 ± 0.75^{a}	34.86 ± 1.51^{a}	44.00 ± 4.45^{b}	54.61 ± 0.63^{c}	55.93 ± 0.46^{c}
	7	34.36 ± 0.80^{a}	35.83 ± 0.90^{a}	36.46 ± 1.02^{a}	56.20 ± 0.95^{c}	55.46 ± 2.28^{c}
De Ritis ratio	1	1.24 ± 0.11^{a}	1.30 ± 0.08^{a}	1.32 ± 0.02^{a}	1.22 ± 0.04^{a}	1.29 ± 0.01^{a}
	7	1.26 ± 0.11^{a}	1.23 ± 0.07^{a}	1.24 ± 0.04^{a}	1.43 ± 0.20^{b}	1.42 ± 0.04^{b}

PWR: Platelet-to-white blood cell ratio, NLR: Neutrophil lymphocytes ratio, CRP: C-reactive protein, $^{a, b,c}$ different superscript letters in the same column indicated significant differences ($p \leq 0.05$).

The TTC staining showed that normal brain tissue has deep red stains after staining (Figure 3a). In contrast, the brain infarct was shown by a pale color of the brain after one day (Figure 3b) and an unstained (white) area of the brain after seven days of reperfusion (Figure 3c). The percentage of infarct area of Group 5 was more significant than the other groups ($p \leq 0.05$). The appearance of an infarct area within the brain parenchyma was consistently shown by Group 5 on days 1 and 7. However, the infarct area of Group 4 is similar to that of Group 5 on day 7 ($p \geq 0.05$). In addition, Groups 1, 2, and 3 did not show any differences in the infarct parameter ($p \geq 0.05$, Figure 3d).

The score of brain histopathology showed that necrosis neurons, microgliosis, secondary hemorrhages, and perivascular edema in Groups 4 and 5 were significantly different compared to the other groups ($p \leq 0.05$, Figure 4). Groups 2 and 3 showed an increase in perivascular edema and secondary hemorrhage score on day one after the EIIS procedure; however, the score decreased on day 7, compared to Group 1 ($p \leq 0.05$). It indicated a decrease in severity within the brain parenchyma in groups 2 and 3. Furthermore, the Nissl bodies were distinctly demonstrated on days 1 and 7 in groups 1, 2, and 3; however, groups 4 and 5 showed the Nissl bodies with unclear morphologies and disappeared in all observation periods (Figure 4).

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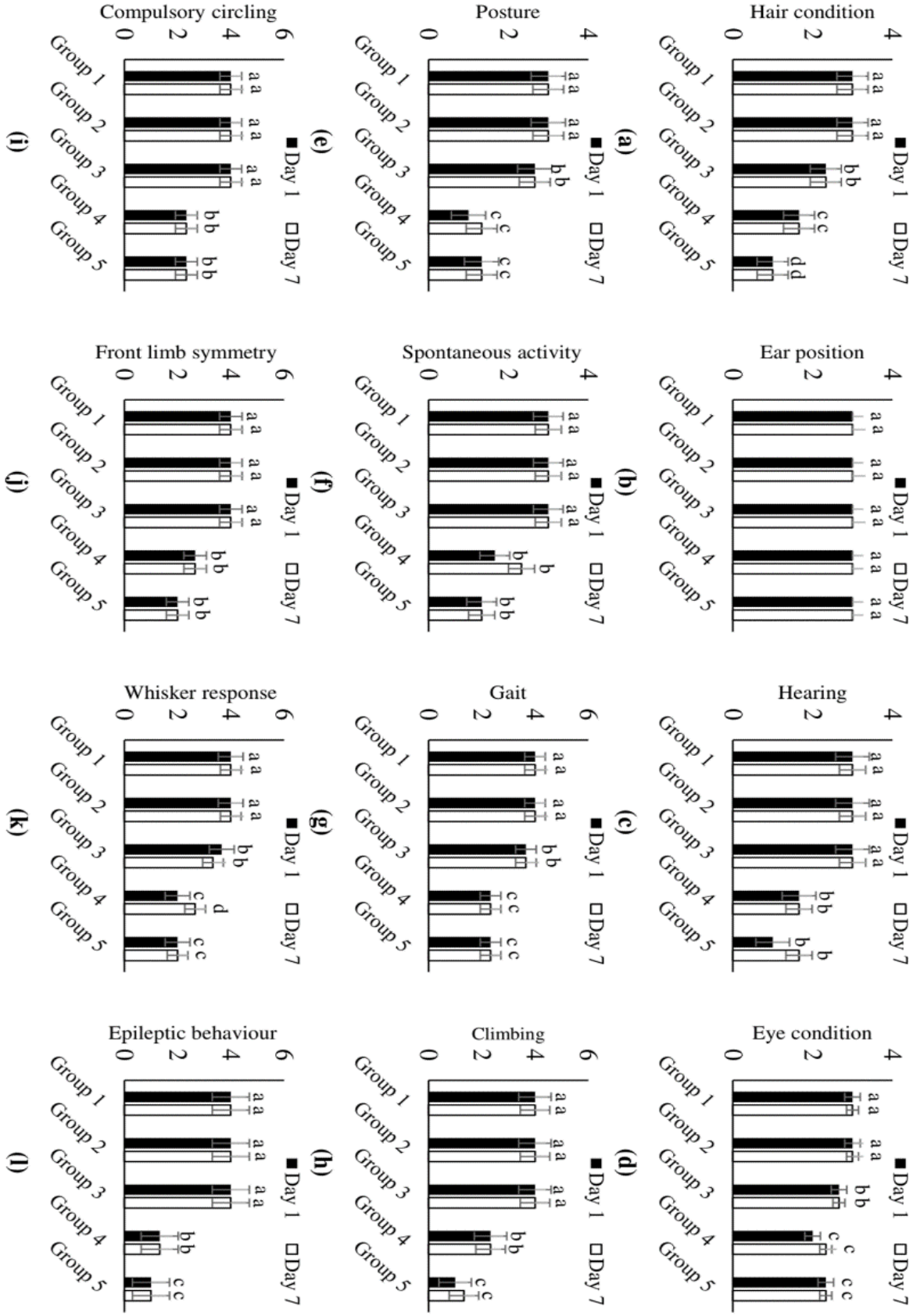

Figure 2. A score of general condition and neurodeficit of the rat after the EIIS procedure. A part of the general condition score consisted of hair condition (**a**), ear position (**b**), hearing (**c**), eye condition (**d**), posture (**e**), and spontaneous activity (**f**). However, the neurodeficit score consisted of gait (g), climbing (**h**), compulsory circling (**i**), front limb symmetry (**j**), whisker response (**k**), and epileptic behavior (l). [a,b,c] Means different superscript letters differ significantly ($p \leq 0.05$).

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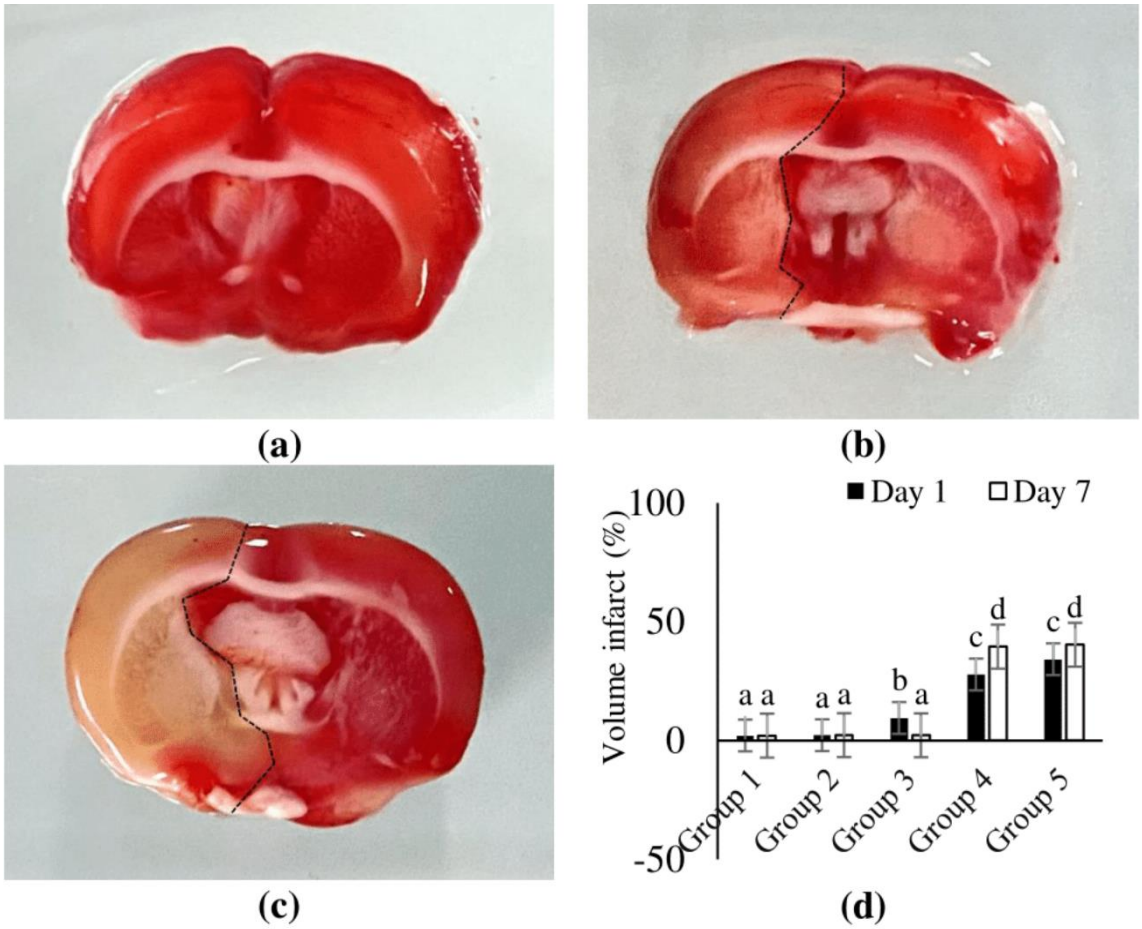

Figure 3. Macro-photograph of the brain after EIIS procedure. Typical appearance of the brain that stained-deep red using TTC staining (**a**), pale coloration (borderline) of the brain after one day of reperfusion (**b**), white coloration (borderline) of the brain after seven days of reperfusion (**c**), the volume of infarct area (%) in days 1 and 7 (**d**). [a,b,c,d] Means different superscript letters in same column and row differ significantly ($p \leq 0.05$).

The CCA is an essential artery that supplies nutrients to brain tissue. The potency of CCA during the physiological aspect makes the researchers utilize it to induce ischemic stroke in rat models. The basic principle to induce ischemic stroke in a rat is the disruption of blood supply to the brain area. One of the standard methods is MCAO. The MCAO can be conducted using two methods. The first method is using inserting the suture within the middle cerebral artery via the CCA, and the second one is using craniotomy. Inserting a sterile operative suture through the CCA can be conducted using a 4/0 and 5/0 nylon suture, and the occlusion can be performed for 30 to 45 minutes (Shvedova et al., 2021). However, this method has a significant limitation, such as a high mortality rate of 71-100% in diver strains of rats (Schulte-Herbrüggen et al., 2006). Another alternative method is using craniotomy (Yeh et al., 2019). A craniotomy is an invasive method that can be applied in rat models. This method correctly exposes the MCA directly, and the occlusion can be performed using coagulation, transection, ligation, and clips (Howells et al., 2010). The previous study described that the modified craniotomy increases the success of ischemic stroke in rat models, proved by the presentation of the infarct area and its inflammatory cytokines (Yeh et al., 2019). However, craniotomy has several difficulties, such as difficulty in finding the precise location of MCA, and cranial bone removal is commonly impeded by a zygomatic arc. It affects the rat's eating ability and physiological function (Theodorsson et al., 2005). Again, all those described methods are brutal to be performed in a research laboratory with minimal veterinary surgery facilities. So, it is necessary to provide a more reliable methodology to induce ischemic stroke in rat models that are simple, cheap, have a low mortality rate, and generate consistent infarction.

This study used a simple interrupted suture to tie up the CCA, ICA, and ECA in the rats. Compared to the other methods, this method provides a more straightforward procedure that can be conducted by veterinarians and researchers with fewer facilities. They need to find the CCA and its bifurcation and ligate it. However, there is a difference between this method compared to the MCAO and craniotomy, including the long ligation time in conducting occlusion. This method requires longer occlusion time compared to the other methods, especially 4 and 8 hours. Using 1 and 2 hours of CCA ligation is not as effective as 4 and 8 hours of CCA ligation because it cannot promote neurological deficit and brain infarction. The failure of infarction forming after 1 and 2 hours of CCA ligation is suspected to be caused by several mechanisms, including the disruption of endothelial cells within CCA and blood crossflow via the anterior communicating artery.

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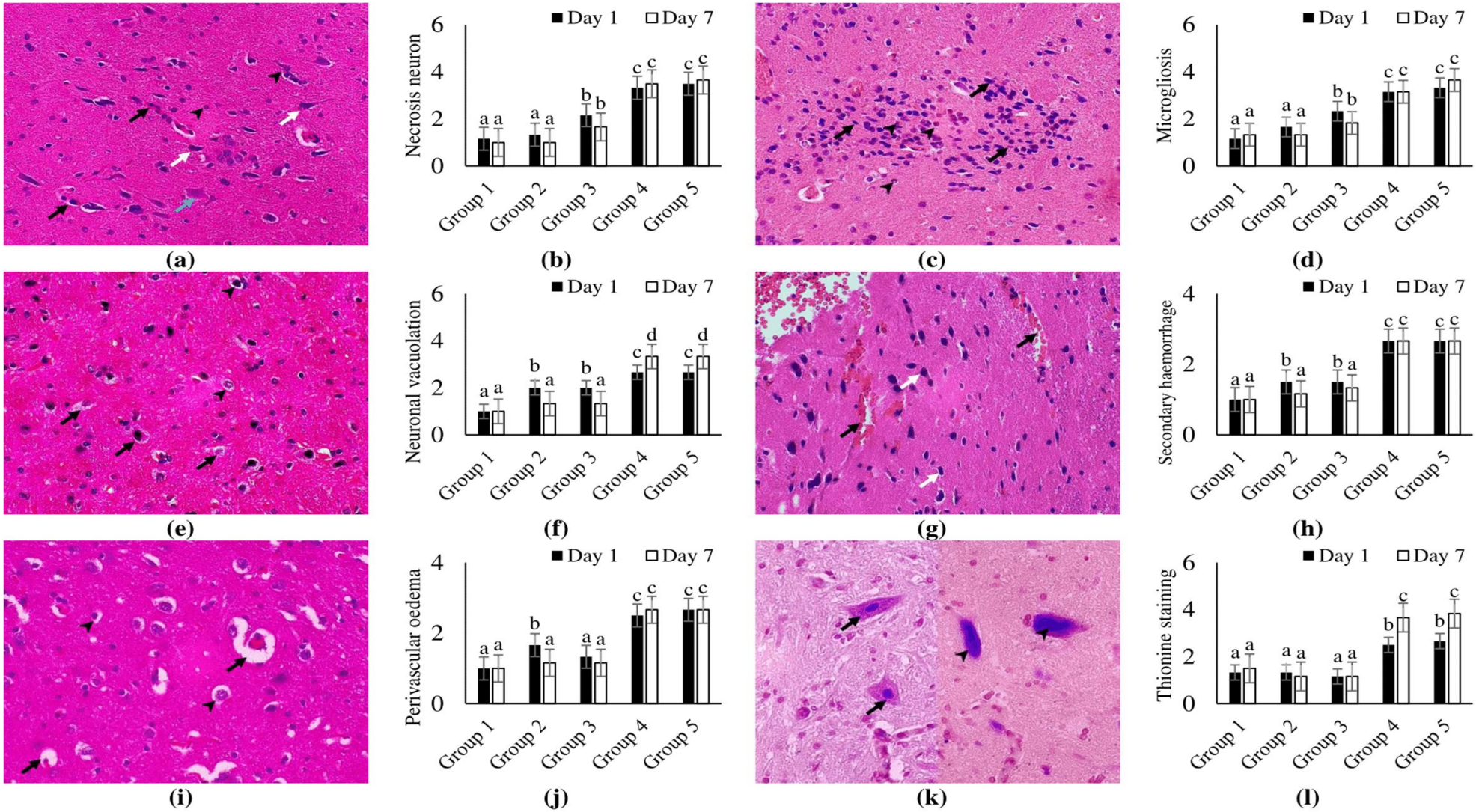

Figure 4. A score of histopathology of the brain after experimentally induced ischemic stroke in rat models. Necrosis neuron that marked by various morphologies, including redness cytoplasm (black arrows), pycnotic nucleus (white arrows), and ghost cell (blue arrow), this area was also surrounded by sateliosis (arrowheads) (**a**), histopathology score of necrosis neuron (**b**), reactive microgliosis (arrows) and glial cells (arrowheads) infiltration within brain parenchyma following the EIIS procedure (**c**), histopathology score of microgliosis (**d**), neuronal vacuolation (arrows) around astroglial (arrowheads) and within the neuronal cytoplasm that indicated by pale cytoplasm (**e**), histopathology score of neuronal vacuolation within the brain parenchyma (**f**), secondary haemorrhage (black arrows) and pycnotic neuron (white arrows) within the infarcted area (**g**), histopathology score of secondary haemorrhage inside the infarcted region (**h**), perivascular edema (arrows) showed the expansion of Virchow Robin space, and its seriously impacts on astroglial (arrowheads) hydropic degeneration (**i**), histopathology score of perivascular edema (**j**), Nissl bodies (arrows) with clear sandy appearance within the neuronal cytoplasm from the control, contrary the infarcted brain showed that the Nissl bodies (arrowheads) disappeared and pycnotic neuron were visible as sharp blue stained (**k**), histopathology score of Nissl bodies using thionine staining (l). $^{a, b, c}$ different superscripts in the same column and row indicated significant differences ($p \leq 0.05$). H&E, 400× **(a, c, e, g, i)**; Thionine staining, 400× (**k**).

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The endothelial cell disruption triggers an increase of pro-inflammatory cytokine and glial cells within the brain that causes an increase in vascularization (Mizuma and Yenari, 2017). Further, increased brain vascularization and blood crossflow during 1 and 2 hours of CCA ligation impact a decreased infarct area. It is supported by a previous study that reported that the blood crossflow after MCAO from the other side of Circle of Willis affects the reduction of infractions (Zarow et al., 1997). Further, another study reported that the blood crossflow and brain preconditioning of rat models could generate ischemic tolerance (IT, Speetzen et al., 2013). The occurrence of IT in this study indicated that noxious stimulus applied to the brain using 1 and 2 hours of CCA ligation induces resistance in brain tissue to prevent severe organ damage. However, did those mechanisms not occur in Groups 4 and 5 with 4 and 8 hours of CCA ligation? The answer is yes. However, longer ligation times in Groups 4 and 5 impact the chaos of the cerebral circulatory system to cover the food and oxygen supplies in brain tissue. It means the infarction within the cerebral tissue cannot be avoided.

Cerebral infractions following 4 and 8 hours of CCA ligation significantly affect the score of general condition, neurodeficit, PWR, NLR, CRP, and De Ritis ratio, compared to the others ($p \leq 0.05$). However, one of the general condition parameters, especially ear position, can be excluded from the observing parameter in EIIS. This parameter does not show any differences in all periods of CCA ligation. The changing of general condition and neurodeficit after EIIS in rat models using 4 and 8 hours of CCA ligation have been observed as the compensatory effects of brain injury, such as necrosis neuronal (Baron et al., 2014), microgliosis, and disappearing of Nissl bodies (Zille et al., 2012). Neurons are the essential cells that integrate all physical activity among living things. Neuron pathological changes cause incoordination of the body movement, as proved by this study. Further, microgliosis during stroke promotes severe neurological defects (Sivadas and Broadie, 2020). Microglia have a significant role, both for inflammatory and non-inflammatory responses. As the inflammatory agent, microglia prevent the spreading of pathogenic agents. However, the microglia act to release inflammatory cytokines that increase synapse loss during ischemic stroke (Colonna and Butovsky, 2017). Furthermore, synapse loss seriously impacts the neurodeficit. It is aggravated by the clumping of the Nissl's bodies. A previous study by Liu et al. (2021) described that ischemic stroke induces an increase in disappearing Nissl's bodies that impacts neurological deficits. In advances, cerebral ischemia influences the local and systemic circulatory system, as proved by brain histopathological changes, including secondary hemorrhage, neuronal vacuolation, and perivascular edema (Chen et al., 2021). The ischemic stroke also consistently impacts the PWR, NLR, CRP, and De Ritis ratio level, which are essential as the ischemic stroke biomarkers (Dagonnier et al., 2021).

CONCLUSION

This study indicated that the simple EIIS procedure using ligation of CCA in 4 and 8 hours is reliable for inducing ischemic stroke in rat models that may represent ischemic stroke in humans. The EIIS using 4 and 8 hours of CCA ligation promotes infarct presentation within the brain that can be observed from day one until day 7. Further, the EIIS using 4 and 8 hours of CCA ligation also changes the score of general condition, neurodeficit, histopathology, thionine staining, PWR, NLR, CRP, and De Ritis ratio. An advanced study must be conducted to elucidate the reproducibility of this method compared to the other methods concomitantly. Moreover, if this method is reproducible, it can be used as a simple method for the pre-clinical study of drugs or therapy for an ischemic stroke in animal models.

DECLARATIONS

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Competing interests
The authors declare that they have no conflict of interest.

Ethical consideration
This paper was written originally by the authors. The authors were not submitting this paper to the other journal or publisher.

Authors' contributions
Prakoso YA was the person in charge of this study. Prakoso YA conceptualized, designed, conducted, monitored, and supervised the research. Sigit M conducted the study and analyzed the statistical data. Aliviameita A conducted the hematological test and interpreted the hematology and serology data. All authors have read and approved the final version of the manuscript for publication in the present journal.

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Availability of data and materials
The data of this study are available upon reasonable request.

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Carrageenan-Induced Acute Inflammation on Back-Skin of Mice: Histopathological Features, Number of Inflammatory Cells, and Expression of COX-2, COX-1, and IL-6

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ABSTRACT

Carrageenan is a sulfated polysaccharide obtained from red seaweed (Rhodophyceae) and can trigger inflammatory activation in both humans and laboratory animals. This study aimed to investigate the expression of cyclooxygenase-2 (COX-2), cyclooxygenase-1 (COX-1), and interleukin-6 (IL-6) and the number of inflammatory cells (neutrophil) involved in a carrageenan-induced acute inflammatory model in the back skin of mice. Paraffin blocks from the back skin of female Swiss mice aged 8 weeks were used in this study. The back-skins of 4 groups of 5 mice in each group were subcutaneously injected with 1%, 2%, and 4% carrageenan powder in 0.9% buffer saline and 0.9% buffer saline as control. Skin samples on paraffin blocks were taken 6 hours after carrageenan injection. Furthermore, paraffin blocks were stained with hematoxylin-eosin (HE) to count the number of inflammatory cells. Immunohistochemistry staining using anti-COX-2, COX-1, and IL-6 antibodies was performed to determine the role of inflammatory mediators. The results showed that the number of inflammatory cells (neutrophils) increased significantly following an increase in carrageenan concentrations. The COX-2, COX-1, and IL-6 expressed by inflammatory cells increased significantly at carrageenan concentrations of 1% to 4%. Histopathological features supported the results obtained from the calculation of the number of inflammatory cells and the expression of COX-2, COX-1, and IL-6. The inflammatory markers consisting of COX-2, COX-1, and IL-6 were expressed on the back skin of mice at 6 hours post-injection with 1% to 4% carrageenan. It can be concluded that carrageenan can be used for an acute inflammatory model of the back skin of a mouse. This inflammation model is intended to facilitate the evaluation or measurement of therapeutic and inflammatory responses when test substances are administered topically or transdermal.

Keywords: Carrageenan, Cyclooxygenase-2, Cyclooxygenase-1, Interleukin-6, Inflammatory cell, Skin inflammation

INTRODUCTION

The development of inflammatory models in experimental animals has been widely carried out to know the release of inflammatory mediators at the inflammation site and determine antiinflammatory activity and efficacy (Fehrenbacher et al., 2012). Previous studies have reported various models to test inflammatory activity using a variety of chemicals and physical agents as inductors (Widyarini et al., 2001; Wei et al., 2016; Wu et al., 2017). Both chemicals and physical agents have been reported to be used in skin and colonic inflammation models in experimental animals (Widyarini et al., 2001; Huang et al., 2014; Wei et al., 2016; Wu et al., 2017). Chemical inductors for inflammatory models are formalin, kaolin, yeast, dextran, croton oil, carrageenan (Fehrenbacher et al., 2012), a combination of oxazolone and carrageenan (Wu et al., 2016), and a combination of 2,4,6-trinitrobenzene sulfonic acid (TNBS) and carrageenan (Wei et al., 2016).

One of the ingredients that are widely used for the activation of inflammatory pathways is carrageenan. Carrageenan is a sulfated polysaccharide obtained from red seaweed named Rhodophycae (Necas and Bartosikova, 2013) and has been widely used as a thickener, stabilizer, and communizing agent in the processing of dairy and cosmetic products (Pereira et al., 2018; Shafie et al., 2022; Kot et al., 2023). Carrageenan has been reported by some researchers as a model for inflammatory research in guinea pigs, rats, and mice (Fehrenbacher et al., 2012; Necas and Bartosikova, 2013). In addition, carrageenan is not only used for inflammatory models but has also been reported to trigger the occurrence of colorectal tumors in the *Apc* $^{min/+}$ mice and colitis in C57BL/6J mice (Bhattacharyya et al., 2017; Shang et al., 2017; Wang et al., 2023).

Subcutaneous injection of carrageenan causes local inflammation characterized by inflammatory signs, namely tumor, color, dolor, rubor, and function laesa (Fehrenbacher et al., 2012). Furthermore, carrageenan injection as a model of paw edema showed macroscopic changes in the form of erythema, swelling, and hyperalgesia (Ben Khedir et al, 2016; Szekalska et al., 2020). Carrageenan injection on the paws of the mice also showed microscopic images in the form of

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acute edema, expanded epidermal thickness, vasculitis, and invasion of immune cells within the epidermis and dermis (Szekalska et al., 2020; Vysakh et al., 2020).

Inflammatory models with carrageenan as an irritant are used to trigger transient inflammation (acute inflammatory model, Fehrenbacher et al., 2012). Furthermore, carrageenan, as an acute inflammatory model, can be used to observe inflammation that occurs in animal models and as a method to monitor the inflammatory response due to the administration of antiinflammatory drugs in experimental animals. Carrageenan injection induces inflammation through a biphasic phase. In the vascular phase, proinflammatory cytokines such as TNF-α, IL-6, and IL-β are involved from the first hour after injection, while prostaglandins play an important role in the cellular phase(second phase), which occurs in the 3-5th hour post injection (Reanmongkol et al., 2009). The mediators mentioned above are responsible for erythema, edema, and pain at the injection site.

Previous studies have stated that more than 400 studies have reported that carrageenan is widely used as a model for inflammatory evaluation of mouse paw edema (Posadas et al., 2004; Suralkar et al., 2008; Fehrenbacher et al., 2012). Evaluation of acute inflammatory models using the back skin of mice has been widely practiced but not by using carrageenan as an inducer (Widyarini et al., 2001; Widyarini et al., 2006; D'Orazio et al., 2013; Kim, 2016). It has been reported that inflammatory models with subcutaneous injection of carrageenan (1-4%) on the back skin of mice result in maximum inflammatory response obtained at a concentration of 4% (Widyarini et al., 2023). The acute inflammatory model using the back skin of mice is expected to facilitate treatment and its evaluation or measurement of inflammatory responses when the test material is administered topically or transcutaneous. Moreover, topical application of the antiinflammatory test material will be easier if carrageenin is injected subcutaneously into the back skin of the mouse than if it is injected into the mouse paw. This study aimed to determine the role of inflammatory mediators in the acute inflammatory process of the back skin of mice triggered by carrageenan. Observations were made by microscopic examination of hematoxylin-eosin (HE) and immunostaining to calculate the number of inflammatory cells and the expression of COX-2, COX-1, and IL-6 in the back skin tissue of mice at 6 hours after injection of carrageenan.

MATERIALS AND METHODS

Ethical approval

This research has received research ethics feasibility from the Research Ethics Commission of the Faculty of Veterinary Medicine, University Gadjah Mada, Indonesia (Number: 024/EC-FKH/Addn./2022).

Study period and location

This study was conducted in January 2022 at the Department of Pathology, Faculty of Veterinary Medicine, University Gadjah Mada, Yogyakarta, Indonesia, and Pathology Laboratory of Prof Dr. Sardjito Hospital, Yogyakarta, Indonesia.

Experimental designs

Paraffin blocks were derived from a previous study (Widyarini et al., 2023). Twenty (20) female Swiss mice aged 8 weeks with an average weight of 30 grams were divided into four groups of five. Mice were purchased from the Integrated Laboratory for Research and Testing, University Gadjah Mada, Indonesia. All animals except the control group were received subcutaneously (SC injection), in the back area with 0.1 mL ƛ-carrageenan in 0.9% buffer saline (Sigma Chemical Co, USA), with concentrations of 1%, 2%, and 4%. The control group was only injected subcutaneously with 0.9% buffered saline. At six hours post-injection with carrageenan, all animals were euthanized by cervical dislocation technique. Subsequently, the skin in the injection area is excised for 1 cm^2 and then fixed in 10% buffer formalin for tissue processing and making paraffin blocks at the Department of Pathology, Faculty of Veterinary Medicine, University Gadjah Mada, Indonesia. Other materials used in this study were microtome for paraffin block cutting (Olympus, Japan), poly-L-lysine-coated slides (Sigma Chemical. Co., USA), and a set of tools for tissue staining with hematoxylin-eosin (HE, Leica Biosystem, USA), as well as anti-COX-2, COX-1 and IL-6 antibodies (Biocare Medical, USA).

Histopathological preparations

Histopathological preparations were carried out according to the standards of the Pathology Laboratory of the Faculty of Veterinary Medicine, University Gadjah Mada Indonesia. Paraffin blocks containing samples (back of mouse skin) were cut to a thickness of 5 μm using a microtome, then placed in a water bath and glued to a glass object. Next, the slides were soaked in xylol I and xylol II solutions each for ten minutes and then placed in alcohol (absolute I, absolute II, alcohol 90%, alcohol 80%, and alcohol 70%) for five minutes each. The slides were then soaked in hematoxylin for ten minutes and rinsed under running tap water for 15 minutes. Subsequently, the slides were immersed in eosin for three to five minutes. After that, the slides were placed into alcohol series (70%, 90%, 100% of absolute

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alcohol I and absolute alcohol II), once for one minute, then the slides were placed into xylol for five minutes, mounted with mounting medium DPX (Merck, Germany), and covered with a deck glass.

Immunohistochemistry staining

The preparation of immunohistopathological examinations was carried out according to the standards of the Pathology Laboratory of Prof. Dr. Sardjito Hospital Indonesia. Paraffin blocks containing samples (back-skin mice) were cut to 5 µm thick using a microtome, placed on a water bath, and affixed to a glass object overlaid with poly-L-lysine (Sigma Chemical. Co., USA). Next, the slides were immersed in a solution of xylol I and xylol II for ten minutes each and then placed into alcohol series (absolute I, absolute II, absolute III, alcohol 90%, alcohol 80%, and alcohol 70%) for five minutes each. Then, slides were immersed in 0.3% H2O2 in methanol for 30 min and washed with pH PBS. 7.2, three times in 5 minutes. Rabbit polyclonal antibodies to COX-2, COX-1, and IL-6 (Biocare Medical, USA) diluted 1:200 was dripped to 100 µl to cover the tissue area to be stained and incubated at room temperature for an hour. After incubation, the slides were washed with PBS three times for 5 min and then incubated with secondary antibodies (Starr Trek Universal HRP Detection System, Biocare Medical, USA) for one hour at room temperature. After washing, slides were incubated with DAB for two minutes at room temperature and then washed under running water. Counterstains were performed by using HE for 10 seconds, followed by washing with running water. In the final stage, slides were immersed in alcohol series (90% alcohol, absolute I, absolute II) once for one minute. Then, the slides were placed into xylol for five minutes, mounted using DPX mounting media (Merck, Germany), and covered with a deck glass.

Statistical analysis

Observations of the number of inflammatory cells were carried out using a binocular light microscope (Olympus CX-23, Japan) with a magnification of 200x. Inflammatory cells in the dermis and subcutaneous areas were calculated from five fields of view using Image Raster 3.7. The average number of inflammatory cells in the treatment group with different percentages of carrageenan was then analyzed using One Way Anova and followed by the Tukey-HSD Test (SPSS Statistics version 26, USA). The p-value less than 0.05 was considered statistically significant. The location of COX-2, COX-1, and IL-6 expression was observed, and the number of inflammatory cells that expressed COX-2, COX-1, and IL-6 was also calculated.

RESULTS

Histopathological examination and inflammatory cell count

Histopathological examination was carried out by taking a skin sample six hours after carrageenan injection for examination under a microscope with hematoxylin-eosin staining. The histopathological pictures show that the group injected with carrageenan at the level of 1-4% showed changes in the dermis and subcutaneous areas. The changes are in the form of edema in the dermis area accompanied by infiltration of inflammatory cells that are increasingly visible along with the increase of the carrageenan concentration (Figure 1), which were in accordance with research conducted by Widyarini et al. (2023). The results of histopathological observation by HE staining in this study showed main changes in the form of edema and inflammatory cell infiltration in the dermis and subcutaneous area (Figure 1). In addition, the results of calculating the number of inflammatory cells in the dorsal skin of mice injected with 1-4% carrageenan can be presented in Figure 2. Figure 2 shows that in normal skin or untreated mice, there were also inflammatory cells were found only in mouse skin. Injections of 1% and 2% carrageenan showed no significant difference in inflammatory cell counts ($p > 0.05$). However, injecting 4% carrageenan into the back skin of mice increased the number of inflammatory cells more than the injection of 1% and 2% carrageenan ($p < 0.05$). Hence, the higher concentration of carrageenin (4%) resulted in more severe tissue damage, and more inflammatory cells were found.

Cyclooxygenase-2, Cyclooxygenase-1 and Interleukin-6

Immunohistochemistry analysis of the back skin of mice injected with 1-4% carrageenan showed an increase in COX-2, COX-1, and IL-6 expression compared with the dorsal skin group without carrageenan treatment (Figures 3, 4, 5, 6, 7, and 8). Expression is characterized by the presence of brown staining in skin epithelial cells and inflammatory cells. The results of this study only calculated the expression of COX-2, COX-1, and IL-6 by inflammatory cells found in the dermis and subcutaneous areas. COX-2 expression on the back skin of mice after injection with carrageenan can be seen in Figure 3. In Figure 3A (normal back skin), no COX-2 expression was found. COX-2 expression was found in the dermis of the group that was given 1% and 2% carrageenin. Interestingly, the injection of carrageenin 4% showed COX-2 expression not only in the dermis area but also in the subcutis area. Figure 4 shows the average number of inflammatory cells expressing COX-2. The number of inflammatory cells expressing COX-2 increased along with the

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increase in carrageenin concentration ($p < 0.05$). Injection of 4% carrageenin showed COX-2 expression in more inflammatory cells (approximately 30%) than those given 1% and 2% carrageenin ($p < 0.05$).

Figure 5 shows the expression of COX-1 on the back skin of mice injected with carrageenan. COX-1 expression is found in normal skin (Figure 5A). There is an increase in COX-1 expression in inflammatory cells of approximately 60%, compared to expression in normal skin ($p < 0.05$, Figure 6). However, there was no significant difference in COX-1 expression in the 1-4% carrageenin group. IL-6 expression in inflammatory cells and the number of IL-6-expressing cells can be seen in Figures 7 and 8. Normal skin does not express IL-6. On the other hand, IL-6 expression increased in the dermis area after 1-4% carrageenin injection (Figure 7). The number of inflammatory cells expressing IL-6 increased in line with the increase in carrageenin concentration (Figure 8). Injection of carrageenin 1%, 2%, and 4% significantly increased the number of inflammatory cells expressing IL-6 with an optimal concentration of 4%.

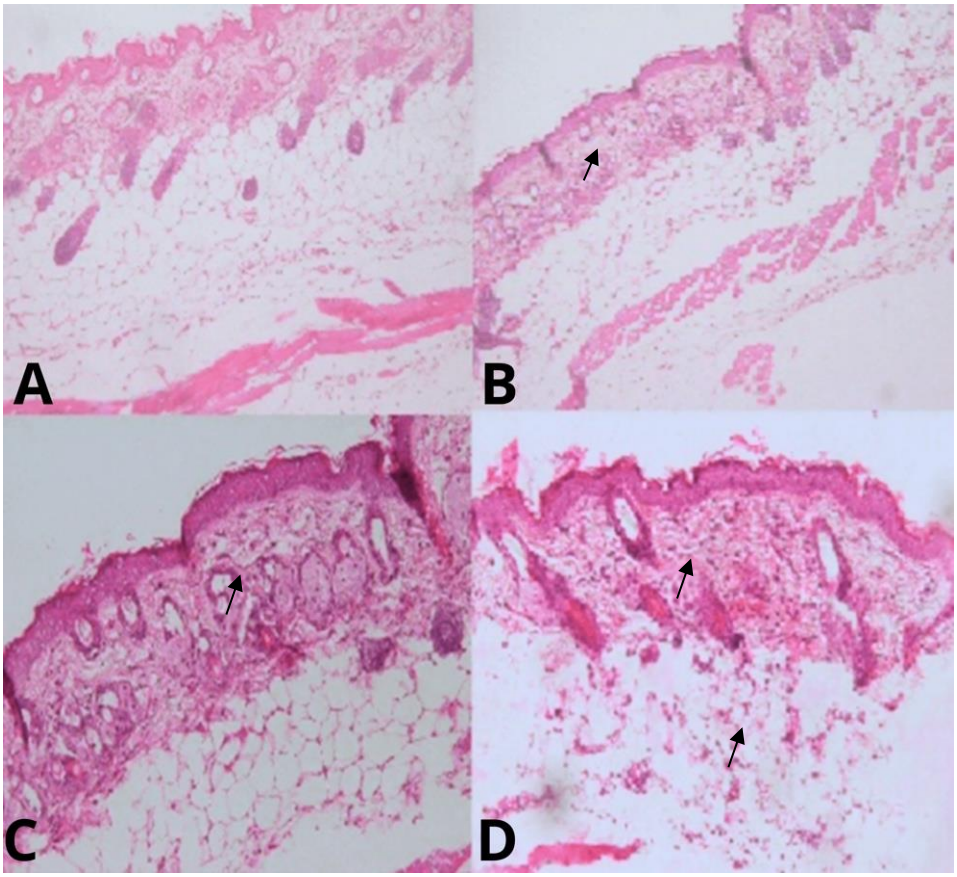

Figure 1. Microphotography of back-skin mice post-injection of 1-4% carrageenan. **A:** Mouse skin without carrageenan treatment. **B, C,** and **D:** Mouse skin with carrageenan treatment at 1-4%. Area's infiltration inflammatory cells of dermal (**B, C**; black arrow) and areas infiltration inflammatory cells (neutrophile) of dermal and subcutaneous edema (**D**; black arrow). HE staining, 200X magnification.

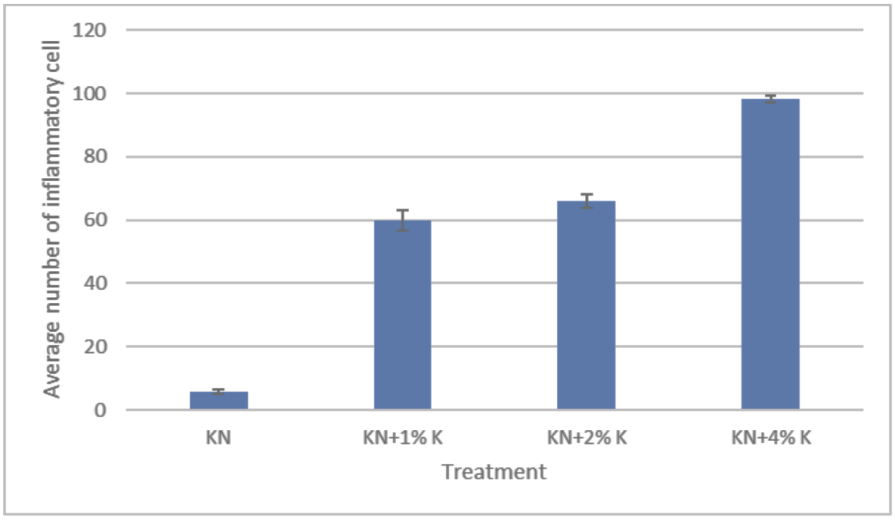

Figure 2. The average number of inflammatory cells (neutrophils) in the back skin of mice injected with 1-4% carrageenan. KN: Untreated mice skin. KN+1%, KN+2%, and KN+4%: Mice skin with carrageenan treatment 1-4%, respectively.

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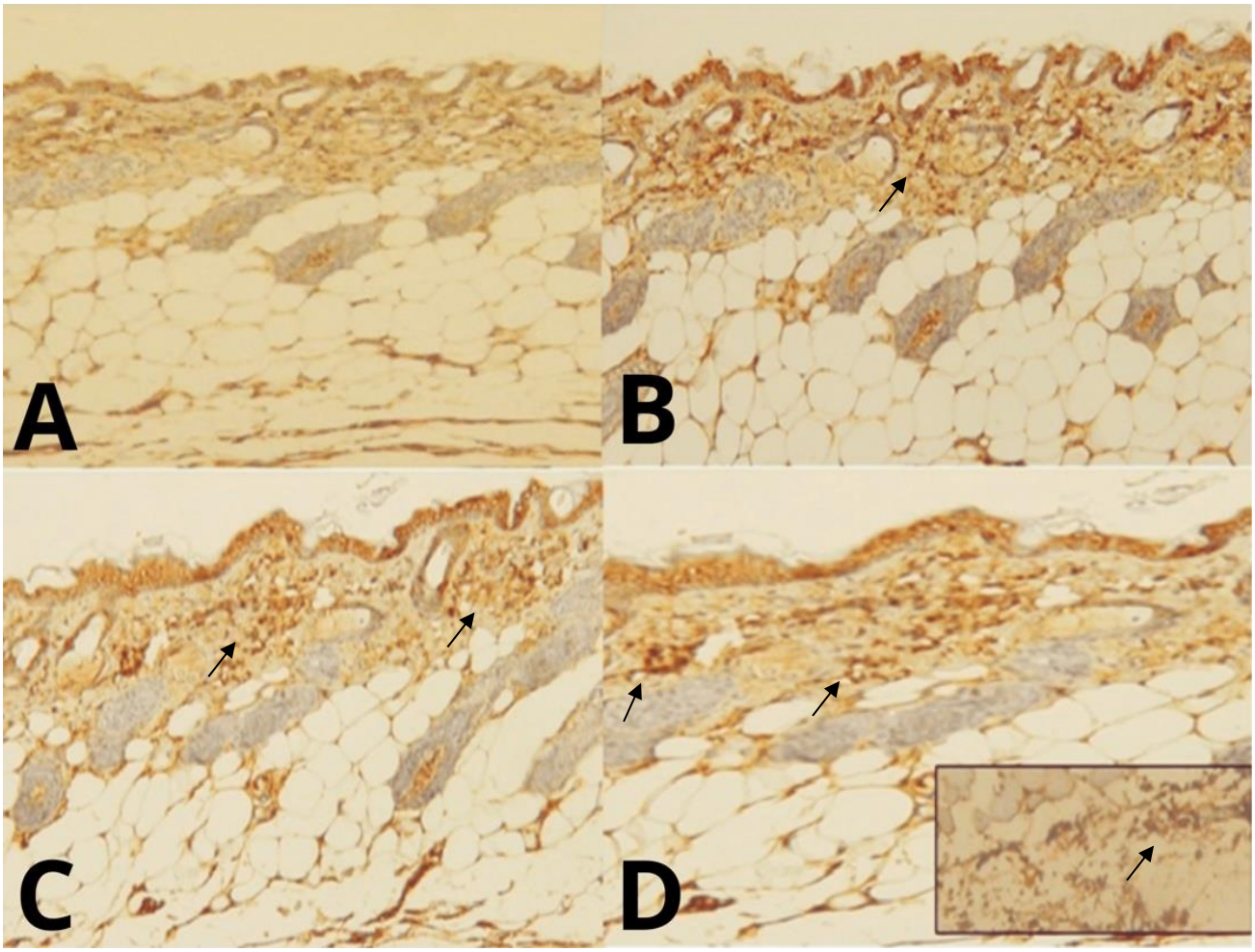

Figure 3. Microphotography expression of COX-2 in the back skin of mice post injection of 1-4% carrageenan. **A:** Mouse skin without carrageenan treatment. **B, C,** and **D:** Mouse skin with carrageenan treatment at 1-4%. COX-2 expression was found in the dermal area (**B, C,** and **D**; black arrow) and in the right corner box-subcutaneous area (**D**; black arrow). Anti-COX-2 antibody staining, 200× magnification.

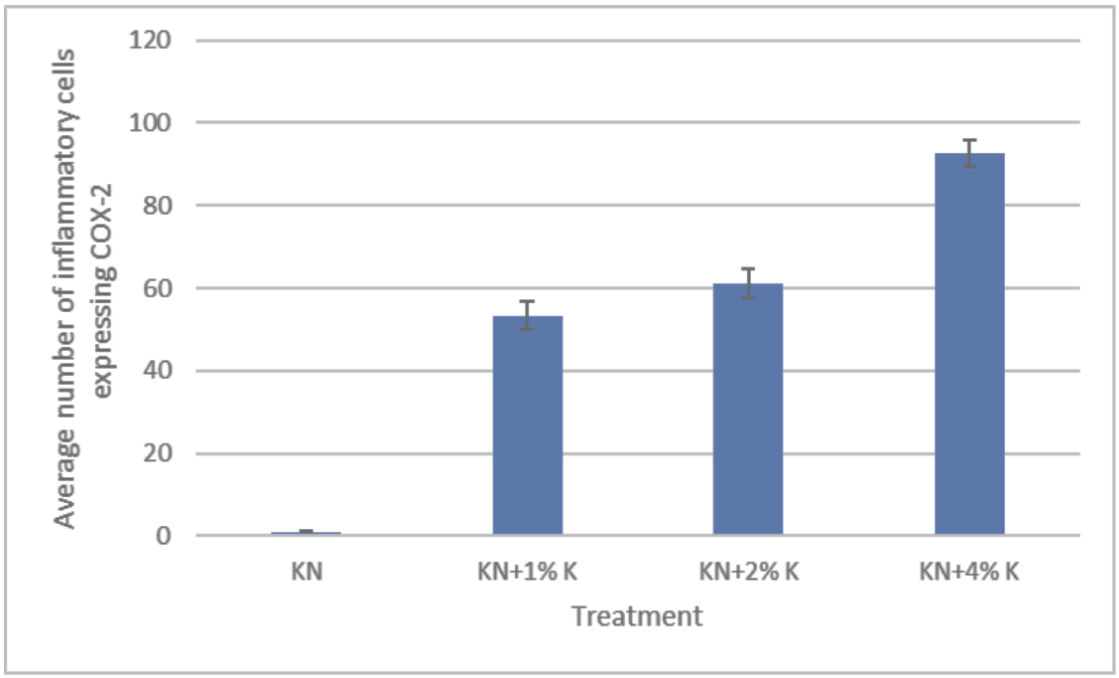

Figure 4. The average number of inflammatory cells in the back skin of mice injected with 1-4% carrageenan expressing COX-2. KN: Untreated mice skin. KN+1%, KN+2%, and KN+4%: Mice skin with carrageenan treatment 1-4%.

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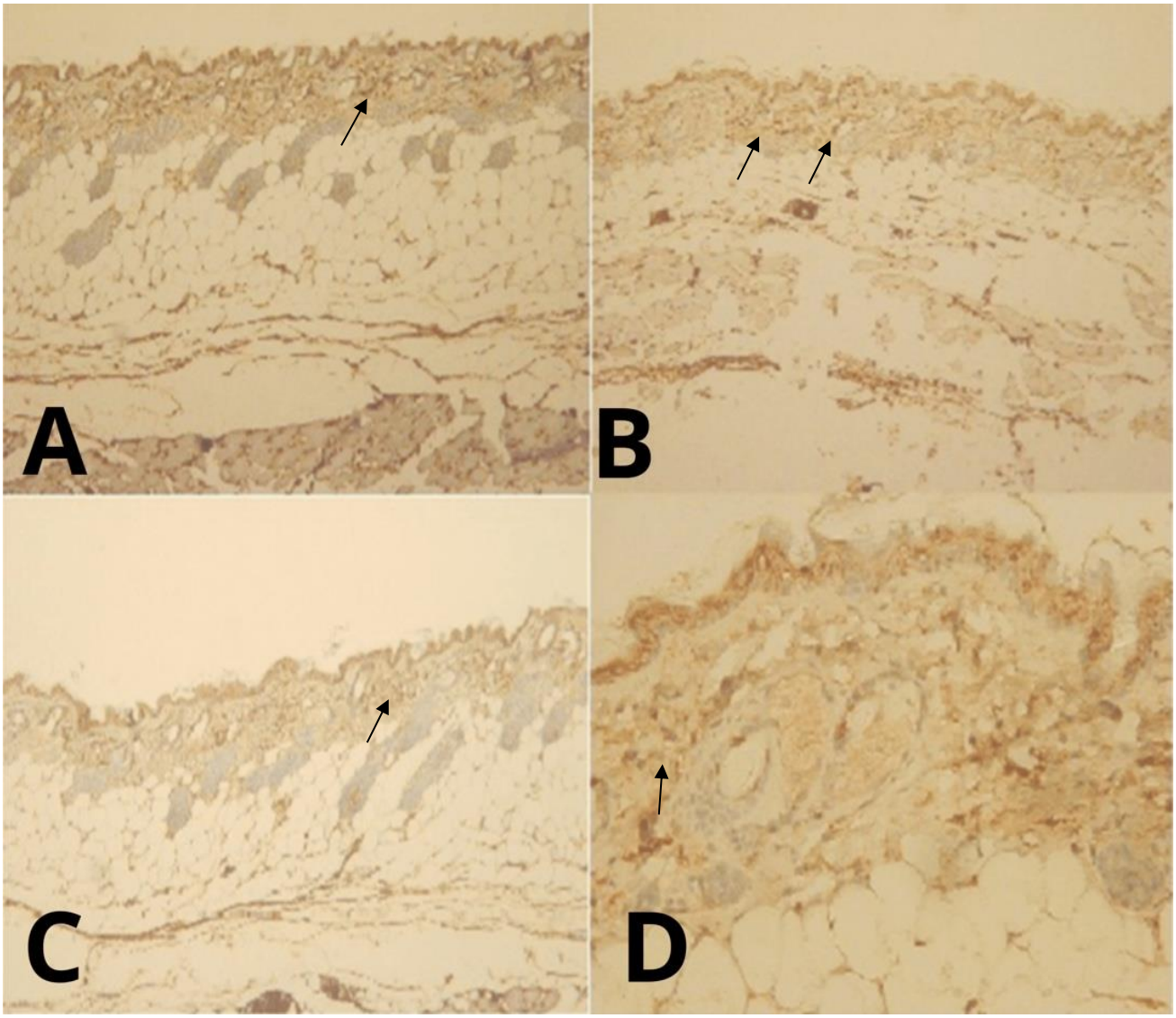

Figure 5. Microphotography of back-skin mice post-injection of 1-4% carrageenan. **A:** Mouse skin without carrageenan treatment. **B, C,** and **D:** Mouse skin with carrageenan treatment at 1-4%. COX-1 expression was found in the dermis area (black arrow). Anti-COX-1 antibody staining, 200× magnification.

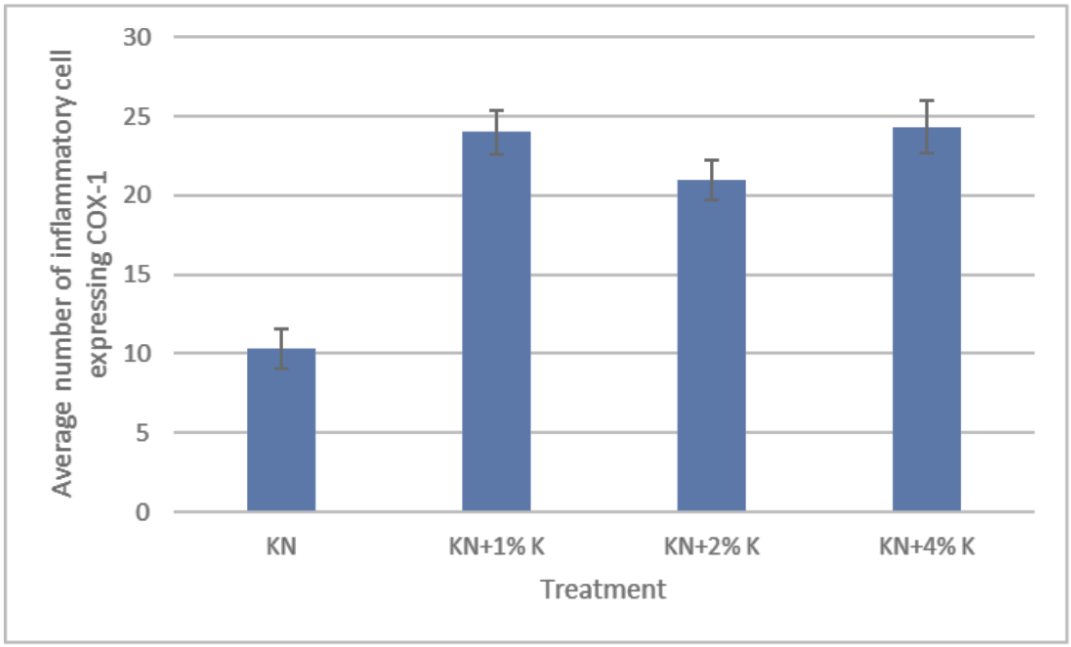

Figure 6. The average number of inflammatory cells in the back skin of mice injected with 1-4% carrageenan expressing COX-1. KN: Untreated mice skin. KN+1%, KN+2%, and KN+4%: Mice skin with carrageenan treatment 1-4%.

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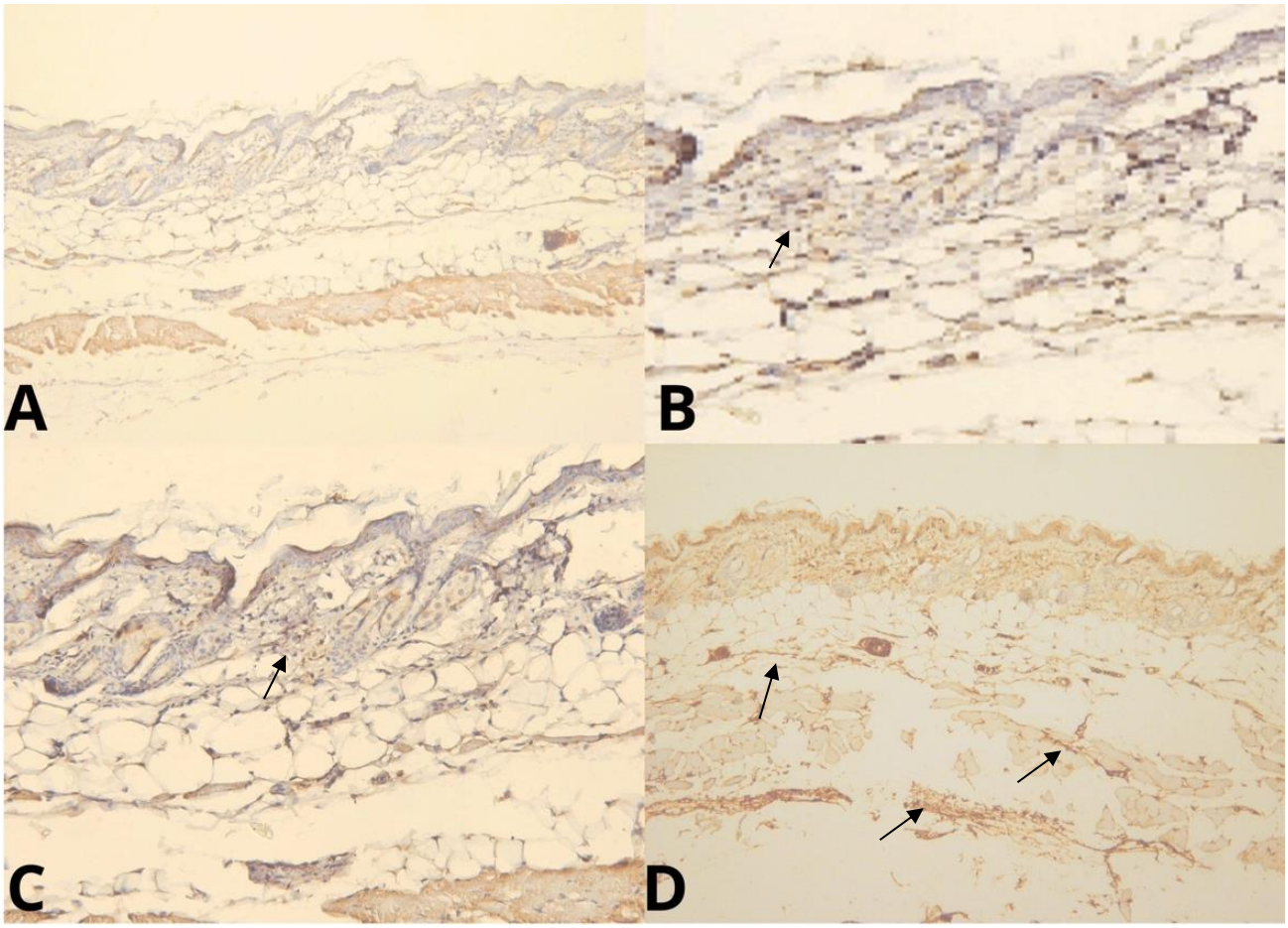

Figure 7. Microphotography of back-skin mice post-injection of 1-4% carrageenan. **A:** Mouse skin without carrageenan treatment. **B, C,** and **D:** Mouse skin with carrageenan treatment at 1-4%. IL-6 expression was found in the dermis area (black arrow). Anti-IL-6 antibody staining, 200× magnification.

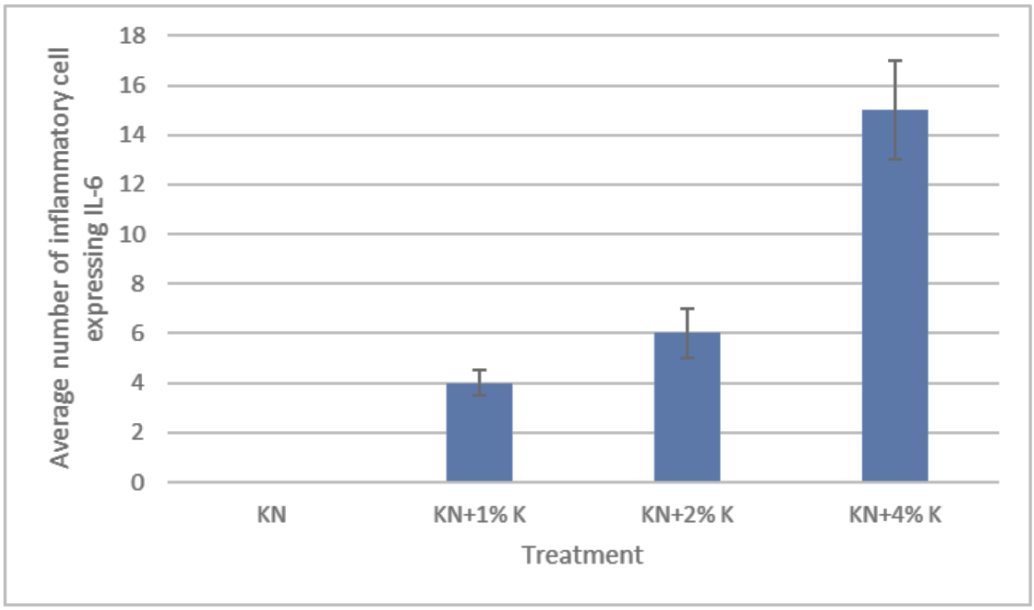

Figure 8. The average number of inflammatory cells in the back skin of mice injected with 1-4% carrageenan expressing IL-6. KN: Untreated mice skin. KN+1%, KN+2%, and KN+4%: Mice skin with carrageenan treatment 1-4%.

DISCUSSION

A previous study by Widyarini et al. (2023) demonstrated that injection with carrageenan concentrations of 1% and 2% produced histopathological changes in the form of mild and moderate inflammation, which were observed as infiltration of inflammatory cells in the dermis accompanied by edema in the dermis. Furthermore, severe dorsal skin inflammation

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of mice was observed in the 4% carrageenan injected group, demonstrating histopathological features in the form of inflammatory cell infiltration in the dermis. Carrageenan can increase vascular permeability and result in edema (Zhang et al., 2022) and panniculus accompanied by edema (Widyarini et al., 2023). A similar histopathological picture is shown in Figure 1.

Figure 1 shows that the higher the concentration of carrageenan results in more severe skin tissue damage. The studies performed by Szekalska et al. (2020) and Vysakh et al. (2020) reported the injection of carrageenan into the paws of mice showed microscopic features in the form of acute edema, increased epidermal thickness, vasculitis, swelling, and infiltration of inflammatory cells in the epidermis and dermis. Injection of different concentrations of carrageenan (1% to 4%) into the dorsal skin of mice revealed microscopic changes in the form of edema and infiltration of inflammatory cells into the dermis and panniculus of varying severity but no microscopic features of hyperemia or vasculitis were found (Figure 1). This is thought to be because the sampling of the back skin of mice in this study was carried out at the sixth hour after carrageenan injection where the effect of carrageenan in the vascular phase has passed, so hyperemia and vasculitis are not found on microscopic examination, as it has been reported by previous studies (Reanmongkol et al., 2009; Ben Khedir et al., 2016; Szekalska et al., 2020).

Previous studies have only reported an association between carrageenan injection on the paws of the mouse (paw edema model) and histopathological images of inflammatory responses (Zhang et al., 2013; Felhi et al., 2017). In this study, quantification of the number of inflammatory cells was performed. The results showed that carrageenan injection at concentrations increasing from 1% to 4% increased the average number of inflammatory cells (Figure 2). Increasing concentrations of carrageenan result in an increased accumulation of inflammatory cells as a result of tissue damage following carrageenan exposure. The increased carrageenan concentrations in this study were consistent with the increased mean inflammatory cell counts (Figure 2) and the histopathological features of edema formation (Figure 1). The results of this study are also consistent with previous studies indicating that some inflammatory cells and edema were observed in the paw tissue of rats after carrageenan injection (Zhang et al., 2013).

Interleukins, such as IL-1, IL-6, IL-12, tumor TNF, interferon, cyclooxygenase, and inducible nitric oxide synthase, are released during the inflammatory process. These cytokines play important roles in the initiation and amplification of inflammatory responses (Calixto et al., 2004). It has been reported that carrageenan injections can trigger acute inflammation due to the influence of various inflammatory mediators (Necas and Bartosikova, 2013). Histamine and serotonin release immediately after induction and decrease after two hours, followed by the release of bradykinin and prostaglandins, and persists in the third hour until the fifth hour after induction (Calixto et al., 2004; Halici et al., 2007). The release of the abovementioned inflammatory mediators affected the increase in the number of inflammatory cells and the expression of inflammatory mediators such as COX-2, COX-1, and IL-6, as well as the histopathological picture in the form of edema in this study.

Injection of 1% carrageenan increased COX-2 expression after 3 hours, according to research conducted by Carvalho et al. (2022). This study demonstrated that carrageenan injection with increased concentrations is in line with the increased expression of COX-2, COX-1, and IL-6 in inflammatory cells. Injection of 4% carrageenan showed a greater number of inflammatory cells expressing COX-2 compared to 1% and 2% carrageenan injections (Figure 4), and this corresponds to the high intensity of COX-2 found in Figure 3D (in the dermal and subcutaneous areas). It is reported that paw edema increased in accordance with the increase in carrageenin concentration (Silva et al., 2010). An increase in inflammatory cells expressing COX-2 after injection of 1-4% carrageenan might be related to the formation of edema and pain around inflammation. A study by Carvalho et al. (2022) reported that neutrophil-derived COX-2 plays an important role in inflammatory pain mediation.

In this study, COX-1 expression is found in the dermis area of normal skin and skin injected with carrageenan. Carrageenan injection at a concentration of 1%-4% increased COX-1 expression by approximately 60% compared to normal skin but did not differ significantly between concentration groups (Figures 5 and 6). COX-1 expressed in the untreated back skin indicates the homeostatic phase prior to inflammatory skin conditions, as previously mentioned by Carvalho et al. (2022).

IL6 is a cytokine that is expressed by cells under stressful conditions such as inflammation, infection, and cancer (Choy and Rose-John, 2017). Carrageenan injection (4%) showed an increase in IL-6 expression in the back skin of mice compared to the carrageenan concentrations of 1% and 2%. IL-6 expression was found in inflammatory cells in the dermal area (Figures 7 and 8). When compared to the expression of COX-2 and COX-1, IL-6 expression appeared to be lower in this study. This is likely due to IL-6 being involved at the beginning of the inflammatory phase, so there is less visible expression in skin tissue compared to COX-2 and COX-1 expressions. In the initial inflammatory phase, the IL-6 concentration increased within 1.5 hours and continued to increase in the next 4 hours after carrageenan injection (Chou, 2003). Increased concentrations of IL-6 facilitate the recruitment of leukocytes and mediate the formation of edema and pain, which are involved in the inflammatory response (Annamalai and Thangam, 2017). In this study, skin samples of the back of mice were taken 6 hours after carrageenan injection in accordance with previous studies (Widyarini et al., 2023). Six hours after injection, carrageenan initiates a cellular phase that will lead to the withdrawal of inflammatory

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cells toward the edematous area (Reanmongkol et al., 2009), corresponding to the results of inflammatory cell counts, COX -2 and COX-1 expression in this study.

The results of this study show that an increase in carrageenan concentration affects the number of inflammatory cells and the expression of COX-1, COX-2, and IL-6 in dorsal skin mice. Histopathological appearance of the skin due to subcutaneous injection of carrageenan at various concentrations supports the result of inflammatory cell count and the expressions of COX-1, COX-2, and IL-6. Thus, the back skin of mice could be used for carrageenin-induced acute inflammation models. Moreover, the back skin area is quite easy and stable to measure and/or evaluate inflammatory responses when the test material is topically applied.

CONCLUSION

Carrageenan injections in the back skin of mice showed significantly different amounts of inflammatory cells at concentrations of 1%-4. Expression of inflammatory mediators was seen in the dermis and subcutaneous areas, where COX-2 expression was indicated at the highest level compared to COX-1 and IL-6. Carrageenan induces inflammation in back skin mice and can be used as a model of acute inflammation for evaluating testing material that will be given topically. The results of this study suggest using carrageenin 2% to 4% as an acute skin inflammatory inducer based on its activity in causing tissue damage, the number of inflammatory cells in the exposure area, and the expression of inflammatory mediators COX-2, COX-1, and IL-6.

DECLARATIONS

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available.

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Authors' contribution

Sitarina Widyarini conceptualized, managed, and supervised the study. Sitarina Widyarini and Sugiyono drafted the manuscript and performed all the experimental procedures. Sitarina Widyarini, Sugiyono, Alsi Dara Paryuni, and Akrom, Alsi Dara Paryuni, conducted data analysis and interpretation. All authors read and approved the final manuscript.

Ethical considerations

The authors carefully examined all ethical issues concerning plagiarism, and double publication, and submission.

Competing interests

The authors declare no conflict of interest concerning the work presented in this report.

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Observational Study on Reproductive Behavior in Semi-Wild Sambar Deer (*Rusa unicolor*) for Wildlife Conservation and Assisted Reproductive Management

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ABSTRACT

Understanding the reproductive behaviors of different wildlife species is essential to unravel their reproductive strategies, ecological adaptations, and conservation requirements. This study delved into the reproductive biology of the sambar deer (*Rusa unicolor*), with a focus on promoting assisted reproductive technology for wildlife conservation and investigating the reproductive behaviors of male and female sambar deer. The study was conducted at Pusat Konservasi Hidupan Liar (PKHL) Sungkai, Perak, Malaysia. The observation focused on one male and two female sambar deer. Direct observations of the deer were conducted for 14 days in September 2022. The direct observations were performed in the morning (Session 1= 8-10 am), afternoon (Session 2 = 10-12 pm), and evening (Session 3= 3-5 pm), using the instantaneous sampling method. A total of 75 behavior instances were recorded, in which male deer exhibited the most reproductive behavior at 58 instances (77.3% of the total reproductive behavior). Successful mating was observed on day 6, elucidating a crepuscular preference in the male animal in exhibiting reproductive behavior. The female's reproductive behavior lasted for a short period, from 24 hours for Female 2 and 72 hours for Female 1. In conclusion, there was a distinct behavior between the male and female deer during the rutting season. Understanding the reproductive behavior to estimate the length of estrus can be useful as a non-invasive tool to detect heat and can be considered to improve breeding management and implement assisted reproductive technology.

Keywords: Breeding, Conservation, *Ex-situ*, Release program, Wildlife

INTRODUCTION

In the study of animal behavior, it is important to understand the species' reproductive strategies, ecological adaptations, and conservation needs. The reproductive biology of the sambar deer (*Rusa unicolor*), an ecologically significant species as a prey source for the critically endangered Malayan tiger (*Panthera tigris jacksoni*) remains to be obscured (Ten et al., 2021). Further decline in the population of sambar deer in their natural habitat increases the urgency of conserving the species (Kawanishi et al., 2014). There has been a growing interest in employing assisted reproductive technology in deer, which highlights a progressive step in wildlife conservation reproductive management (Rola et al., 2021). This initiative aligns with the Malaysian government's dedicated efforts to elevate the genetic standards of the nucleus herd population, thereby enhancing the overall resilience and viability of these deer for subsequent release programs into their natural habitat (Munisamy et al., 2022).

Assisted reproductive technology, such as artificial insemination, is an important technique to explore wildlife species. The technique has been proven to be an effective tool in improving the genetic exchange in livestock (Van Doormaal and Kistemaker, 2003). However, before this technology can be applied, it is important to understand the reproductive biology and behavior of the sambar deer. Due to the limitation in obtaining new genetic resources for breeding, deer was shown to be predisposed to inbreeding, which has been shown to contribute to fertility decline (Yahaya et al., 2020). Poor reproductive performance in deer was also associated with the low exchange of genetic materials (Zakaria et al., 2016). Stress and reproductive behavior studies of the sambar deer with fecal metabolites analysis have been performed (Abdul Hamid et al., 2022; Selvarajah et al., 2022). It is important to collate this specific knowledge in the reproductive biology of the sambar deer due to the species-specific differences that can limit the efficiency of assisted reproductive technology (Morrow et al., 2009).

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The sambar deer exhibit a distinct sexual dimorphism. Physically, male and female deer can be distinguished by the presence of antlers in the male animal (Leslie, 2011). The function of antlers and reproductive performance has long been an enigma and center of attention in deer reproductive physiology studies (Monfort et al., 1993). The breeding selection through fitness and genetic superiority in the antler confirmation contribute to the gene being passed down the generation and the survivability of the offspring (Kruuk et al., 2002). The dynamic antler cycle and social dominance also mean varying opportunities for the male to breed (Savanth et al., 2011). The drop of the antler signifies the end of the breeding activity in an Elk deer; however, a different male may continue the mating activity (Johnson et al., 2005). Thus, the behavioral patterns and sexual preferences require a further understanding in elucidating the main component contributing to reproductive success. This study aimed to investigate the sambar deer reproductive behaviors, aiming to shed light on contrasting patterns exhibited by male and female individuals in promoting active conservation management of the species.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Department of Wildlife and National Parks (DWNP), Peninsular Malaysia, with approval number JPHLTN.600-6/1/4 JLD (78) and Institutional Animal Care and Use (IACUC), Universiti Putra Malaysia, Serdang, Selangor, Malaysia, approval number UPM/IACUC/AUP-U041/2022.

Study site

The study was conducted at Pusat Konservasi Hidupan Liar (PKHL) Sungkai, Perak, Malaysia (4.0353° N, 101.3658° E) including a total of 2468 acres of lowland dipterocarp forest. It is one of the 35 protected areas managed by the Department of Wildlife and National Parks (DWNP), Peninsular Malaysia, and is situated on the western side of Perak in the Batang Padang district.

Management of sambar deer

The sambar deer at the conservation center were managed under a semi-wild system. They were all kept in the paddocks with shelter and provided with food and water daily. The deer were fed with cut and carried various leaves, including *charcoal tree*, *Trema orientalis*, and turn-in-the-wind, *Mallotus paniculatus* given *ad libitum*. The pasture was planted with Koronivia grass, *Brachiaria humidicola*, and guinea grass, *Megathyrsus maximus*. Besides that, the deer were fed with concentrates, mineral lick blocks (Solsel®, European Salt Company [ESCO], Hannover), and multivitamins (Vita-Stress, Sunzen, Malaysia) every two weeks. The grass was provided *ad libitum*. The grass quality varies with season; thus, the concentrates fed were adjusted based on the performance of the body condition score which was assessed weekly. The deer were fed once daily in the morning, and the keeper utilized this time to observe the animals for any sign of poor health as well as to check the perimeter of the fence for integrity. The average temperature in the district during the study period was 27˚C, the temperature during the observation was 28.5˚C ranging from 23˚C to 34˚C. There was no artificial lighting provided at the conservation center.

Ethogram behavioral observation

In this study, the deer were managed in a herd of mixed ages (2-6 years old) and varied distribution of sexes (male = 4, female = 22, and juvenile = 4). The observation focused on one male and two females. The sambar deer is housed in a paddock enclosure that is 75 meters long and 36 meters wide. A preliminary behavioral observation for three days was conducted to confirm that the deer were all healthy. Direct observations of the deer were conducted for 14 days, in September 2022. The direct observations were separated into three sessions: in the morning (Session 1= 8-10 am), afternoon (Session 2 = 10-12 pm), and evening (Session 3 = 3-5 pm), using the instantaneous sampling method (Altmann, 1974). In brief, during each session, the observations were done in 15 minutes sub-session time block, which is equivalent to eight slots over a span of two hours. Only a single frequency will be noted for each behavior under a one-time slot. Each slot only corresponds to a single occurrence of behavior despite multiple repetitions. The observation sheet to collect the behavioral data was described in the ethogram (Table 1). The ethogram was created from a slight modification following a preliminary observation period of three days before data collection (Abdul Hamid et al., 2022; Selvarajah et al., 2022).

Data analysis

The data is presented in frequency. The percentage was derived by dividing the number of reproductive behaviors by the total number of reproductive behaviors exhibited by the individuals. Preparation, tabulation, and presentation of data and graphs were made using Microsoft Excel Version 2310 from Microsoft 365 Apps for Enterprise.

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Table 1. Ethogram of sambar deer (*Rusa unicolor*) reproductive behavior in Pusat Konservasi Hidupan Liar Sungkai during 2 weeks of observational study in September 2022

Behavioral grouping	Behavioral subgrouping	Code	Description
Reproductive behavior (Male-specific)	Follow	FO	Male follow female
	Smelling of female urine or feces	SM	Smelling urine or feces, followed by flehmen response
	Anal sniffing	AS	Male smells female vaginal area
	Flehmen response	FR	Male raised their head and curled his upper lip
	Chin resting	CR	Male rests its chin on the rump of the female
	Grooming	GR	Male licking the female's body
	Mounting	MO	Male climbs on the female's back with the thorax resting on her rump. Front limbs hang and the hind limbs on the ground
	Mating	MA	Male executes successive movements of flexion and extension of the vertebral spine while mounting on the female.
Reproductive behavior (Female specific)	Follow	FO	The female follows the male
	Smelling of male urine or faeces	SM	Smelling urine or feces, followed by flehmen response
	Anal sniffing	AS	The female smell male rectal/penis area
	Flehmen response	FR	The female raised her head and curled her upper lip
	Chin resting	CR	The female rests its chin on the rump of the male
	Grooming	GR	Female licking the male's body

FO: Follow, SM: Smelling of urine or feces, AS: Anal sniffing, FR: Flehmen response, CR: Chin resting, GR: Grooming, MO: Mounting, MA: Mating

RESULTS AND DISCUSSION

A total of 84 hours of behavioral observations were made during the fourteen days, capturing the reproductive behavior of three sambar deer individuals (M1, F1, and F2). Out of the recorded observations, M1 exhibited reproductive behavior in 58 instances, while F1 and F2 displaced reproductive behavior in 14 and 3 records, respectively. The frequency of reproductive behavior is represented in Graph 1. Notably, the male (M1) showed a high frequency of sexual behavior, accounting for 77.33% of the total incidences, compared to F1 and F2 at 18.67% and 4%, respectively. Further analysis revealed that M1 primarily engaged in anal sniffing (AS), which constituted the highest observed frequency at 32.8%. Conversely, both females, F1 and F2, exhibited a high frequency of grooming (GR) behavior at high frequencies of 50% and 66.7%, respectively.

Due to the high frequency of reproductive behavior shown by the male sambar deer, deeper insights were needed to assess the activity pattern across different observation sessions. The reproductive behavior of male sambar deer is summarized in Table 2. It was found that the male displayed reproductive behavior preference towards the crepuscular periods during the evening and morning as compared to the afternoon session. Out of the total 58 reproductive behaviors displayed, 51% occurred during the evening session (3 pm to 5 pm), followed by 40% during the morning session (8 am to 10 pm), while only 9% during the afternoon (10 am to 12 pm).

Throughout the 14-day observation period, the individual sambar deer reproductive behavior was visualized (Graph 2). The male consistently displayed active reproductive behavior throughout the observation period. However, two peaks in reproductive behavior were observed during days 6 and 10. Additionally, successful mating occurred on day 6, during the S1 morning observation session between M1 and F1 (Figure 1). The F1 reproductive behavior steadily increased from zero on day 3 to day 5, peaked on day 6, and quickly reduced on day 7 until it finally ceased activity on day 8. The reproductive behavior decreased and remained plateaued post-mating. In the case of F2, reproductive behavior remains at zero, with a peak activity observed on day 10. However, no successful mating was observed and the reproductive behavior reduced on day 11 and finally ceased for the following observation. The reproductive behavior in the females lasted for a short period, from 24 hours for F2 and 72 hours for F1. The male sexual behavior displayed a

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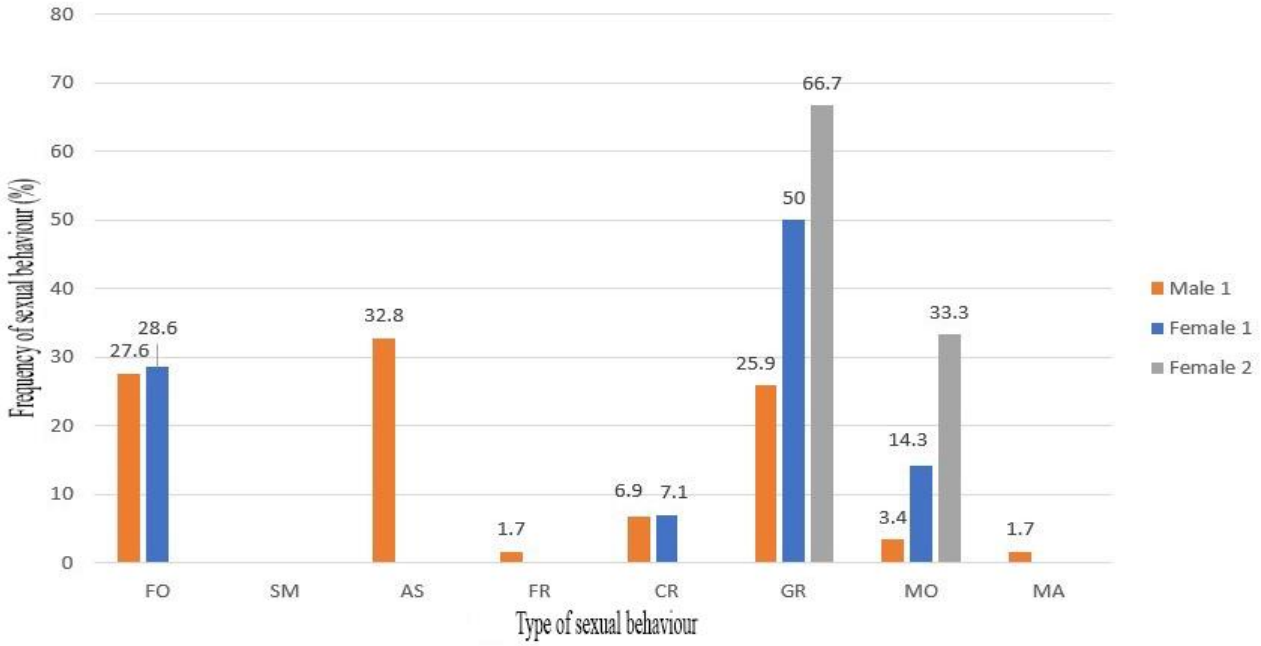
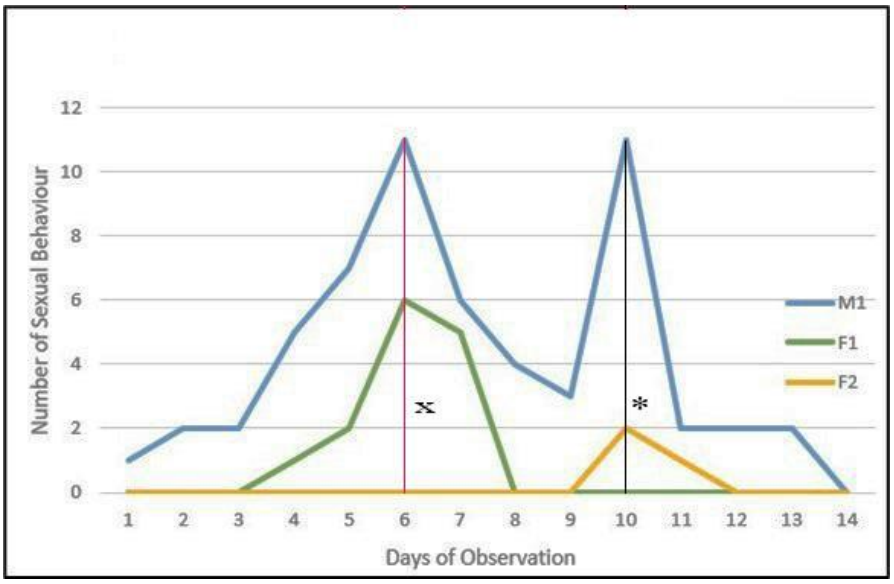
sharp decline from day 11 to zero reproductive behavior on day 14, coinciding with the shedding of the final and second sides of the antler on day 13.



Graph 1. The frequency of reproductive behavior was recorded from the three deer (*Rusa unicolor*) in Pusat Konservasi Hidupan Liar (PKHL) Sungkai during the two weeks of observational study in September 2022. FO: Follow, SM: Smelling of urine or feces, AS: Anal sniffing, FR: Flehmen response, CR: Chin resting, GR: Grooming, MO: Mounting, MA: Mating.

Table 2. The three-session frequency of the male sambar deer (*Rusa unicolor*) exhibiting behavior reproductive behavior in Pusat Konservasi Hidupan Liar (PKHL) Sungkai during the two weeks of observational study in September 2022

Time of the day	Frequency of sambar deer exhibiting reproductive behavior
8 am - 10 am	40% (22/58)
10 am - 12 pm	9% (5/58)
3 pm - 5 pm	51% (31/58)



Graph 2. Frequency of sexual behaviors exhibited by all three deer (*Rusa unicolor*) in Pusat Konservasi Hidupan Liar (PKHL) Sungkai during the two weeks of observational study in September 2022. M1: Male 1, F1: Female 1, F2: Female 2. X: Successful mating observed, * shedding of the final and second side of the antler observed

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Figure 1. Successful mating involving the observation of successful penile insertion on day 6 in sambar deer (*Rusa unicolor*) in Pusat Konservasi Hidupan Liar Sungkai during 2 weeks of observational study in September 2022

The male sambar deer demonstrated a significantly higher frequency of sexual behaviors compared to the two female individuals. This aligns with previous studies indicating that male deer often exhibit more active and varied sexual behaviors as part of their reproductive strategies (Bazyan, 2013). In the present study, the male sambar deer predominantly engaged in anal sniffing (AS), which represented the most frequent sexual behavior observed. This behavior is known to play a crucial role in scent marking and gathering of olfactory information related to female receptivity and reproductive status (Apfelbach et al., 2015). The high frequency of AS exhibited by the male individual suggests its importance in male sambar deer's reproductive strategies.

Conversely, the females, F1 and F2, exhibited grooming (GR) behavior at notably high frequencies towards the male deer. Grooming behavior in the female sambar deer and its role in reproduction is poorly understood. A study on the sika deer found that there was no correlation in grooming following the rutting season, as it only reduces the tension in the group (Matsuno and Urabe, 1999). However, a recent study observed a high grooming frequency, suggesting this behavior is a pre-courting behavior for the male to assess female receptivity in sambar deer (Selvarajah et al., 2022). This further strengthens the finding in the present study suggesting that grooming behavior plays an important role during the mating process and is aligned with a successful mating event. Female deer grooming male deer may indicate their interest and availability for mating, contributing to courtship dynamics within the population. The present study suggests that female deer may groom male deer as a form of sexual attraction and to signal their reproductive readiness. The minimal sexual behaviors displayed by the female individuals, except during the peak periods, may indicate a more selective and receptive mating strategy (Ciuti and Apollonio, 2016). The minimal sexual behaviors exhibited by the female deer suggest the possibility of behavioral observation in females to estimate estrus reliably (Mahre et al., 2013)

Estimating the estrus period is crucial for successful reproductive management since the fertilization window is limited. The result from this study indicated that there are two distinct peaks in male sexual behavior, which coincided with the estrus behaviors of the female deer. The result from this study further enhances the findings suggested in another study on sambar deer, which found that the frequency of sexual behavior in males can be used to estimate the presence of females in estrous in sambar deer (Putranto et al., 2010). The observed peaks in reproductive behavior on days 6 and 10 further highlight the temporal dynamics of sexual activity in male sambar deer.

The female reproductive behavior in this study offers intriguing insights into the female receptivity pattern. Notably, the presence observations revealed a noteworthy occurrence. After successful mating in F1, there was a pronounced decline in her reproductive behavior. Simultaneously, the reproductive behavior of F2 remained absent until day 10, and even then, it diminished shortly afterward. This sequence coincided with the male shedding its final antler, and consequently, his reproductive behavior also ceased. From these observations, it becomes apparent that the females' estrus cycles might not synchronize. This reproductive strategy could potentially serve to stagger the timing of female receptivity, affording the male ample opportunity to fertilize multiple females. This adaptive strategy may enhance the chances of successful fertilization, contributing to the species' reproductive success. The present findings further strengthen the idea that the more dominant and older females mate earlier in the rut than the younger and less dominant

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females (Farrell et al., 2011). Further exploration of these intricacies could provide valuable insights into the underlying mechanisms of sambar deer reproductive behaviors.

The occurrence of successful mating on day 6 during the morning session indicated the importance of timing and synchrony between male sexual behavior and female receptivity. The mating frequency is unknown in the sambar deer. This study only observed one successful mating once during the whole observation period. Deer is known to mate only once during the rutting season, accounting for 75% of the breeding female population (Say et al., 2003). Hence, the successful mating status of F2 remains ambiguous, given its alignment with the male deer's antler-shedding period. While it remains a plausible scenario that successful mating transpired, it is possible that the decline in mating behavior could have been influenced by the male's breeding ability. Considering that breeding entails resource competition among females, the dynamics of female-female interactions and dominance hierarchies emerge as pivotal factors in evaluating female fitness for breeder selection. This aspect gains even greater significance when applied to breeding programs aimed at conserving and reintroducing wildlife into their natural habitats. By monitoring the frequency of sexual behavior and specific copulatory behaviors, the timing and length of the estrous period can be estimated (Higuchi et al., 2013). This information is essential for identifying the optimal time for insemination, as ovulation typically occurs 24 to 28 hours after standing heat is observed in deer (Asher, 1985).

The antler cycle of male deer also appears to influence sexual behavior (Malo et al., 2012). This study indicates an arrest in male sexual behavior after the second antler was cast down. The antler stage is useful for determining the breeding season in deer (Fitri et al., 2017). Testosterone levels were observed to peak during the period of highest semen quality and subsequently decrease following the casting of the antler (Jaafar et al., 2017). The sudden drop in testosterone concentration during the antler casting stage contributes to the reduced frequency of sexual behaviors in the male deer (Bartos, 2012). Antler shedding is a physically demanding process, requiring energy allocation and physiological changes, which may temporarily reduce the male's reproductive activity (Kavčić et al., 2019). This suggests a potential interplay between hormones, antler development, and sexual behavior in sambar deer.

The relationship between sexual behavior and feeding patterns is an interesting aspect of this study. Most male sexual behaviors occurred during the evening, coinciding with the time when grazing activities were the most predominant. This finding aligns with the crepuscular nature of sambar deer, which are most active during dawn and dusk (Comte et al., 2022). Additionally, environmental factors such as temperature and food availability may influence the timing of sexual behavior. Higher water-soluble carbohydrate concentrations in the grass during the evening hours may affect the grazing decisions of deer (Wang et al., 2020). The timing of sexual behavior is crucial as it may optimize reproductive success by synchronizing with the receptivity of females or minimizing predation risks (Pérez-Barbería and Walker, 2018). The higher frequency of sexual behaviors during the evening and morning sessions suggests that male sambar deer allocate their reproductive efforts during these periods, potentially reflecting optimal conditions for mating and courtship. This was observed in the population of jungle fowl (*Gallus gallus domesticus)*, which changed its preference to mate in the evening, particularly in male-biased groups (Løvlie and Pizzari, 2007). Therefore, late afternoon or evening could be considered the optimal period to observe estrous signs and monitor sexual behaviors in the sambar deer based on presence observation.

CONCLUSION

The present study offers valuable insights into the reproductive behaviors of the sambar deer, illuminating the distinct patterns displayed by male and female individuals. Notably, the present study highlights a crucial aspect of the estimation of female estrous. This estimation hinges on a composite analysis of factors including antler stage, male reproductive behavior, and the corresponding responses from female individuals. Given the fleeting receptivity of female oocytes to fertilization, present findings assume significance in refining the accuracy of reproductive interventions such as artificial insemination. The noteworthy decline in reproductive behavior observed during antler casting serves to deepen the comprehension of the antler cycle and mating ability. This, in turn, prompts essential considerations for semen collection procedures when not in the rutting season. The recommendation for future study is to consider performing estrous synchronization response and artificial insemination in sambar deer based on behavioral observation to evaluate the efficacy of the heat detection technique.

DECLARATIONS

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Availability of data and material
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Authors' contribution

Aiman Murad was centered on the acquisition of data and fieldwork. Hartini Ithin was integral in the conceptualization of the sampling. Tengku Rinalfi Putra was involved in the manuscript revision and intellectual discussion. Cosmas Ngau was the key field person who ensured that the project ran smoothly and assisted immensely in data analysis and interpretation. Wan Nor Fitri contributed to the write-up of the manuscript and the overall form of the article. All authors have read and approved the final draft of the manuscript.

Ethical consideration

Ethical issues, such as the integrity of the data and permission to conduct and publish the study, have been checked by all the authors before publication in this journal.

Competing interests

The authors have no conflict of interest.

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Efficiency Evaluation of Silica Nanoparticles as a Pesticide against *Ctenocephalides felis*

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ABSTRACT

The increasing resistance of arthropods to many insecticides has encouraged researchers to search for new alternatives to combat harmful insects. The present study aimed to evaluate the effectiveness of silica nanoparticles (NPs) on *Ctenocephalides felis* (*C. felis*), a prevalent species among cats and a known vector for diseases. The killing efficacy of SiO2-NPs against *C. felis* was tested at three different concentrations (50, 100, and 150 mg/ml) over three different time intervals (10, 20, and 40 minutes), alongside positive and negative control groups (distilled water and cypermethrin). The results of the current study indicated that all concentrations had a fleacidal effect, with SiO2-NPs demonstrating increased efficacy with higher concentrations and longer exposure periods. The concentration of 150 mg/mL of SiO2-NPs led to the highest effect at 96% upon exposure for 40 minutes. The results of the current study revealed significant differences between the control groups and all the groups treated with Sio2-NP concentrations. It can be concluded that Sio2-NPs are a practical approach to flea control although it is necessary to search for environmentally friendly pesticides. The current results indicate that SiO2-NPs have anti-parasitic effects against *C. felis*.

Keywords: Cat fleas, Iraq, Nanoparticles, Pesticide, Silica

INTRODUCTION

Nanotechnology is an important technology recently used against various pathogens, such as bacteria, fungi, and parasites. Recent studies have indicated the possibility of using nanomaterials as effective pesticides against insect pests and plant pathogens (Thabet et al., 2021). Nanopesticides are soluble in water and have no environmental risks, compared to traditional insecticides (Goswami et al., 2010; Abbasi et al., 2020; Croissant et al., 2020).

Silica nanoparticles (NPs) have received great attention as a potential alternative to traditional insecticides due to the direct effects of silica NPs on many pest insects. In addition, other studies investigated the field effects of silica on some insect pests (El-Samahy and Galal, 2012; Mousa et al., 2014; El-Samahy et al., 2015).

Ctenocephalides felis (*C. felis*) are external parasites infecting domesticated cats and dogs (Rust, 2017). It is one of the most common types of fleas, and its spread has increased in recent years due to people's interest in domesticating cats (Paterson, 2008; Rust, 2016). Cat fleas, specifically *C. felis*, play a crucial role in transmitting various diseases, such as plague, murine typhus, and tapeworms, to humans and animals. Moreover, *C. felis* is known as an intermediate host for *Dipylidium caninum* (Rousseau et al., 2022). The salivary secretions of *C. felis* can lead to a severe infection called flea dermatitis (Manvell et al., 2022), and consequently, hyperpigmentation, alopecia, and pyoderma (Noli, 2020). Given the resistance of fleas to insecticides and the role that nanoparticles play in treating many diseases, the current study aimed to examine the effectiveness of silica oxide nanoparticles (SiO2-NPs) on fleas *in vitro.*

MATERIALS AND METHODS

Ethical approval

The current study was approved ethically by the Committee of Department of Biology, Faculty of Education, University of AL-Qadisiyah, Iraq.

Sample collection

An examination was conducted on 51 domestic cats (*Felis catus*) admitted to the General Veterinary Hospital in Diwaniyah, among which 12 cats were infected with fleas. Samples of adult species *C. felis* were collected from the cat's hair using Flea combs from different body areas, including the head, under the tail, and abdomen. The fleas were then

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removed using forceps and placed in plastic boxes. These samples were transported directly to the Parasitology Laboratory in the Department of Life Sciences, College of Education, Al-Qadisiyah University, Iraq.

Preparation of Silica nanoparticles

Silica nanoparticles were acquired in the form of ready-made oxide from an American company (Sky Spring Nanoparticles). The material was presented as a fine white powder composed of Sio2NPs ranging in size from 10 to 30 nm with a purity of 99.5%.

A stock solution of Sio2NPs was prepared by dissolving 3 g of Sio2NPs in a liter of distilled water, and it was sterilized by autoclave. After that, the solution was mixed using an ultrasonic homogenizer for 20 minutes, and then the three concentrations (50, 100, and 150) were prepared and placed in the refrigerator (Haghi et al., 2012).

The anti-flea effect of Silica nanoparticles

To investigate the anti-flea effect of Sio2-NPs, *C. felis* flea samples were collected, and their diagnosis was confirmed using a compound light microscope (Olympus, Japan) at 40× magnification. The fleas were distributed on 15 petri dishes, with 5 fleas placed in each dish. For dishes 1-3, 0.5 ml of distilled water was added as a positive control group. Dishes 4-6 received Cypermethrin 10% (Mainland, China). They were left as a negative control group, while dishes 7 to 15 received three different concentrations of Sio2-NPs at levels of 50 mg/ml, 100 mg/ml, and 150 mg/ml, with three replicates for each concentration. The examination of the dishes was repeated after 2-6 hours to ensure the death of the fleas.

The petri dishes were then examined under a light microscope to observe any expected movement of the fleas after 10, 20, and 40 minutes for each concentration, and the immobile fleas were considered dead (Meinking et al., 1986).

Statistical analysis

A completely randomized design was used in a two-factor experiment (concentrations and time periods) and for three replicates. The results that were obtained were subjected to statistical analysis using SPSS software version 24. Least Significance Differences (LSD) was performed, and the probability level was $p < 0.05$.

RESULTS

The results of testing the effectiveness of Sio2-NPs on *C. felis* at three different concentrations of 50, 100, 150 mg/ml and for three-time intervals of 10, 20, and 40 minutes revealed that all the mentioned concentrations had pesticidal effects to varying degrees (Table 1). It was noted that the effectiveness of Sio2-NPs increased with increasing concentration and duration of exposure to the pesticide. The 150 mg/ml concentration indicated the best pesticide effect of 96% after 40 minutes, which was the highest percentage, compared to other concentrations and time intervals ($p < 0.05$). The lowest mortality rate was recorded for the group treated with a 50 mg/ml concentration, reaching 22.3% at 10 minutes.

After 40 minutes, all concentrations of Sio2-NPs had pesticidal effects, reaching 78%, 79.81, and 96%, respectively. The effectiveness of Sio2NPs began to decrease with decreasing time, as the percentage of *C. felis* deaths in the three concentrations after 20 minutes reached 45%, 74.69%, and 89.91%, respectively. At the same time, the lowest flea mortality rate was recorded for the three concentrations after 10 minutes, with percentages reaching 22.3%, 68%, and 81%, respectively. Statistical analysis showed significant differences between the treatment groups and the control group ($p < 0.05$).

Table 1. Anti-activity of SiO2-NPs against cats' fleas at different time intervals

Concentrations (mg/ml)	10 minutes	20 minutes	40 minutes
50	22.3 ± 0.0	45 ± 4.76	78 ± 4.89
100	68 ± 0.0	74.69 ± 4.62	79.81 ± 4.89
150	81 ± 0.0	89.81 ± 4.89	96 ± 4.76
Cypermethrin 10%	90 ±3.0	92 ± 4.91	99 ± 4.89
Negative control	0	0	0

Time (minutes) / mortality rates of *C. felis* (%)

DISCUSSION

In recent years, concern has increased as a result of the resistance of many arthropods to pesticides, coupled with the persistence of these pesticides in soil, groundwater, and food. This escalating issue necessitates the exploration of novel

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alternatives. Nanoparticles, receiving significant attention in arthropod control, have emerged as a promising avenue to address these challenges (Marimuthu et al., 2010; Norouzi et al., 2022).

The results of the current study indicated that the three concentrations of SiO2-NPs led to the inhibition and paralysis of flea movement and then their death in varying time intervals. It was observed that the flea mortality rate increased with increasing concentration and period of exposure to SiO2-NPs. Observations, conducted by calculating the percentage of deceased *C. felis* at three different time points (10, 20, and 40 minutes) for each concentration revealed a consistent trend. The highest percentage of dead fleas treated with SiO2NPs occurred after 40 minutes, indicating an increased efficacy with prolonged exposure. In contrast, the lowest percentage of dead fleas was noted after 10 minutes of treatment. Notably, these percentages were comparatively lower than those recorded after 20 and 40 minutes of treatment.

The concentration of 150 mg/ml was the most efficient, compared to the two concentrations of 50 mg/ml and 100 mg/ml, as it led to a killing rate of 96% after 40 minutes. Generally, the results of the current study align with those reported by other researchers who have examined the effect of SiO2-NPs on various arthropods. For instance, Norouzi et al. (2022) examined the effects of SiO2-NPs on ticks using different concentrations. Moreover, Thabet et al. (2021) focused on their influence against different insect pests. Both studies, similar to the current research, observed that SiO2-NPs possess the ability to kill or repel pests and predatory insects, whether directly or indirectly. The effects of SiO2-NPs on some insect pests belonging to Lepidoptera were also investigated in the laboratory by Mousa et al. (2014). It was found that Sio2NPs could be used to combat *Mythimna separate*.

In another study, Vani and Brindhaa (2013) found that amorphous Sio2-NPs had an effective toxic effect against the stored grain pest *Corcyra cephalonica*, leading to a 100% kill rate. On the other hand, Sio2NPs are promising insect control materials that have been tested as alternatives to pesticides and chemical compounds (Salem, 2020). In the same context, Biradar et al. (2021) noted the superiority of silica nanoparticles over zinc and copper nanoparticles in eliminating *Corcyra cephalonica*, indicating that an increase in concentration and exposure period led to an increase in the mortality of the insect in its various phases

The high percentage of fleas killed by silica particles is due to several reasons. It is plausible that these particles may erode the insect's skin or be absorbed through the skin layers. Alternatively, Sio2NPs penetrate the insect's outer shell and affect the nervous system of *C. felis*, leading to its death. Silica NPs may have an indirect insecticidal effect by blocking the digestive system and causing deformation of the external morphology (Thabet et al., 2021).

CONCLUSION

It is concluded that nanoparticles can be applied as an effective approach to control flea. Results of the current study indicate that SiO2-NPs have anti-parasitic properties and effects against *Ctenocephalides felis*, particularly at a concentration of 150 mg/ml after 40 minutes of exposure.

DECLARATIONS

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Competing interests
The authors declared that we have no conflict of interest.

Authors' contributions
Sadiya Aziz Anah and Saad Aziz Anah contributed to collecting samples, implementing the experiment, and statistically analyzing data. All authors checked and confirmed the final draft of the manuscript.

Ethical consideration
Ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal.

Availability of data and material
The data of the current study are available by reasonable request from authors.

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Hematological Profile and Aminotransferase Activity in Kintamani Bali Puppies Injected with High Doses of Ivermectin

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ABSTRACT

Ivermectin toxicity is known to cause harmful side effects or even death in dogs intolerant to the medication. Intolerant dogs have a mutation in the MDR-1 (Multi-Drug Resistance) gene, so they lack the P-glycoprotein gene that removes drugs from the brain. Therefore, this study aimed to determine ivermectin toxicity in Kintamani Bali puppies by examining physiological responses based on hematological profiles and aminotransferase activity after a high-dose injection. A laboratory observational approach was used, and the samples were 25 healthy female Kintamani puppies based on a veterinary examination, aged 3-6 months, weighing 6.32 ± 1.18 kg, randomly divided equally into five treatment groups. The treatments included a placebo (1ml Aqua Pro Injection) as a control, as well as a single dose of ivermectin injection sequentially 200, 400, 800, and 1600 µg/kg subcutaneously. Blood samples were collected before treatment and after 7 and 14 days post-treatment. The hematologic parameters observed included levels of hemoglobin, erythrocytes, hematocrit, total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, as well as blood biochemistry, namely aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. Observation results after 4 hours of administration of ivermectin at doses of 800 and 1600 µg/kg of puppies showed changes in behavior, restlessness, depression, tremors, mydriasis, hypersalivation, anorexia, and polydipsia. Meanwhile, the results of hematological examination on the seventh day after ivermectin treatment showed a trend of erythropenia, leukocytosis, a decrease in hemoglobin levels, and an increase in aminotransferase enzyme activity. This condition continued until day 14, but the physiological parameter values showed that the puppy's condition gradually improved compared to the seventh day after treatment. There were significant differences in the blood profile, AST, and ALT of Kintamani puppies injected with ivermectin at doses of 800 and 1,600 ug/kg compared to controls on days 7 and 14 after and before treatment. It was concluded that high-dose ivermectin injections in Kintamani Bali puppies caused toxicity with clinical signs of erythropenia, decreased hemoglobin, leukocytosis, and increased aminotransferase activity.

Keywords: Aminotransferase, Blood profile, Ivermectin, Kintamani dogs, Toxicity

INTRODUCTION

Kintamani Bali dogs, a native Indonesian breed, are well-known for their attractive appearance, medium size, trainability, and loyalty, culminating in frequent adoption among dog enthusiasts (Putra and Darmayanthi, 2023). The constantly growing number of enthusiasts has also led to an increase in their population. However, a high population density without a balanced environment poses risks of viral, bacterial, and parasitic diseases (Everman et al., 2011; Short et al., 2017). Parasitic diseases can be attributed to both endoparasite and ectoparasite infestations, with the most commonly reported endoparasitic diseases in dogs being infestations with worms such as *Toxocara canis*, *Ancylostoma caninum*, *Trichuris vulvis*, *Dipylidium caninum*, and *Necator sp*. (Pesavento and Murphy, 2014). Meanwhile, frequently mentioned ectoparasites include the *Rhipicephallus sanguineus* tick (Dantas-Torres, 2010), *Heterodoxus spiniger* and *Thrichodectes canis* fleas, *Ctenocephalides (C.) felis*, and *C. canis* lice (Rinaldi et al., 2007), as well as the *Sarcoptes scabei* and *Demodex canis* mites (Vladimirovna Moskvina, 2017). Parasitic infections require serious attention due to their ability to cause a deteriorated appearance in dogs and their zoonotic nature (Chomel, 2014; O'Neil, 2018).

Nematode worm infections in Kintamani Bali dogs have been reported in their natural habitat (Evayana et al., 2017). Prevention and management of worm infestations require specific medication tailored to the diagnosis. Deworming is generally performed by administering prescribed drugs every 3-4 months regularly. Some common deworming drug options include albendazole, febendazole, pyrantel pamoate, and praziquantel. As for the management of ectoparasite cases, it is accomplished topically using shampoos, sprays, anti-parasitic creams, or by administering oral medications (Plumb, 2008; Riviere and Papich, 2018). This method is considered less effective as it requires a longer duration and the diligence of the dog owner in care (Riviere and Papich, 2018). Systemic drugs capable of addressing

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both endoparasites and ectoparasites include ivermectin and fipronil. Ivermectin is a macrocyclic lactone compound belonging to the chemotherapeutic class. This active compound, often referred to as endectocide, is produced from the fungus *Streptomyces avermitilis* and has a broad spectrum of action (Prichard et al., 2012; Campbell, 2016; Lotfalizadeh et al., 2022).

Ivermectin toxicity has been reported to have a negative impact on the host animal by causing damage to various organs such as the liver, kidneys, brain, reproductive organs, and endocrine glands (El-Saber Batiha et al., 2020). Several studies report that ivermectin has induced nephrotoxicity in rats, rabbits, mice, and dogs (Al-Jassim et al., 2016; Wilson Magdy et al., 2016; Dey et al., 2017). Ivermectin is lipophilic, with high concentrations in the liver causing oxidative stress and hepatotoxicity (Zhu et al., 2013; Celis-Giraldo et al., 2020). Neurotoxicity events are associated with P-glycoprotein deficiency (Kiki-Mvouaka et al., 2010; Merola and Eubig, 2018), so ivermectin accumulates in the brain, causing damage and dysfunction of the cerebral cortex (Bates, 2020). According to previous studies, some dog breeds such as collies, Australian shepherds, Shetland sheepdogs, old English sheepdogs, longhaired whippets, German shepherds, and some mixes of these breeds are intolerant to ivermectin, leading to acute toxicity and death (Merola and Eubig, 2018). Various clinical symptoms of intolerance are associated with neurotoxicity, namely depression, hypersalivation, tremors, mydriasis, ataxia, seizures, coma, and ultimately death (Yas-Natan et al., 2003; Bates, 2020). The recommended dosage for oral use is 100-600 µg/kg, while the dosage for subcutaneous injection is 200-400 µg/kg body weight, depending on the diagnosis and therapeutic purpose (Plumb, 2008; Riviere and Papich, 2018). The use of macrocyclic lactone drugs, including ivermectin, has shown resistance in endoparasites (Yanuartono et al., 2020). Field observations found that a high dose of 1.000-2.000 µg/kg of ivermectin therapy practices exceed the therapeutic dose, posing toxic risks (Unpublish data). Therefore, this study aimed to find the possible toxicity of high doses of ivermectin in Kintamani Bali puppies by assessing behavioral changes, hematological profiles, and aminotransferase activity.

MATERIALS AND METHODS

Ethics approval

The Animal Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Badung, Indonesia, gave ethical approval for this research with certificate number B/247/UN14.2.9/PT.01.04/2021.

Experimental animal

The Kintamani Bali puppies were obtained from Sukawana Village, Kintamani Subdistrict, Bangli Regency, Bali, Indonesia, as the native habitat. Purposive sampling was used to select healthy female puppies by veterinarian, aged 3-6 months, body weight 6.32±1.18 kg, and subsequently acclimatized for a minimum of 14 days in the experimental facility. This research was carried out from September to November 2022 at the Veterinary Pharmacy and Pharmacology Laboratory, Faculty of Veterinary Medicine, Udayana University, Indonesia. This was accomplished to adapt the test animals to the new environment and ease the handling by the study team. The experimental animals were placed in individual cages equipped with food and water containers. Commercial feed and fresh drinking water were provided *ad libitum*. The experimental room was maintained at a temperature ranging from 25 to 27°C, 60-70% humidity, and a 12-hour light-dark cycle.

Study design

This study used a complete randomized design with a laboratory observational analysis setting. A total of 25 Kintamani Bali puppies were randomly divided into five treatment groups, each having five replications. Ivermectin 1% (Intermectin, manufactured by Interchemie werken “De Adelaar” B.V. The Netherlands) was administered as a single dose, referring to the therapeutic dosage of 200-400 µg/kg (Plumb, 2008). In this study, the therapeutic dose was doubled for P3 and P4 groups. The treatments were as follows, P0 as the control group received a 1 ml placebo using free pyrogen aqua dest (Aqua Pro Injection, PT. Ikapharmindo Putramas, Indonesia) while P1, P2, P3, and P4 were each given ivermectin at doses of 200, 400, 800, and 1,600 µg/kg body weight through a single subcutaneous injection. Changes in the behavior of puppies were observed during the first three days, including depression, hypersalivation, mydriasis, tremors, ataxia, seizures, appetite, and drinking. Subsequently, blood samples were collected for hematological profile (Hemoglobin, erythrocytes, hematocrit, leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils) and aminotransferase activity examinations before treatment, and on day 7, and 14 days after ivermectin injection.

Blood collection

Dog blood collection was carried out aseptically through the cephalic vein using a venoject. In this regard, 1 ml was collected into a blood vacutainer with Ethylenediamine Tetra-acetic Acid (EDTA) for routine blood examinations

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and 2 ml into a vacutainer without anticoagulant for blood biochemical examinations. The blood samples were taken to the laboratory using a coolbox provided with ice gel cooling for further examination.

Hematology and blood chemistry examination

Routine blood tests were performed using the LICARE 3-Part Vet Auto Hematology Analyzer (Licare Biomedical Limited, China). Parameters observed included hemoglobin levels, erythrocytes, hematocrit, total leukocytes, and leukocyte differential counts. The Seamaty Veterinary Automatic Biochemical Analyzer (Chengdu Seamaty Technology Co., Ltd, China) was used for blood chemistry examination. The observed parameters included alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. ALT and AST examination using the Seamaty Vet Chemistry Reagent kit (Chengdu Seamaty Technology Co., Ltd, China). Seamaty veterinary reagent disc adopts microfluidic technology. It contains optical and mechanical components, which can be used with the instrument to participate in each stage of blood analysis.

Statistical analysis

Research data includes erythrocytes, hemoglobin, hematocrit, leukocytes, and differential of leukocytes, AST, and ALT were presented in mean and standard deviation. Then, the research data was analyzed using the ANOVA and Duncan tests using the IBM SPSS Statistics version 26 device for Windows. If the P value is below 0.05, it is declared statistically significant ($p < 0.05$).

RESULTS AND DISCUSSION

Results

Observations of changes in the behavior of Kintamani Bali puppies aged 3-6 months after subcutaneous injection of various doses of ivermectin are summarized in Table 1. All puppies felt pain, grumbling, restlessness, and feelings of fear immediately after the ivermectin injection treatment. Behavioral changes indicating toxicity were found in the group receiving high doses of ivermectin (800 and 1,600 µg/kg), including restlessness, depression, tremors, mydriasis, hypersalivation, anorexia, and polydipsia. No clinical signs of ataxia or seizures were found in all treatment groups. Visually, it appears that higher doses show stronger changes.

The results of the blood examination of Kintamani puppies are presented in Table 2. As can be seen in the table, total erythrocytes, hemoglobin, and hematocrit decreased significantly ($p < 0.05$). Total leukocytes and monocytes increased significantly in animals treated with high doses of ivermectin compared to the control group ($p < 0.05$). These significant changes occurred on 7 and 14 days after treatment compared to the first day before treatment.

Table 3 summarizes measurements of the aminotransferase enzyme activity of Kintamani Bali puppies, which appeared to increase after injection of high doses of ivermectin. AST and ALT enzyme activities increased significantly 7 and 14 days after treated ivermectin, compared to the control group and the first day before treatment ($p < 0.05$).

Table 1. Behavior change of Kintamani puppies aged 3-6 months after being given different doses (200, 400, 800, and 1600 µg/kg) of ivermectin

Behavioral changes	Control	Dose of Ivermectin (µg/kg)				Explanation
		200	400	800	1600	
Restlessness	No	Yes	Yes	Yes	Yes	All puppies exhibited pain, grunted, and became restless immediately after receiving a subcutaneous injection of ivermectin.
Depression	No	No	No	Yes	Yes	Puppies show anxiety and seem to want to avoid being held while feeding. This behavior began to appear after 12 hours of ivermectin injection.
Hypersalivation	No	No	No	Yes	Yes	The puppy appears to be producing more saliva than usual.
Mydriatic	No	No	Yes	Yes	Yes	Pupils were measured after 4 hours of ivermectin injection, showing that mydriasis occurred compared to pupil size before and after treatment.
Tremor	No	No	No	No	Yes	Tremors were observed after 4 hours of subcutaneous injection of ivermectin at a dose of 1,600 µg/kg, and disappeared after the third day of treatment.
Ataxia	No	No	No	No	No	None of the pups showed movement incoordination after ivermectin injection.
Seizures	No	No	No	No	No	None of the puppies had seizures
Appetite	Normal	Normal	Normal	Anorexia	Anorexia	Puppies show nausea and reduced appetite.
Drinking	Normal	Normal	Normal	Polydipsia	Polydipsia	There was an increased water consumption in puppies treated with ivermectin at doses of 800 and 1.600 µg/kg subcutaneously.

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Table 2. Hematological profile of Kintamani puppies aged 3-6 months after subcutaneous injection of different doses (200, 400, 800, and 1600 µg/kg) of ivermectin

Parameter	Day	Control (Placebo)	Dose of ivermectin (µg/kg)			
			200	400	800	1600
Erythrocyte (10^6/µL)	day 1	6.48 ± 0.70	6.99 ± 1.23	7.12 ± 1.48	6.81 ± 1.72^{A}	6.83 ± 1.52^{A}
	day 7	6.47 ± 0.71^{b}	6.65 ± 1.11^{b}	6.69 ± 1.32^{b}	6.22 ± 1.56^{Ba}	6.08 ± 0.88^{Ca}
	day 14	6.54 ± 0.77^{b}	6.80 ± 1.12^{b}	6.94 ± 1.35^{b}	6.47 ± 1.30^{Bab}	6.21 ± 0.92^{Ba}
Hemoglobin (g/dL)	day 1	14.12 ± 2.17	13.96 ± 2.11	14.02 ± 2.50	14.28 ± 2.25^{A}	14.02 ± 2.90^{A}
	day 7	14.13 ± 2.18^{c}	13.52 ± 1.90^{bc}	13.10 ± 2.62^{b}	12.78 ± 1.87^{Bb}	11.66 ± 1.19^{Ba}
	day 14	14.02 ± 1.96^{c}	13.88 ± 2.07^{c}	13.62 ± 2.41^{c}	12.86 ± 1.79^{Bb}	12.02 ± 1.83^{Ba}
Hematocrit (%)	day 1	42.62 ± 6.48	42.54 ± 7.79	42.16 ± 7.06	44.48 ± 9.63^{A}	43.02 ± 6.49^{A}
	day 7	42.04 ± 7.11^{b}	41.70 ± 7.25^{b}	40.24 ± 5.90^{b}	37.62 ± 3.71^{Bab}	34.70 ± 4.17^{Ba}
	day 14	43.24 ± 8.01^{b}	42.30 ± 7.68^{b}	41.58 ± 6.40^{b}	37.46 ± 3.42^{Bab}	35.36 ± 4.37 ^{Ba}
Leucocyte (10^3/µL)	day 1	15.16 ± 2.02	15.75 ± 1.88	16.26 ± 3.28	15.28 ± 3.07^{A}	15.06 ± 3.09^{A}
	day 7	15.15 ± 2.06^{a}	15.67 ± 1.69^{a}	16.05 ± 3.07^{ab}	17.29 ± 2.63^{Bb}	19.02 ± 3.11^{Cb}
	day 14	15.36 ± 3.05^{a}	16.03 ± 1.91^{ab}	16.16 ± 3.05 ^{ab}	17.71 ± 2.18^{Bb}	18.61 ± 2.85^{BCb}
Neutrophil (%)	day 1	54.90 ± 4.77	50.32 ± 6.10	49.66 ± 7.90	51.06 ± 8.43	49.62 ± 5.38
	day 7	53.04 ± 3.70	49.62 ± 6.17	48.26 ± 6.69	48.14 ± 9.33	45.96 ± 4.11
	day 14	52.64 ± 9.33	50.14 ± 5.90	48.52 ± 5.13	47.48 ± 4.38	47.10 ± 5.02
Lymphocyte (%)	day 1	36.14 ± 3.50	41.10 ± 6.53	40.76 ± 6.77	39.70 ± 7.18	39.02 ± 4.34
	day 7	37.88 ± 2.16	41.14 ± 6.19	41.96 ± 6.29	40.80 ± 8.22	41.26 ± 6.10
	day 14	38.28 ± 2.55	40.90 ± 6.23	41.56 ± 4.52	40.82 ± 3.77	41.14 ± 3.33
Monocyte (%)	day 1	4.94 ± 0.79	4.92 ± 1.14	5.84 ± 2.22	5.12 ± 1.47^{A}	6.53 ± 1.92^{A}
	day 7	4.92 ± 0.79^{a}	5.14 ± 1.30^{a}	6.04 ± 1.88^{ab}	6.94 ± 2.15^{ABb}	8.24 ± 1.20^{Bc}
	day 14	4.88 ± 0.75^{a}	5.12 ± 1.33^{a}	6.24 ± 1.61^{ab}	7.14 ± 1.98^{Bb}	7.68 ± 0.79^{Abb}
Eosinophil (%)	day 1	3.30 ± 1.68	3.03 ± 1.65	3.16 ± 1.09	3.52 ± 1.27	4.10 ± 1.39
	day 7	3.34 ± 1.70	3.26 ± 1.48	3.12 ± 1.09	3.32 ± 1.11	3.62 ± 0.97
	day 14	3.34 ± 1.70	3.06 ± 1.68	2.84 ± 1.06	3.30 ± 1.14	3.26 ± 1.11
Basophil (%)	day 1	0.72 ± 0.47	0.64 ± 0.49	0.58 ± 0.47	0.60 ± 0.41	0.71 ± 0.47
	day 7	0.82 ± 0.48	0.84 ± 0.30	0.62 ± 0.44	0.80 ± 0.40	0.92 ± 0.33
	day 14	0.86 ± 0.44	0.78 ± 0.38	0.84 ± 0.37	0.96 ± 0.34	0.82 ± 0.31

^{abc} Different superscript letters towards each row indicate significant differences ($p < 0.05$), ^{ABC} Different superscript letters towards the same column indicate significant differences ($P<0.05$)

Table 3. Enzymes activity of Kintamani puppies aged 3-6 months given different doses (200, 400, 800, and 1600 µg/kg) of ivermectin

Parameter	Day	Control (Placebo)	Dose of ivermectin (µg/kg)			
			200	400	800	1600
AST (µ/L)	day 1	38.62 ± 5.50	38.40 ± 7.64	36.20 ± 5.76	35.20 ± 8.58^{A}	33.80 ± 8.44^{A}
	day 7	38.80 ± 5.45^{a}	38.20 ± 6.76^{a}	42.20 ± 5.85^{a}	63.80 ± 12.83^{Bab}	113.20 ± 13.68^{Cb}
	day 14	37.40 ± 5.55^{a}	43.60 ± 5.32^{a}	43.00 ± 6.08^{a}	60.80 ± 12.48^{Bab}	82.80 ± 10.83^{BCb}
ALT (µ/L)	day 1	22.80 ± 3.42	27.60 ± 5.08	26.60 ± 6.69	28.80 ± 6.30^{A}	27.20 ± 4.44^{A}
	day 7	23.60 ± 3.05^{a}	29.02 ± 7.22^{a}	40.60 ± 7.54^{ab}	67.40 ± 8.26^{Bb}	108.20 ± 15.12^{Cc}
	day 14	23.20 ± 3.11^{a}	33.00 ± 5.70^{a}	34.20 ± 7.16^{a}	46.60 ± 7.64^{Bbc}	69.80 ± 12.23^{Bc}

^{abc}Different superscript letters towards the row indicate significant differences ($p < 0.05$); ^{ABC}Different superscripts letters towards the same column indicate significant differences ($P<0.05$), AST: Aspartat aminotransferase, ALT: Alanin aminotransferase

DISCUSSION

Ivermectin can cause harmful side effects when administered above the maximum dose or given to non-target animals (Siroka and Svobodova, 2013). Cases of ivermectin toxicity have been reported in pigs, cows, dogs, cats, horses, and turtles (Jourdan et al., 2015; Dey et al., 2017; Celis-Giraldo et al., 2020). In this study, clinical observations of behavioral changes were conducted before and after treatment with high doses of ivermectin up to the third day. Kintamani puppies were found to exhibit toxicity symptoms after treatment, including restlessness, depression, tremors, mydriasis,

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hypersalivation, anorexia, and polydipsia (Tabel 1). These symptoms are similar to clinical ivermectin poisoning findings reported in dogs (Hopper et al., 2002; Epstein and Hollingsworth, 2013), calves (Patel et al., 2018), horses (Norman et al., 2012), lions (Saqib et al., 2015), and rabbit (Branco et al., 2021). Until now, ivermectin is still the drug of choice in parasite control practices because of its broad spectrum of action. However, caution in its use is necessary, considering that this drug works well against parasites and has an impact on the host animal. Side effects found in various animals include nephrotoxicity, hepatotoxicity, neurotoxicity and reproductive toxicity (Salman et al., 2022).

The hematological profile of Kintamani puppies given high-dose ivermectin subcutaneously significantly differed from the control and therapeutic dose groups (200-400 µg/kg). Kintamani puppies experienced erythropenia, leukocytosis, and decreased hemoglobin after injection of ivermectin 2-4 times more than the recommended dose. These results are in line with Salman et al. (2022), who reported that male albino rats treated with Abamectin experienced a decrease in total red blood cells, an increase in total white blood cells, a decrease in hemoglobin, changes in serum enzyme levels, and a decrease in the number and motility of spermatozoa. Erythropenia and decreased hemoglobin presumably resulted from damage to the blood cells due to the presence of ivermectin in the bloodstream. The decrease in total erythrocytes can be caused by hemorrhage, hemolysis, low production, or other factors such as nutrition (Maglaras et al., 2017; Martinez et al., 2019). Deficiency in folic acid and Vitamin B12 impairs erythrocyte maturation during erythropoiesis, resulting in a decrease in their number in the bloodstream (Widyanti et al., 2018). Meanwhile, leukocytosis refers to increased leukocytes, typically found in patients with infections, inflammation, tissue necrosis, or leukemic neoplasia. Two researchers have reported hemorrhage in experimental mice after administration of ivermectin and Abamectin. A single injection of 50 mg/kg dose of ivermectin causes hepatic congestion and hemorrhage accompanied by centrilobular necrosis (Dadarkar et al., 2007). Oral administration of Abamectin, 2 mg/kg for 5 days, causes edema, hemorrhage, inflammatory cell infiltration, and tubular necrosis (Abdel-Daim and Abdellatief, 2018). Meanwhile, subcutaneous injection of ivermectin in rabbits at a dose of 0.4 mg/kg and in goats at 2 mg/kg causes congested blood vessels, tubular degeneration, desquamation and necrosis of tubular epithelium, glomerular necrosis, and infiltration of leucocyte (GabAllh et al., 2017). The same oxidative damage allegedly occurred in the puppies in this study, resulting in erythropenia and a significant decrease in hemoglobin. Tissue damage increases autophagy to clear damaged organelles and proteins formed by damaged hepatocytes (Zhu et al., 2013). This autophagy process involves many white blood cells, leading to inflammatory cell infiltration and leukocytosis in the animal (Salman et al., 2022).

The biochemical blood tests showed a significant increase in aminotransferase enzymes in the group administered a high dose of ivermectin. The enzymes reflect the integrity or integration of liver cells, and a significantly high increase may reflect the level of damage. The high activity in this study was due to increased drug metabolism in the liver, leading to hepatocyte damage (Senior, 2012). Ivermectin is highly lipophilic and can be distributed with a wide volume and induce oxidative stress (Salman et al., 2022). This leads to the accumulation of fat tissues, serving as a drug reservoir. The highest accumulation of ivermectin was found in the liver and fat tissues, while the lowest was detected in the brain and bone marrow (Zemkova et al., 2014; Juarez et al., 2018).

Ivermectin works by releasing and binding to Gamma-Aminobutyric Acid (GABA) neurotransmitters, which block nerve impulse transmission at the peripheral endings and smooth muscle cells of parasites, resulting in paralysis and death (Canga et al., 2009; Eraslan et al., 2010). The affinity for Glutamate-gated Chloride channel receptors at certain neuron synapses increases chloride ion permeability, leading to decreased appetite, fecundity, and parasite movement due to paralysis (Wolstenholme, 2011; El-Saber Batiha et al., 2020). Ivermectin metabolism occurs in the liver and is primarily excreted through feces, with a small portion released through urine. According to a previous study, several dog breeds are intolerant to ivermectin, including Collies, Australian Shepherds, as well as Shetland and Old English Sheepdogs (Merola and Eubig, 2018). These intolerant dogs have genetic abnormalities in the MDR-1 (multi-drug resistance) gene, resulting in a deficiency of P-glycoprotein (Kiki-Mvouaka et al., 2010; Mueller et al., 2020). This protein is responsible for removing residual drugs and their metabolites from the central nervous system (Riviere and Papich, 2018). Accumulation of ivermectin in the brain potentially causes acute toxicity and various disorders in the central nervous, gastrointestinal, and cardiovascular systems, which leads to an increased risk of death (Merola and Eubig, 2018).

The recommended therapeutic dose of ivermectin falls in the range of 200-400 µg/kg (Plumb, 2008; Martin et al., 2021). Associated toxicity in dogs without the MDR-1 gene mutation was reported with an acute oral LD50 of 8000 µg/kg (Plumb, 2008), while those with the MDR-1 gene defect have an acute oral LD50 of 200 µg/kg (Plumb, 2008; Woodward, 2012). Ruminant livestock farmers often increase the dose to tenfold the recommended level or about 2000 µg/kg due to a lack of understanding regarding the effective dosage (Yanuartono et al., 2020). Other researchers reported the LD50 of a single dose of ivermectin injection in rats at a dose of 50 mg/kg (Dadarkar et al., 2007). Furthermore, young animals are more sensitive to ivermectin toxicity than adults (Patel et al., 2018). In this study, indications of toxicity in puppies were shown by the increase in aminotransferase activity at a dose of 800-1600 µg/kg starting on day 7, and the levels remained high after 14 days of administration. It also indicated that the pharmacodynamics of high-dose ivermectin led to a tendency for erythropenia, leukocytosis, and a decrease in hemoglobin levels (Johnson-Arbor, 2022).

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CONCLUSION

In conclusion, subcutaneous injection of high doses of ivermectin (800-1.600 µg/kg) in Kintamani Bali puppies led to toxicity with clinical signs of erythropenia, leukocytosis, a decrease in hemoglobin levels, and an increase in aminotransferase activity. The results of the present study can serve as a reference for veterinary practitioners to properly administer ivermectin therapy to prevent toxicity and resistance.

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Availability of data and materials

The authors confirm that the data presented is currently available upon reasonable request.

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Authors' contribution

Luh Made Sudimartini conceptualized and designed the study. Luh Made Sudimartini, Romy Muhammad Dary Mufa, and I Made Merdana conducted the study and collected and analyzed data. Luh Made Sudimartini wrote the manuscript. I Made Merdana provided advice and a scientific review. All authors checked and confirmed the last edition of the article.

Competing interests

The authors declare that there are no competing interests in the writing and processing of this article.

Ethical consideration

All the authors have checked ethical issues such as the plagiarism index, double publication, and any important publication ethics before the submission of this article.

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Effects of *Histomonas*, *Trichomonas*, and *Eimeria* Co-infection on Productivity and Macro-morphological Indicators of Eggs in Laying Hens

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ABSTRACT

The study of macro-morphological changes is important for recognizing disturbances in egg formation that cause pathologies, especially co-infection. The current study aimed to evaluate the level of egg productivity and macro-morphological parameters of eggs in domestic chickens of the Rhode Island breed with co-infection of *Histomonas*, *Trichomonas*, and *Eimeria*. Clinical and parasitological, coproscopic, morphometric research, and statistical analysis methods were used for this research. Pathogens of *Histomonas* and *Trichomonas* were detected by microscopy of smears of fresh feces, and *Eimeria* oocysts were identified by flotation according to the Fullenborn method. During 30 days of research, there was a significant decrease in egg production (52%), a decrease in egg weight by 16.8%, and a decrease in the shell thickness by 30.43% during spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection in laying hens. The eggshell indicated noticeable macro-morphological changes, including deformations and defects resulting from insufficient calcification. These changes manifest as combined damage to the shell, characterized by small cracks, roughness, bumpy or spilled thickenings, and complete or partial depigmentation. When evaluating the internal content of eggs in 12% of their samples, there were bloody spots, relatively smaller and lighter yolks, thinning of the protein part. Thus, the specified macro-morphological changes and egg defects were the result of the negative impact of co-infection on the processes of egg formation, which indicates the systemic nature of the lesion and the morphofunctional insufficiency of the egg-forming organs.

Keywords: Comorbidity, Egg defect, Egg production, Eimeriosis, Histomonosis, Laying hen, Trichomoniasis

INTRODUCTION

Recently, protozoan diseases, particularly avian *Eimeria*, have emerged as a significant obstacle to increasing demand for chicken meat and egg production, as recognized by the United States Department of Agriculture (Godfray et al., 2010; USDA, 2023). In terms of importance, this disease is one of the top three (Dalloul and Lillehoj, 2006). The global poultry industry economy loses more than 14.5$ US billion annually (Blake et al., 2020). In adult chickens, cases of protozoan infestations (eimeriosis, histomoniasis, trichomoniasis) became more frequent (Dolka et al. 2015). They have become widespread in most countries of the world, particularly following the prohibition of protistocidal drugs (CEC, 2002), such as nitroimidazoles, nitrofurans, and arsenic drugs (Hess et al., 2015). Enterohepatitis of *Histomonas* and *Trichomonas* etiology (Dolka et al., 2015) and granulomatous liver lesions (Araújo et al., 2015; Lopes et al., 2022) are often detected in adult chickens. Despite being asymptomatic, these conditions are characterized by signs of a decrease in the reproductive capacity and periodic manifestations of diarrhea symptoms (Mehlhorn, 2016). Protozoan diseases significantly threaten the health of chickens (Chen et al., 2022; Tuska-Szalay et al., 2022; Saikia et al., 2023). They are widely distributed worldwide among different species of agricultural and wild bird species (Badparva et al., 2020). The prevalence of trichomoniasis epizootics among wild birds of ecoparks (Fadhil et al., 2020) often leads to a significant decrease in their populations and even threatens the disappearance of certain bird species in natural ecosystems (Forzán et al., 2010; Feng et al., 2021). Possible variants of cross-infection of birds with causative agents of trichomoniasis and histomoniasis from wild birds and vice versa have been proven (Tuska-Szalay et al., 2022). The development of *Eimeria*, *Histomonas*, and *Trichomonas* co-infection in adult chickens leads to chronic inflammation of the intestine, resulting in damage to the liver (necrotic hepatitis) and cecum (diphtheria typhitis) as noted by Shchebentovska and Holubtsova (2020). Spontaneous trichomoniasis in birds involves damage to the oral cavity, pharynx, small and large parts of the intestines, and the formation of granulomas with localization in the liver and cecum (Landman et al., 2019). According to

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Liulin et al. (2023), during co-infection eimeriosis, histomonosis, and trichomonosis in domestic chickens, lesions extended to other organs and tissues, including spleen, bursa of Fabricius, peritoneum, and even skin. The causative agents of avian trichomoniasis can cause damage to the organs of the reproductive system of birds, resulting in reduced or halted egg production (Falkowski et al., 2020). The productivity of chickens and a decrease in egg production and egg mass are also significantly affected by invasions of *Eimeria* spp. and *Histomonas meleagridis* (Dolka et al., 2015; Vakili et al., 2021), which needs further study. With this in mind, this study aimed to determine the level of egg productivity and classify macromorphological changes in the eggs of domestic laying hens of the Rhode Island breed with a spontaneous combination of eimeriosis, histomoniasis, and trichomoniasis infection.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in compliance with the ethical norms and principles of scientific research specified by the European Convention on the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes. The approval of the Bioethics Commission of the State Biotechnology University (SBTU), Ukraine, was not required since the object and material of the study were the population of adult laying hens and their freshly laid eggs.

Stages of research

At the first stage of the research, a comprehensive examination was conducted, including general clinical, parasitological, and special coproscopic assessments of laying hens. A population of laying hens (n = 254) of the 1.5-year-old Rhode Island breed of a private farm in the Kharkiv region with a free-range organic housing system was investigated. Based on the results of the examinations, an experimental group (n = 35; average chicken weight of 2.20 ± 0.062 kg) with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection and a control (n = 35; average chicken weight 2.64 ± 0.056 kg) group of non-infested laying hens were selected. The chickens were placed in separate sections of the room with a maximum density of 6 hens per 1 m^2. They were provided free range with a pasture area of 10 m^2 per head. The lighting schedule involved a 13-hour day, with an illumination level of 20 lux at the feeders. The air temperature in the poultry house was +18°C, and the relative humidity was 70%.

Coproscopic studies

Smears were prepared from fresh feces (immediately after defecation) to identify the causative agents of Histomonas and Trichomonas. These smears were then fixed with methyl alcohol for 3-5 minutes and stained according to the Romanovsky-Hiems method. To identify *Trichomonas*, the smears were air-dried and stained with methylene blue. Pathogens were identified by morphological features (Menezes et al., 2016) using light microscopy on an Axioscop-40 microscope (Zeiss Germany), magnification ×400.

Flotation method

Eimeria oocysts were detected following the Fulleborn flotation method (Halat et al.,2004) . Individual fecal samples were collected, primarily during defecation. Feces (3 g) were placed in a 100 ml glass, and a saturated solution of NaCl in a ratio of 1:20 was added while stirring with a glass rod. The resulting suspension was filtered through a metal filter (hole size 0.8-1.0 mm) into similar cups and left for 30 minutes. After settling, 3 drops of the surface film were collected using a metal loop (0.8 cm in diameter). These drops were then transferred to a glass slide and subjected to microscopy for the presence of *Eimeria* oocysts. Invasion intensity (the number of oocysts in 1 g of feces) was determined according to the McMaster method (Vadlejch et al., 2011). The species of *Eimeria* oocysts was determined according to Pellerdy (1974).

Accounting of egg productivity and macromorphological indicators of eggs

The productivity of experimental and control groups of laying hens was recorded daily for 30 days. The gross collection, the average laying capacity, and the number of rejected (%) eggs were recorded. Evaluation of macromorphological changes in eggs, and their analysis was carried out visually. Egg mass was determined by weighing on an Adventurer electronic laboratory scale. The egg shell thickness was examined using an ultrasonic thickness gauge (from 0 to 300 mm) Co. Ltd., Echometer 1061 Co. Ltd, Karl Deutsch, Wuppertal, Germany (Tsaruk and Dikhtiaruk, 2014).

Statistical analysis

Statistical processing of the obtained results was carried out using the descriptive statistics tool. For comparing the data, the Data Analysis package in MS Excel 2019 was utilized, specifically utilizing the two-sample t-test with different variances and correlation analysis. The average values of the main feature, including egg mass, were determined. Mean

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error and absolute error at a given confidence level p > 95%, which corresponds to the level of statistical significance p < 0.05 according to the Student's test (Lebed'ko et al., 2022).

RESULTS

The results of intravital clinical-parasitological and special coproscopic studies of laying hens of the Rhode Island breed (n = 254) indicated the spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection (EI-23.22%). Co-infection was caused by pathogens *Eimeria acervulina* (19.4%), *Eimeria brunetti (*7.9%), *Eimeria maxima (*16.5%), *Eimeria mitis* (5.1%), *Eimeria necatrix* (12.5%), *Eimeria praecox* (3.2%) and *Eimeria tenella* (35.4%), at the intensity of 49.6 ± 4.8-106.4 ± 5.7 oocysts in a gram of feces and *Histomonas meleagridis* and *Trichomonas gallinae* at intensity 1-3 of the pathogen in the field of view of the microscope (p < 0.05). Table 1 shows the results of the effect of *Eimeria-Histomonosis-Trichomonosis* co-infection on the level of egg productivity and egg culling of the experimental and control groups for 30 days.

Compared to the control group, egg production decreased by 52% in the chickens of the experimental group with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection during the observation period (30 days). In addition, due to structural defects of the shell, 58.57% of eggs obtained from hens of the experimental group were rejected. Morphometric indicators of eggs obtained from experimental and control groups of chickens and the results of statistical processing are presented in Table 2.

At the same time, it was found that all morphometric egg parameters of the control and experimental groups differed significantly. Tables 3 and 4 tabulate the correlation coefficients of the morphometric indicators of eggs in the experimental and control groups.

Table 1. Indicators of productivity and hatching of Rhode Island chickens' eggs for 30 days

Indexes	Research group (n=35)	Control group (n=35)
Productivity for 1 laying hen in 30 days (eggs)	12	25
The average egg mass (g)	51.70	62.14
Gross collection of eggs for 30 days (pieces)	420	875
Eggs culled (%)	58.57	2.23

Table 2. Results of primary processing of morphometric parameters of Rhode Island chicken eggs

Statistical indicators	Average egg mass (g)	Egg shell thickness in the middle part (mm)	Egg shell thickness at the sharp end (mm)	Egg shell thickness at the blunt end (mm)
Control group (n = 35 chickens)				
M	62.14	0.322	0.337	0.322
M	0.24	0.001	0.002	0.001
ΔM	0.49	0.002	0.004	0.002
s^2	2.01	$5.176 \cdot 10^{-5}$	0.000129	$5.176 \cdot 10^{-5}$
Experimental group (n = 35 chickens)				
M	51.70	0.224	0.237	0.227
m	0.49	0.005	0.005	0.005
ΔM	1.00	0.010	0.011	0.009
s^2	8.41	0.001	0.001	0.001

M: Selective average, m: Error of the mean; ΔM: Absolute measurement error with a reliable probability of p > 0.95 (that is, the measurement result X falls within the interval X = M ± ΔM); s2: Average sample variance.

Table 3. Correlation matrix between morphometric indicators of eggs in the research group of Rhode Island laying hens

Indexes	Egg mass	Egg shell thickness in the middle part	Egg shell thickness at the sharp end	Egg shell thickness at the blunt end
Egg mass	1			
The thickness of the egg shell in the middle part	0.916	1		
The thickness of the egg shell at the sharp end	0.886	0.992	1	
The thickness of the egg shell at the blunt end	0.889	0.979	0.967	1

Table 4. Correlation matrix between morphometric indicators of eggs in the control group of Rhode Island laying hens

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Indexes	Egg mass	Egg shell thickness in the middle part	Egg shell thickness at the sharp end	Egg shell thickness at the blunt end
Egg mass	1			
The thickness of the egg shell in the middle part	0.750	1		
The thickness of the egg shell at the sharp end	0.762	0.950	1	
The thickness of the egg shell at the blunt end	0.750	0.999	0.950	1

When examining these correlation matrices, a reliable correlation was established between the indicators of egg mass and shell thickness at the level of very high and high correlations in the experimental (0.886–0.916) and control (0.750-0.762) groups. This finding underscores the close interdependence between these variables in the studied groups.

The following macromorphological changes were revealed during the external examination of the eggs. A softened (insufficiently mineralized) shell was found in some of the eggs, which could often be destroyed when the eggs were collected from the nest (Figures 1, 2). The eggs of chickens in the experimental group indicated damage to the shell in the area of the blunt end-small cracks in the form of spider-like branches and bumpy thickenings on its surface (Figure 2). Some eggs laid by chickens in the experimental group retained an intact shell, characterized by small, fragile layers of white color on the outer surface, as depicted in Figure 3. The hens in the experimental group displayed noticeable signs of defects in the eggshell texture. These included complete or partial depigmentation, the emergence of roughness marked by spilled thickenings, and calcareous nodules across the entire surface. Additionally, local contamination of the shell surface with blood was observed (Figure 4). Hens of the control group laid conditioned eggs, which had a characteristic monoasymmetric shape with an apical narrowed end and a blunt lower end, as well as a solid, smooth, clean shell, uniformly colored in a light beige color (Figure 5). Pigmented and depigmented spots, as well as deformations in the form of bumps or areas of thickening and depressions, mainly in the area of the blunt end, were found on the egg shell of the chickens in the experimental group (Figure 6). In 80% of eggs from hens in the experimental group, contamination of the shell with traces of feces and/or blood was observed, particularly in combination with its roughness and pigmented speckled spots (Figure 7). Some of the chickens in the experimental group laid eggs without calcified shells. Such eggs were easily broken, so when inspecting the territory of the poultry farm, only their fragments were often found, in particular, the remains of the inner shell. There was also a rupture of this shell in the middle part (Figure 8). Some of the eggs of the chickens in the experimental group had significant deformations. Their shells were weakly calcified and poorly designed, with violations of the usual egg shape, with layers of white granular substrate on their outer surface and partial minor contamination with blood (Figure 9). When examining the internal content of the eggs of the experimental group, bloody spots were found in the protein part in 12% of cases. Compared with the indicators of the internal content of eggs of hens of the control group, the yolk of the eggs of the experimental group was smaller in size and lighter in color, and the protein part was rare (Figure 10). The specified defects of the shell and egg contents of the chickens of the experimental group became the basis for their culling as unsuitable for transportation and/or incubation.

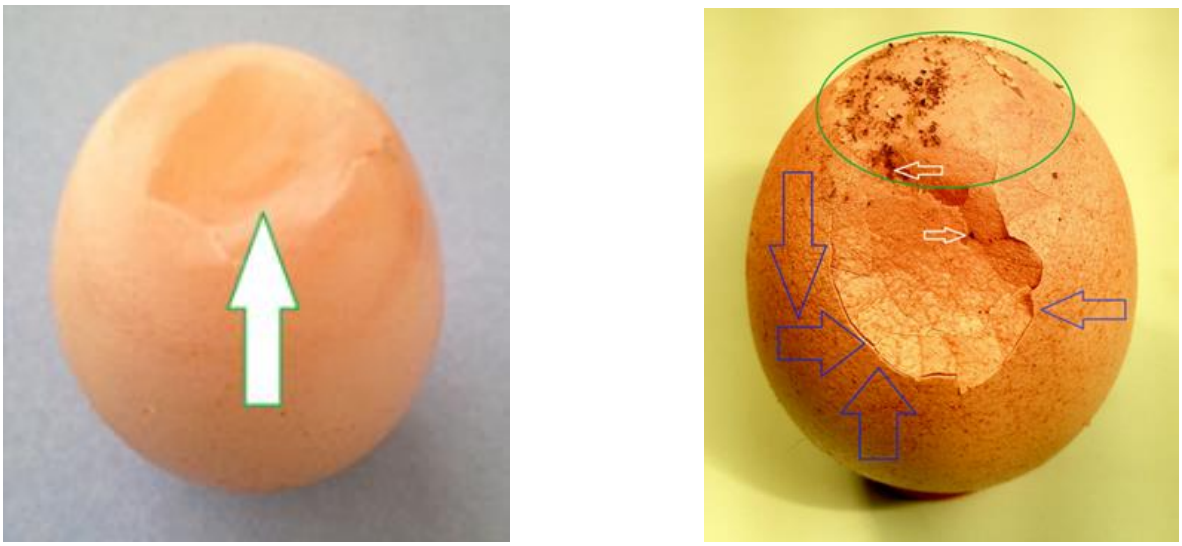

Figure 1. Cull eggs with a defect of insufficient calcification- a thin shell (arrow) with small tubercles on the outer surface (blue arrows), blood stains (white arrows) and contamination (oval) from a domestic hen (Rhode Island, age 1.5 years, experimental group).

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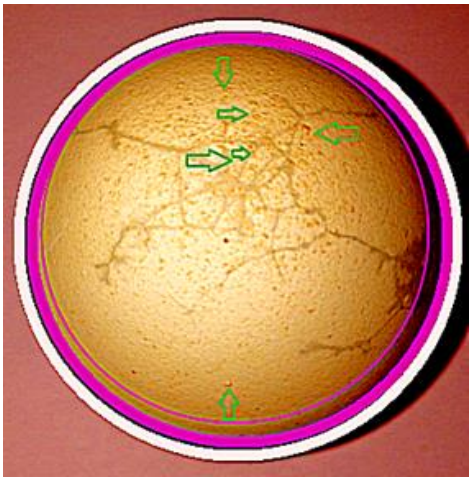


Figure 2. Substandard egg with combined shell defects (fine cracks and small bumpy thickenings–green arrows) in the blunt end region from a domestic hen (Rhode Island; age 1.5 years, experimental group).

Figure 3. The appearance of an egg with white brittle layers on the outer surface of the shell from a domestic chicken (Rhode Island breed; age 1.5 years, experimental group).

Figure 4. An unconditioned egg with a bumpy surface defect of a depigmented shell, unusually light-color (oval), and blood stains (arrow) from a domestic chicken (Rhode Island breed; age 1.5 years, experimental group).

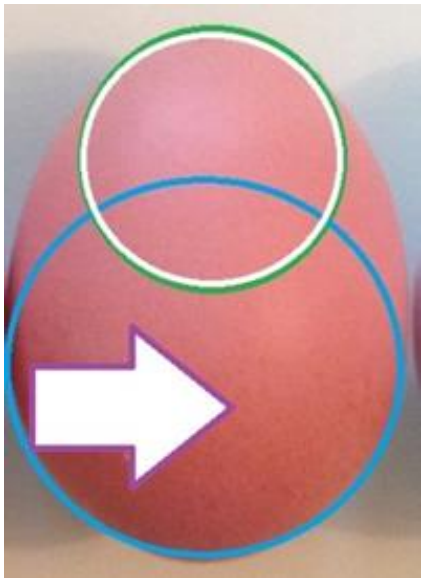
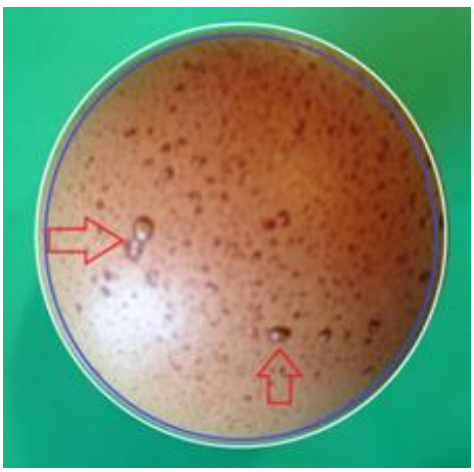


Figure 5. A conditioned egg of a preserved form from a hen (Rhode Island breed; 1.5 years old) of the control group: the surface of the shell is intact, smooth, clean, and uniformly colored in a light beige color (arrow).



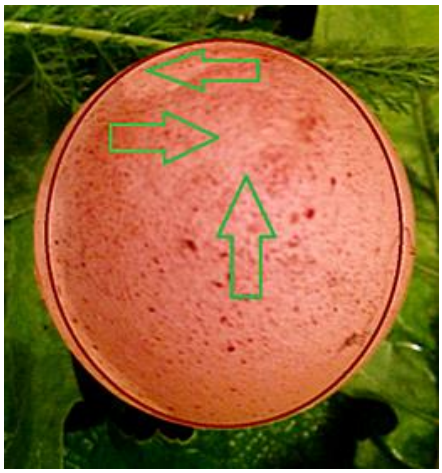


Figure 6. The surface of substandard eggs from the extended (blunt) end side with the presence of small pigmented and depigmented spots, diffuse thickenings and indentations on the surface of the shell (arrows in the oval) from a domestic chicken (Rhode Island, 1.5 years, experimental group).

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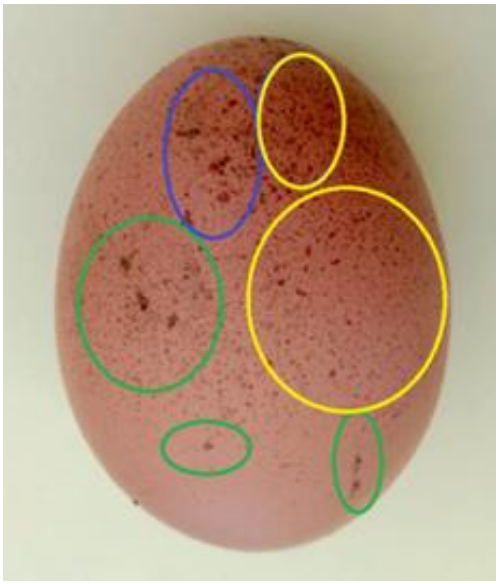

Figure 7. The surface of an unconditioned egg with the presence of pigmented spots (yellow ovals), contamination with blood (blue oval), and droppings (green ovals) in a domestic hen (Rhode Island breed, 1.5 years old, experimental group).

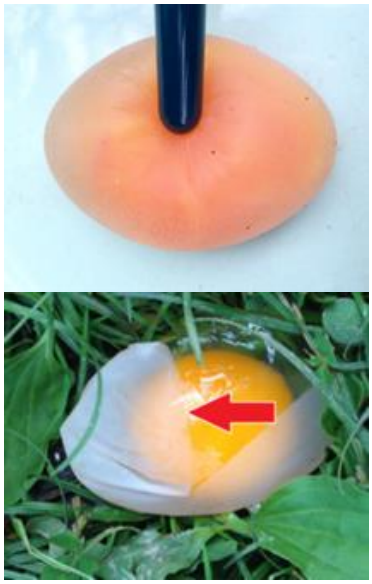

Figure 8. View of substandard eggs without calcified and torn shells in the middle part (arrow) laid on a pasture by a domestic hen (Rhode Island; 1.5 years old, experimental group).

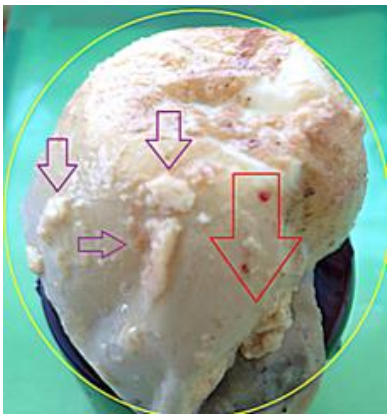

Figure 9. A fragment of a weakly calcified eggshell of a domestic chicken (Rhode Island breed; 1.5 years old) with the presence on the surface of layers of white color (purple arrows) with blood impurities (Two red spots in the red arrow).

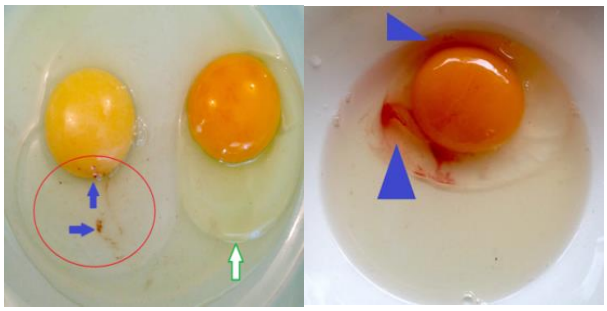

Figure 10. The internal content of eggs of domestic chickens (Rhode Island breed; 1.5 years old) of the experimental (left and right eggs) and control group (the egg in the center). The eggs of the experimental group have a rarefied protein, bloody content (blue arrows in a red oval), and a comparatively lighter yolk. An egg in the center of the control group with a bright yellow yolk and dense white (white arrow).

DISCUSSION

Considering the obtained results on the reduction of egg productivity in chickens with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection, it should be noted that many poultry diseases are characterized by a decrease in egg production and/or egg mass. This is confirmed by research conducted on adult chickens and turkeys (Richter et al., 2010; Landman et al., 2016) regarding the negative impact on the body and threats to the health of the bird and, accordingly, the impact on its productivity (Saikia et al., 2023). The *Eimeria-Histomonosis-Trichomonosis* co-infection in the experimental group of chickens exhibited a chronic latent course without prominent clinical signs, consistent with observations by other researchers (Amin et al., 2011). As the intensity of co-infection, especially involving its components, such as *Trichomonas gallinarum* and *Histomonas meleagridis*, increases, there is a heightened risk of invasion outbreaks (Amin et al., 2014). These outbreaks can lead to severe consequences, including high mortality, which was confirmed by Feng et al. (2021) for the study populations of certain species of wild birds. It is known that birds often suffer from eimeriosis, histomoniasis, and trichomoniasis (Tuska-Szalay et al., 2022). Co-infection undeniably exerts a negative impact on the health of laying hens, with a particular emphasis on production rates, ultimately leading to a decrease in egg production (McDougald, 2005; Hess et al., 2015). In the present study, egg production was reduced by 52%, and egg mass decreased by 19.37% during eimeriosis, Histomonasis, and trichomoniasis co-infestation in laying hens. These findings align with the observations reported by Amin et al. (2011)

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and Landman et al. (2021), particularly in the context of trichomoniasis in hens. Both studies noted decreased egg production and a loss of average egg weight without any clinical signs. Indicators of reduced egg production and/or loss of average egg mass in hens, even in the absence of apparent disease symptoms, could serve as a basis for suspecting co-infection and require a comprehensive, including parasitological, investigation.

Together with a decrease in the level of egg production (gross collection of eggs) and egg weight in infested hens, the changes in the macromorphological indicators of eggs were determined. Their manifestation was influenced by the combined destructive action of pathogens of *Eimeria-Histomonosis-Trichomonosis* co-infection in relation to various structural targets of the chickens' bodies. *Eimeria acervulina* (Tyzzer, 1929), *Eimeria brunetti* (Levine, 1939), *Eimeria maxima* (Tyzzer, 1929), *Eimeria mitis* (Tyzzer, 1929), *Eimeria necatrix* (Jonson, 1927), *Eimeria praecox* (Jonson, 1927), and *Eimeria tenella* (Raillet and Lucet, 1891) caused the development of pathological processes in the intestinal canal, which were characterized by damage to the epithelial cells of the mucous membrane of the wall of the intestinal tube along its entire length (Dalloul and Lillehoj, 2006). Damage to the intestinal canal is caused by the pathogens *Histomonas meleagridis* and *Trichomonas gallinarum*, negatively affecting its terminal part (Lee, 1972) and causing granulomatous damage (Landman et al., 2019) to internal organs (Liebhart et al., 2014; Fadhil et al., 2020). Researchers reported a decrease in egg production, egg weight, shell condition, and changes in the internal content of eggs in birds with trichomoniasis and associate this with the development of degenerative changes in its genital organs due to liver damage (Narcisi et al., 1991). This finding is also consistent with the results of studies by Fitz-Coy and Edgar (1992).

In chickens with *Eimeria-Histomonosis-Trichomonosis* co-infection, there was a high probability of a decrease in the absorptive function of the intestinal mucosa. This refers to insufficient intestinal absorption of various substances in infested chickens, especially those involved in forming the egg and its shell (Dalloul and Lillehoj, 2006). Researchers (da Costa Freitas, 2014) experimentally proved the occurrence of changes in the structure of intestinal villi during eimeriosis in chickens, which prevented the absorption of nutrients, such as calcium, phosphorus, magnesium, proteins, and lipids, leading to decreased weight and productive qualities of chickens. The deficiency in calcium ions affects the quality of the eggshell (Yan et al., 2016; Oikeh et al., 2019). Egg shell formation largely depends on Vitamin D content (Babazadeh et al., 2022). Partly due to the mentioned reasons, the eggs of the chickens of the experimental group showed signs of shell formation disorders, which, according to the morphological evaluation of the egg, was manifested by the presence of its textural defects (softening/insufficient mineralization), cracks and bumpy thickening on the shell. As a result of calcium deficiency, infested hens laid eggs with insufficiently formed, softened shells or without them (Yan et al., 2016; Oikeh et al., 2019).

Contamination of the eggshells of experimental chickens with droppings indicated the presence of inflammatory processes in their intestinal tract, and it can result in diarrhea. Slight contamination of the eggshell surface with blood indicated the presence of probable inflammatory processes in the egg-forming organs. It is important that the egg yolks of chickens with spontaneous *Eimeria-Histomonosis-Trichomonosis* co-infection have a lighter color, compared to the yolks of eggs of healthy chickens. The intensity of the color of the egg yolk depends on the level of carotenoids. This is confirmed by the report of Ruff and Fuller (1975), who reported a decrease in the absorption of carotenoids, resulting in light pigmentation of egg yolks during eimeriosis in chickens. The risk of such a phenomenon naturally increases when the intestines of chickens are affected by *Eimeria*, *Histomonas*, and *Trichomonas*. The rarefaction of protein in the eggs of co-infested chickens in the current study is consistent with previous studies (Teng et al., 2021; Kim et al., 2022) and is logically explained by a general protein deficiency due to a decrease in the intestinal absorption of proteins, amino acids and other nutrients protein (Vakili et al., 2021).

CONCLUSION

According to the results of the current research, spontaneous *Eimeria-Histomonas-Trichomonas* co-infection had a negative effect on the productive qualities of laying hens. The findings indicated a decrease in the egg-laying rate, egg mass, a significant decrease in shell thickness, and manifestations of macromorphological changes in eggs (defects in the shell and internal contents). The specified macromorphological changes and egg defects result from the negative impact of co-infection on oogenesis and the processes of egg formation and indicate the systemic nature of the lesions and the morphofunctional insufficiency of the egg-forming organs. The prospect of further research requires studying the state of the reproductive organs of domestic laying hens during co-infestation.

DECLARATIONS

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Authors' contribution

Petro Liulin conducted coproscopic studies and described his results, and together with the owners of the farm, he formed experimental and control groups of chickens. Lyubov Lyakhovych conducted macromorphological studies of the eggs of chickens of the experimental and control groups, took photographs, and analyzed the obtained data. Mykola Bogach performed the determination of the species relationship of co-infection pathogens. Alla Petrenko participated in the determination of egg productivity of chickens and morphometric indicators of eggs. Inna Kostyuk took part in the detection of deformations of eggs. Petro Lyulin, Lyubov Lyakhovych and Mykola Bogach conducted the literature analysis and wrote the manuscript. All authors have reviewed and approved the final version of the manuscript for publication in this journal.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

All authors reviewed the manuscript for ethical issues such as plagiarism, consent to publish, misconduct, forgery and/or falsification of data, re-publication and/or submission, and redundancy.

Availability of data and materials

Authors of this article confirm the availability of data and materials of valid research and availability upon reasonable requestS.

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Effects of Adding Glutathione to AndroMed Diluent on Intact Plasma and Acrosome Membranes and Progressive Motility of Cattle Spermatozoa During Freezing Processes

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ABSTRACT

Adding endogenous antioxidants to the diluent is significantly associated with semen quality during the freezing process. This study aimed to investigate the effects of adding glutathione to AndroMed diluent on the preservation of crucial sperm attributes, namely, intact plasma membrane (IPM), intact acrosome membrane (IAM), and progressive motility of Bali cattle spermatozoa. A completely randomized design was used, and spermatozoa samples were obtained from a Bali cattle and divided into two diluent treatment groups (36 diluent samples in each group with six replications), namely pure AndroMed as the control and a group with the addition of glutathione (1 mmol) to AndroMed. Each treatment was replicated six times and evaluated at three freezing stages, including post-dilution, post-equilibration, and post-thawing, for crucial sperm properties. The results indicated that fresh Bali cattle spermatozoa had progressive motility, IAM, and IPM of 75%, 89%, and 88%, respectively. During the freezing process, there was a significant decrease in semen quality, including progressive motility, IAM, and IPM of spermatozoa after dilution to post-equilibration and post-equilibration to post-thawing in both treatment groups. Meanwhile, the addition of 1 mmol of glutathione to AndroMed diluent had a significant difference in increasing progressive motility, IAM, and IPM of Bali cattle spermatozoa at each stage of semen freezing, including post-dilution, post-equilibration, and post thawing when compared with controls. Based on the results, it can be concluded that adding 1 mmol of glutathione to the AndroMed diluent enhanced the quality and integrity of Bali cattle semen, including progressive motility, IAM, and IPM during the freezing process.

Keywords: Bali cattle, Freezing Process, Glutathione, Progressive Motility

INTRODUCTION

The success of an artificial insemination program using frozen semen depends on the quantity and quality of semen ejaculated by a male and the freezing process. This process begins with semen collection, evaluation, dilution, filling and sealing, equilibration, aerating over liquid nitrogen vapor, immersion in liquid nitrogen, and thawing (Bebas et al., 2018; Bebas and Agustina, 2022).

During the semen freezing process, spermatozoa are subjected to extremely low temperatures of up to -196°C, which affects the integrity of the cell membranes. At these low temperatures, the interior of cells undergoes physical and chemical changes, including increased intracellular electrolyte concentrations and the formation of ice crystals, causing cold shock (Anwar et al., 2015; Liu et al., 2021; Carriço et al., 2023). Cold shock damages the plasma and acrosome membranes (Ax et al., 2000; Bebas et al., 2018). Another primary issue faced during semen freezing is the exposure of spermatozoa to free radicals (Zhang et al., 2021; Bebas and Agustina, 2022). During this process, spermatozoa undergo peroxidation that generates free radicals such as hydroxyl (-OH) and singlet oxygen or $^1[O]_2$ (Bansal and Bilaspuri, 2011; Park and Yu, 2017). These radicals are highly reactive and potentially induce lipid peroxidation in the plasma and acrosome membranes (Douard et al., 2003; Bebas and Agustina, 2022). Lipid oxidation in the plasma membrane produces malondialdehyde (MDA) as a marker for the presence of toxic free radicals, which reduces motility and causes DNA damage (Dutta et al., 2019).

The plasma membrane regulates the influx and efflux of all electrolytes and substrates the spermatozoa needs (Delgado-Bermúdez et al., 2022). The physiological integrity is closely associated with protecting and maintaining spermatozoa motility during storage, within the female reproductive tract, capacitation, and fertilization. This is particularly significant due to the direct or indirect interactions and adhesion between the plasma membrane and cumulus oophorus (Arvioges et al., 2021; Syafi'i and Rosadi, 2022).

The acrosome membrane is a structure that covers two-thirds of the anterior head. It contains acrosin, hyaluronidase, and corona-penetrating enzyme, which function to lyse the zona pellucida as a pathway for spermatozoa

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to enter the cytoplasm of the ovum during fertilization (Nofa et al., 2017). This membrane is vital in assessing spermatozoa quality, as it is crucial for successful fertilization. Damage to the membrane leads to the leakage of enzymes and a decrease in fertilization capacity (Cahya et al., 2017; Arvioges et al., 2021).

AndroMed is one of the commercial semen diluents devoid of egg yolk, which is prone to microbial contamination. It is user-friendly, and the composition includes sugar, tris hydroxy-aminomethane, and glycerol as an energy source, a buffer, and cryoprotectant, respectively, alongside antibiotics to prevent bacterial growth (Juniandri and Isnaini, 2014). AndroMed also contains soybean extract rich in lecithin, minerals including sodium, magnesium, calcium, chloride, phosphorus, potassium, and manganese, as well as carbohydrates, proteins, citric acid, glycerol lecithin, fats, and glyceryl phosphoryl choline (Lestari et al., 2014; Aji et al., 2019).

Glutathione is a primary antioxidant that prevents the formation of new free radicals (Ansari et al., 2021). It converts existing free radicals into less membrane-impactful molecules. Free radicals are atoms or molecules with one or more unpaired electrons and are highly unstable (Zulaikhah, 2017). To obtain a pair, free radicals react with other atoms or molecules, such as unsaturated fatty acids, proteins, nucleic acids, or lipopolysaccharides, resulting in the creation of abnormal compounds (Pizzorno, 2014; Ansari et al., 2021). The addition of glutathione to the spermatozoa diluent medium reduces or prevents the formation of free radicals that could damage plasma and acrosome membranes as well as motility. Consequently, the fertility of frozen semen increases, ultimately leading to higher pregnancy rates. The recommended concentration of glutathione suitable as a diluent for cattle semen freezing is approximately 1 mmol/L (Syarifuddin et al., 2012), and for dairy goats is 2 mmol/L (Zou et al., 2021). Based on the information provided, this study aimed to determine the impact of adding glutathione to AndroMed diluent on the intact plasma membrane, intact acrosome membrane, and progressive motility of Bali cattle spermatozoa in the post-dilution, post-equilibration, and post-thawing processes. This research reveals new information regarding the role of glutathione as an antioxidant added to Andromed semen diluent on the quality of Bali cattle semen during the freezing process.

MATERIALS AND METHODS

Ethical approval

All procedures have been reviewed by the Experimental Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University, and have received approval No. B/252/UN14.2.9/PT.01.04/2023.

Materials and Equipment

The materials used included 4-year-old Bali cattle semen, AndroMed (REF:13503/0200 Minitub, Hauptstrasse-Germany), Glutathione (248-170-7 Merck, Darmstadt-Germany), NaCl crystals (1.06404.0500 Merck, Darmstadt-Germany), Sodium citrate (1.06448.0500 Merck, Darmstadt-Germany), Fructose (1.05323 Merck, Darmstadt-Germany), Alcohol 70%, distilled water, liquid nitrogen, and Formalin solution 37% (Merck, Darmstadt-Germany).

The equipment utilized included binocular microscope, spectrophotometer, micropipette, Pasteur pipette, 10 ml volumetric pipette, glass slides, cover slips, pH meter, measuring cylinder, Erlenmeyer flask, straws, straw rack, water bath, 1 cc and 10 cc syringes, livestock crush, artificial cattle vagina, refrigerator, Styrofoam box, filling and sealing machine, straw printing machine, and liquid nitrogen container.

Semen shelter for Bali cattle

The experimental animal utilized in this research was a 4-year-old male Bali cattle, in healthy condition according to veterinary examination. The research was conducted from May to September 2023 at the Artificial Insemination and Livestock Breeding Center, Baturiti District, Tabanan Regency, Bali Province, Indonesia. Before semen collection, flushing of the preputium was performed to ensure cleanliness, followed by drying with a clean cloth. The teaser cattle were introduced into the mating pen, then the male was brought closer, allowed to mount the teaser cattle, and subsequently dismounted (Mappanganro, 2020). This process was repeated 2-3 times to enhance the libido of the male. Once an erection occurred, the penis was directed towards the artificial vagina by holding the preputial region, followed by ejaculation. The collected ejaculate of approximately 5-6 ml was promptly transported to the laboratory and placed for 3-5 minutes in a water bath at 37^{o}C for immediate semen quality evaluation at the Laboratory of Artificial Insemination and Livestock Breeding.

Semen evaluation

The collected semen from a single male was subjected to both macroscopic and microscopic examinations. The research design used a completely randomized design, where 72 spermatozoa samples were divided into two treatment groups, with three stages of the freezing process and each with six replications. The macroscopic examination entailed evaluations of volume, pH, consistency/viscosity, color, and odor. Meanwhile, the microscopic examination using Microscope Binoculer Olympic CX-23 (Olympus Corporation, Japan) with magnification 400x included analyses of

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mass motility, individual motility, spermatozoa concentration, abnormality, IPM, and IAM (Cocchia et al., 2011; Susilawati, 2011).

AndroMed diluent preparation

AndroMed in powdered form (grams) was placed into a measuring glass (50 ml) containing distilled water with a ratio of 1 (gram) to 4 (ml), then homogenized. Afterward, the sample was placed in a water bath for 3-5 minutes at 37ºC, and the diluent was prepared for use (Susilawati, 2011). For the test group, glutathione was added to AndroMed diluent at a concentration of 1 mmol, according to previous studies (Syarifuddin et al., 2012; Maleki et al., 2023).

Cement dilution

The collected semen and the AndroMed diluent were placed for 3-5 minutes in a water bath at a temperature of 37°C. This step was taken to equilibrate the temperature of the diluent with that of the semen. Furthermore, semen that underwent evaluation was diluted using the diluent prepared according to the formula for calculating the diluent quantity (Wishart, 2009; Syarifuddin et al., 2012).

Straw printing

For liquid semen that fulfilled the quality standards before freezing, the next step entailed labeling the packaging using a straw printing machine (Minitub, Hauptstrasse-Germany) (Syarifuddin et al., 2012; Maleki et al., 2023).

Filling and sealing

Liquid semen that fulfilled the standards was filled into mini straws with a volume of 0.25 ml, containing a concentration of 25 million spermatozoa (Syarifuddin et al., 2012; Setiono et al., 2015). Subsequently, the packaging was sealed using a filling and sealing machine (Minitub, Hauptstrasse-Germany).

Equilibration

The semen that underwent filling and sealing was then stored in a refrigerator at a temperature of 4°C for 4 hours (Murphy et al., 2018; Swarna et al., 2023).

Aerating in liquid nitrogen vapor

The pre-freezing process was conducted by rapidly lowering the semen temperature from 4°C to -140°C to prevent cold shock (sudden temperature change affecting sperm cells). It was accomplished by placing straws, arranged on a straw rack, into the liquid nitrogen vapor approximately 4 cm above the surface for 9 minutes (Syarifuddin et al., 2012; Baharum et al., 2017).

Freezing

The freezing process was carried out by immersing the semen product into liquid nitrogen until fully submerged, resulting in the temperature dropping to -196°C (Ansari et al., 2021).

Post-thawing motility evaluation

In the Post-thawing motility (PTM) process, frozen semen was subjected to quality testing to determine its suitability for distribution according to the Indonesian National Standards, which required a minimum of 40% progressive motility and a minimum concentration of 25 million cells per dose. Frozen semen that did not fulfill these standards was subsequently discarded. In this research, the assessment of semen quality was achieved by observing progressive motility, IPM, and IAM. These observations were conducted on fresh semen post-dilution with respective treatments, post-equilibration, and post-thawing (Zhang et al., 2021).

Progressive motility examination

Diluted semen of 0.05 ml was gently dropped onto a warm glass slide (37°C), covered with a glass cover, and observed under a light Microscope Binoculer Olympic CX-23 (Olympus Corporation, Japan) at 400x magnification. The counting of spermatozoa exhibiting progressive motility was performed and expressed as a percentage. This observation was carried out across five fields of view (Wishart, 2009; Manehat et al., 2021).

Intact plasma membrane examination

The percentage of spermatozoa IPM was assessed using the hypoosmotic swelling (HOS) test method (Nur et al., 2012). The composition of the hypoosmotic solution consisted of 0.9g of fructose and 0.49g of sodium citrate dissolved in distilled water to reach a volume of 100 ml (100 mOsm/kg). About 20 ml of the solution was combined with 0.2 ml of semen, mixed to homogeneity, and then incubated at 37°C for 45 minutes. Thin smears were prepared on glass slides,

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followed by evaluation under a light microscope Binoculer Olympic CX-23 (Olympus Corporation, Japan) at 400x magnification on a minimum of 200 spermatozoa. Spermatozoa with IPM were characterized by coiled or swelling tails, while damaged ones exhibited straight tails.

Intact acrosome membrane examination

About 0.9 grams of NaCl was dissolved in distilled water to make 100 ml, then 1 ml of formalin was added to 99 ml of the physiological NaCl solution and shaken until homogeneous. A part of semen was mixed with three parts of the NaCl and formalin mixture. The mixture was left for approximately 3 minutes, and thin smears were created on glass slides. Spermatozoa were examined under a light microscope at 400x magnification. The assessment was conducted by counting the proportion with IAM within 100 spermatozoa. Samples with IAM were marked by the presence of a dark acrosome membrane (Cocchia et al., 2011; Zhang et al., 2021).

Data analysis

The obtained data were analyzed using analysis of variance (ANOVA) through SPSS version 25 for Windows. In cases of significant differences among treatments, further analysis was conducted using the Duncan test. P value < 0.05 is declared statistically significant.

RESULTS

The macroscopic evaluation of fresh Bali cattle semen yielded normal characteristic aroma, moderate-normal consistency, creamy color, and pH of 6.5. Meanwhile, the microscopic evaluation showed mass motility (+++), spermatozoa concentration of $1358x10^6$ ml^{-1}, 75% progressive motility, 86% viability, 5% abnormality, IPM at 86%, and IAM at 89%. The comprehensive results of the semen evaluation are presented in Table 1. The addition of glutathione with a concentration of 1 mmol to the AndroMed diluent led to an improvement in semen quality during the freezing process stages, including dilution, post-equilibration, and post-thawing. Statistical analysis indicated that the treatment and freezing stages significantly ($p < 0.05$) affected the quality of Bali cattle semen, compared to control (Table 2). The effect of adding glutathione to the AndroMed diluent on the semen quality during the freezing process stages is shown in Figures 1, 2, and 3. Microphotograph of sperm motility and integrity are shown in Figures 4, 5, and 6.

Table 1 shows that fresh Bali cattle spermatozoa have progressive motility, IAM, and IPM of 75%, 89%, and 88% respectively. Meanwhile, Table 2 shows that the stages of the freezing process had a significant effect ($p < 0.05$) on reducing progressive motility, IAM, and IPM of spermatozoa after dilution compared to post-equilibration and post-equilibration, compared to post-thawing in all treatment groups. Next, the obtained showed that the addition of 1 mmol gluthation to Andromed diluent had a significant difference ($p < 0.05$) in increasing progressive motility, IAM, and IPM of Bali cattle spermatozoa at the post-dilution, post-equilibration, and post thawing compared to control.

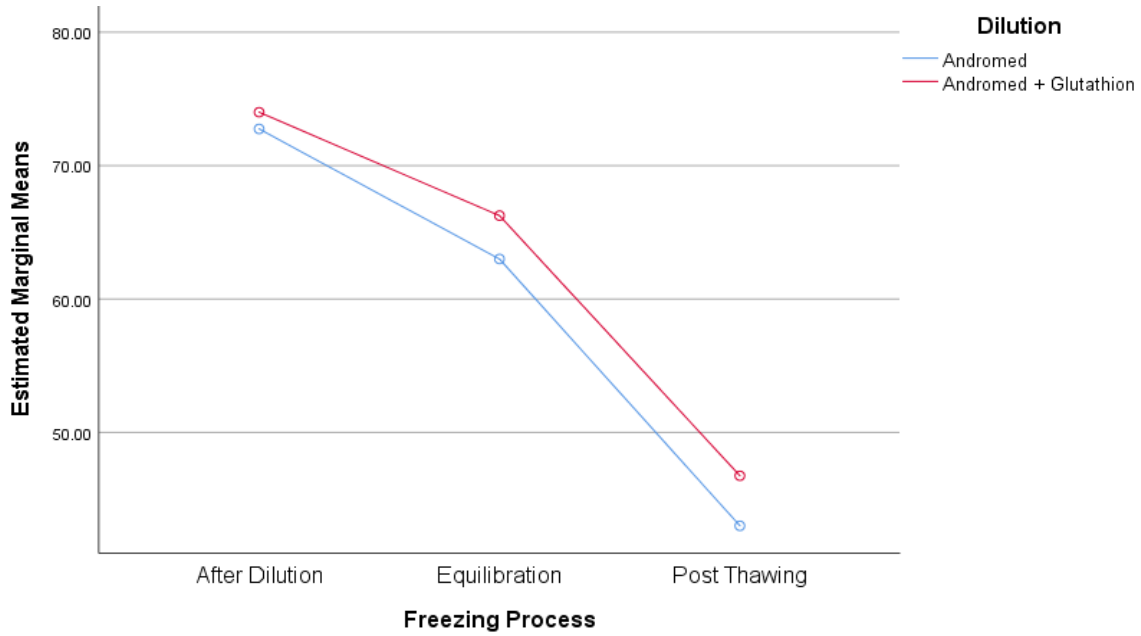

Figure 1. Motility of 4 years old Bali cattle spermatozoa due to the effect of adding 1 mmol glutathione to AndroMed diluent during the semen freezing process

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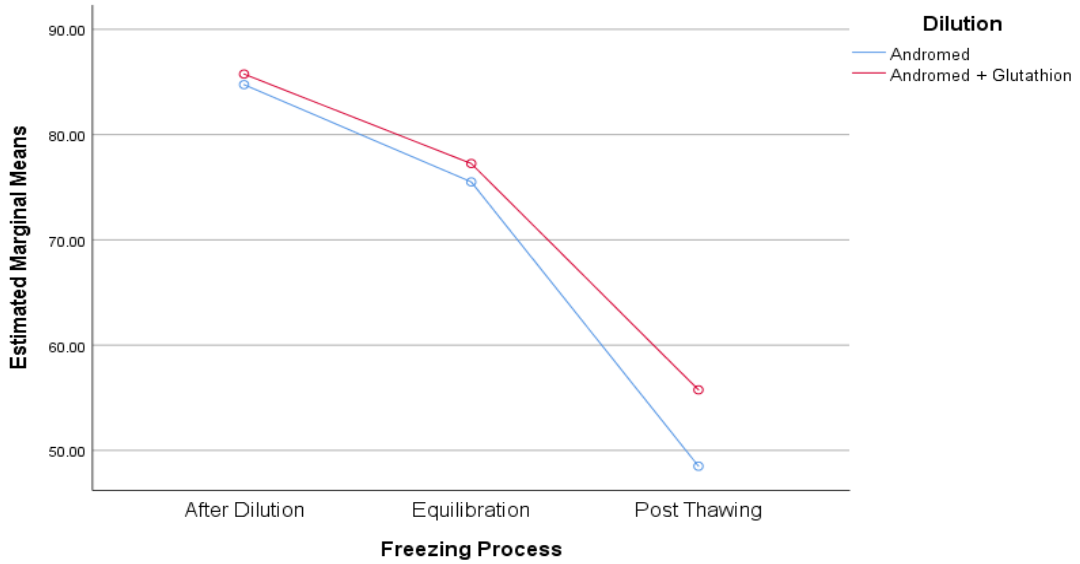

Figure 2. Intact acrosome membrane of 4 years old Bali cattle spermatozoa due to the effect of adding 1 mmol glutathione to AndroMed diluent during the semen freezing process

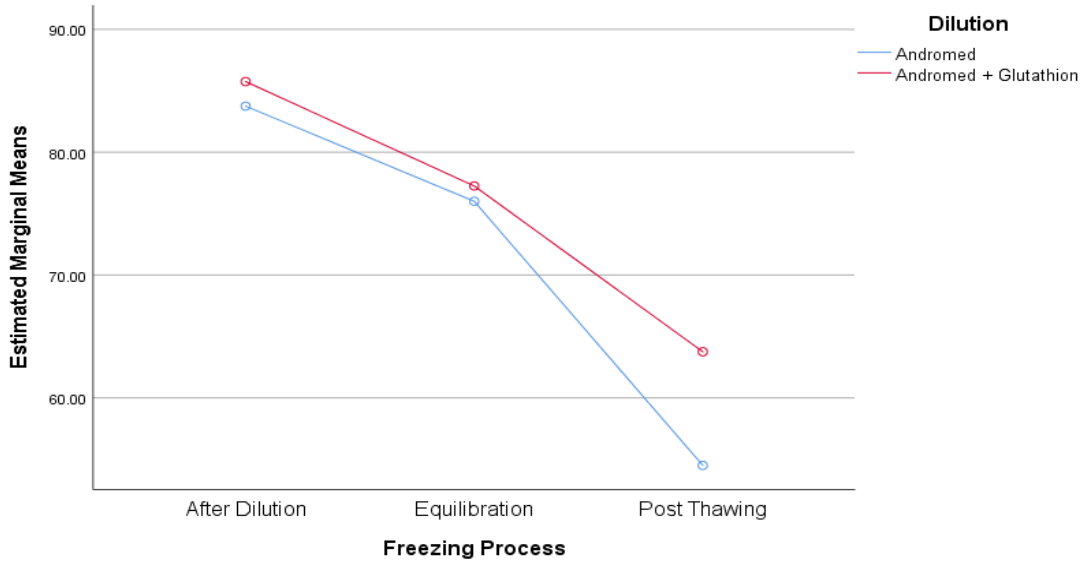

Figure 3. Intact plasma membrane of 4 years old Bali cattle spermatozoa due to the effect of adding 1 mmol glutathione to AndroMed diluent during the semen freezing process

Figure 4. Micropothograph of 4 years old Bali cattle spermatozoa with normal morphology (400x).

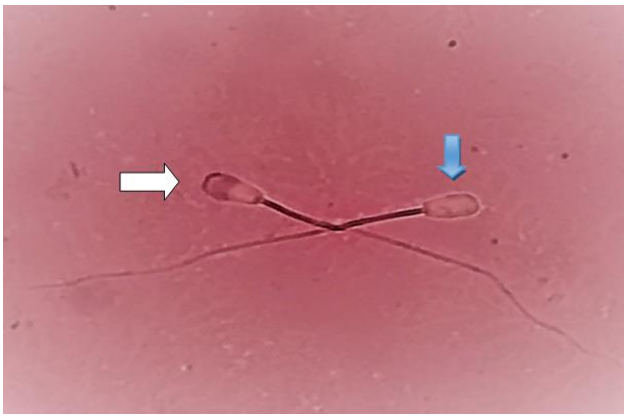

Figure 5. Microphotograph of 4 years old Bali cattle spermatozoa with intact acrosome membrane (white arrow) and detached acrosome membrane (blue arrow, 400x)

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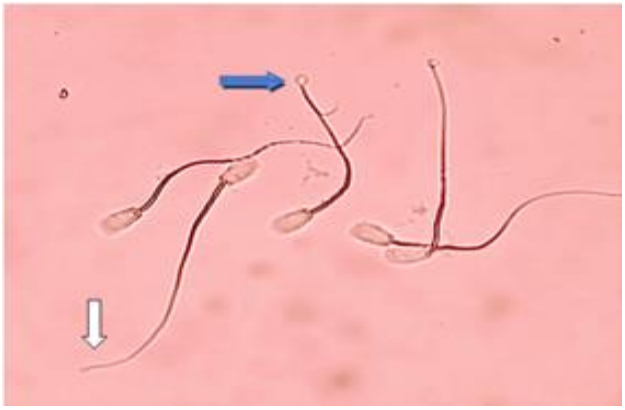

Figure 6. Microphotograph of 4-year-old Bali cattle spermatozoa with intact plasma membrane and curled tail (blue arrow) and detached plasma membrane with straight tail (white arrow, 400x)

Table 1. Macroscopic and microscopic fresh semen quality of Bali cattle aged 4 years

Parameter		Value
Macroscopic	Volume (ml)	5.8
	Odor	Normal
	Consistency	Moderate-thick
	Color	Cream
	pH	6.5
Microscopic	Mass Motility	+++
	Spermatozoa Concentration ($x10^6$)	1358
	Progressive Motility (%)	75
	Viability (%)	86
	Abnormality (%)	5
	Intact Acrosome Membrane (%)	89
	Intact Plasma Membrane (%)	88

+++: Mass motility was very good

Table 2. Effect of freezing stages on semen quality of Bali Cattle aged 4 years diluted using AndroMed with the addition of 1 mmol Glutathione

Parameter	Freezing stages	Diluted	
		AndroMed	AndroMed + 1 mmol Glutathione
Progressive motility (%)	Post-Dilution	72.75 ± 1.25^{Aa}	74.38 ± 1.15^{Ba}
	Post-Equilibration	63.00 ± 1.82^{Ab}	66.25 ± 0.95^{Bb}
	Past-Thawing	43.00 ± 0.81^{Ac}	46.75 ± 0.95^{Bc}
Intact acrosome membrane (%)	Post-Dilution	84.75 ± 0.95^{Aa}	85.75 ± 1.71^{Ba}
	Post-Equilibration	75.50 ± 1.29^{Ab}	77.25 ± 1.70^{Bb}
	Past-Thawing	48.50 ± 1.29^{Ac}	55.75 ± 2.06^{Bc}
Intact plasma membrane (%)	Post-Dilution	83.75 ± 0.95^{Aa}	85.25 ± 0.95^{Ba}
	Post-Equilibration	76.00 ± 1.41^{Ab}	77.25 ± 1.50^{Bb}
	Past-Thawing	54.50 ± 1.29^{Ac}	63.75 ± 1.89^{Bc}

^{abc}Superscript letters that differ towards the column show a significant difference ($p < 0.05$); ^{ABC}Different superscripts letters towards the row indicate significant differences ($p < 0.05$)

DISCUSSION

The results from this research (Table 1) were consistent with Bebas et al. (2022), where macroscopic evaluation such as volume, odor, consistency, color, and pH was reported as 5.98±1.35 (Bebas and Agustina, 2022). Meanwhile, microscopic evaluation, including mass motility, concentration, progressive motility, abnormality, and IPM was noted as +++, 1104 ± 202.21 x 10^6 ml^{-1} of ejaculate, 69.58±0.30%, 4.66±1.58%, and 83.22±1.64%, respectively. Hafez and Hafez (2000) stated that normal semen appeared whitish-gray to pale cream in color, with a thick consistency. The consistency correlates with the color; for instance, creamy-colored semen tends to have a thick or viscous consistency, while clear or light-colored semen is usually less viscous (Islam et al., 2018). The distinct aroma of cattle semen indicates normalcy and the absence of contamination. This aligned with Ilaria (2011), which categorized semen odor as distinctive. According to Hafez and Hafez (Hafez and Hafez, 2000), the concentration of cattle spermatozoa ranges between 800-2000 million/ml. The observed mass motility was very good (+++), characterized by large, numerous, dark, thick, active, and fast-moving waves. In general, the cattle semen fell within the normal concentration with good quality and integrity, in line with reports by Garner and Hafez (2000) and Prastowo et al. (2018).

During the semen freezing process, including dilution and equilibration, spermatozoa encounter extremely low and extreme temperatures, reaching -196°C. Freezing significantly affects the cell membrane of spermatozoa, with subzero temperatures below the freezing point causing physical and chemical changes, including the formation of ice crystals and an increase in intracellular electrolyte concentration, resulting in cold shock (Pratiwi et al., 2014; Çelik et al., 2020). According to previous research, cold shock culminates in damage to both plasma and acrosome membranes (Liu et al., 2021; Carriço et al., 2023). The main challenge encountered during semen freezing was not only cold shock but also the exposure of spermatozoa to free radical attacks such as hydroxyl and singlet oxygen (Park and Yu, 2017; Angrimani et al., 2018). These highly reactive radicals can induce lipid peroxidation on plasma and acrosome membranes (Bansal and

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Bilaspuri, 2011; Syafitri et al., 2022). The spermatozoa plasma membrane consists of double lipids containing unsaturated fatty acids which are very susceptible to peroxidation (Douard et al., 2003). Free radicals work to oxidize lipids in the membrane so that the unsaturated fatty acid chains are broken and produce MDA, which is toxic to spermatozoa cells (Zulaikhah, 2017; Zhang et al., 2021). The presence of MDA is an indicator of the existence of free radicals (Dutta et al., 2019) that damage the plasma membrane, resulting in a decrease in the quality and integrity of spermatozoa, including concentration, motility, and DNA damage (Prihantoko et al., 2022).

Research related to antioxidants as cryoprotectants has reported success in reducing oxidative damage due to reactive oxygen species (Zulaikhah, 2017; Liu et al., 2021). Glutathione is a significant antioxidant that neutralizes free radicals for cryopreserved in dogs (Angrimani et al., 2018), Indian red jungle fowl (Ansari et al., 2021), Mithun cattle (Perumal et al., 2021), goat (Zou et al., 2021) and sheep (Syafitri et al., 2022). It is a naturally occurring substance present in the body since birth and is found both within and outside cells (Zou et al., 2021). Blood glutathione levels range from 5 to 8 mM/L, with the highest concentration being present in the liver, which serves as the most essential organ for detoxification in the body. This antioxidant is also present in the spleen, kidneys, lungs, heart, brain, and stomach (Zulaikhah, 2017).

The glutathione antioxidant system serves as a primary endogenous protection mechanism, actively participating in the neutralization of reactive oxygen species (ROS) and maintaining the reduced (active) forms of vitamins C and E (Biswas et al., 2020). Glutathione is also called the "master antioxidant" as it could lead to the activity of other antioxidants (Chakravorty et al., 2020). For instance, vitamins C and E capture free radicals, followed by the transfer to glutathione, which then cycles to capture more ROS (Zeitoun and Al-Damegh, 2014). This antioxidant neutralizes and eliminates free radicals through urine. Its efficacy in protecting cells surpasses other antioxidants, including vitamins C and E. Furthermore, glutathione protects DNA and RNA chains from degradation and shields the nucleus from free radicals. The glutathione binds and expels unwanted substances through urine and bile (Solihati et al., 2018). The presence of glutathione protects cells from toxic ROS effects and acts as a scavenger for hydroxyl (-OH) and superoxide (O_2^-) radicals. This antioxidant also plays roles in maintaining cell survival, DNA replication, protein thiolation, enzyme catalysis, membrane transport, receptor action, intermediary metabolism, and cell maturation (Zhang et al., 2005). According to a previous study, glutathione captures peroxides that could harm cells. Due to the mitochondrial respiration process, all aerobic organisms naturally face oxidative stress. Compounds, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), induce the production of oxygen radicals, which are toxic and capable of causing lipid peroxidation and cellular injury (Zeitoun and Al-Damegh, 2014; Bollwein and Bittner, 2018). Based on the results, adding glutathione at a concentration of 1 mmol to the AndroMed diluent significantly improved semen quality, including spermatozoa motility, IPM, and IAM, during the freezing process stages.

CONCLUSION

The addition of glutathione with a concentration of 1 mmol to the AndroMed diluent significantly improved the frozen semen quality characterized by progressive motility, intact acrosome membrane, and intact plasma membrane during the freezing process stages, including post-dilution, post-equilibration, and post-thawing. Furthermore, research needs to be carried out on fertility tests on semen quality through artificial insemination technology.

DECLARATIONS

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Availability of data and materials

The data of the current study are available.

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Authors' contributions

Wayan Bebas is the Conception and design of the study. Wayan Bebas and I Wayan Gorda conducted an Acquisition of data. Wayan Bebas and I Made Merdana Analysis and interpretation of data, and wrote the manuscript.

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Kadek Karang Agustina and I Wayan Gorda give the Critical review and revision. All authors checked and confirmed the data analysis and the final version of manuscript.

Competing interests

We declare there is no any conflict of interest in the publication of this article.

Ethical consideration

Ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal.

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Epidemiology, Molecular, and Phylogenetic Characterization of *Echinococcus granulosus* Cysts in Slaughtered Farm Animals in Al-Jouf Province, Saudi Arabia

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ABSTRACT

Echinococcosis, or hydatidosis, is a serious veterinary disease and public health issue worldwide, particularly in rural areas in which dogs have frequent contact with local herbivores. This study assessed the frequency of hydatidosis found among farm animals slaughtered in Al-Jouf Province in northern Saudi Arabia in 2021. A total of 156754 sheep, 36337 goats, 8590 camels, and 986 cattle were inspected for hydatidosis infection by comprehensive evaluation involving meticulous visual inspection and manual exploration of the internal organs through palpation. The cysts were subjected to molecular and phylogenetic analysis. The overall prevalence rates of hydatid cysts were 0.43%, 0.19%, 0.54%, and 0.51% in the inspected sheep, goats, camels, and cattle, respectively. The highest disease prevalence rates among sheep (27.8%) and goats (30.9%) occurred in the spring, and the highest prevalence rates among camels (41.3%) and cattle (80%) were in the summer. Regarding the prevalence of the disease in four slaughterhouses in the Al-Jouf Province, the highest prevalence in sheep, goats, and camels was in the Tabarjal slaughterhouse (1.43%, 0.81%, and 1.08%, respectively), although the Al-Qurayat slaughterhouse had the highest prevalence rate in cattle (1.98%). Complete molecular analysis indicated that cytochrome c oxidase subunit 1 (*cox1*) sequences from cyst isolates belonged to *Echinococcus granulosus* (*E. granulosus*). Moreover, there was high homology (98-100%) with associated Genbank sequences of *E. granulosus* isolated from sheep in the Kingdom of Saudi Arabia (KSA). Sheep and camels were a significant source of hydatidosis transmission to dogs and helped to maintain disease incidence in the Al-Jouf Province. Thus, significant efforts should focus on preventing cyst transmission from abattoirs and infected stray dogs.

Keywords: *Echinococcus granulosus*, Epidemiology, Molecular characterization, Farm animals

INTRODUCTION

Echinococcosis, or hydatidosis, is a parasitic disease caused by infection with a larva from the *Echinococcus* genus of the family Taeniidae (WHO, 2016). It is a widespread zoonotic disease affecting several areas of the world, including Saudi Arabia, posing a serious risk to humans and livestock and having a severe economic impact on the farm industry (Eckert and Deplazes, 2004; WHO, 2015). There are multiple species of *Echinococcus (E.)*, including *E. granulosus, E. multilocularis, E. oligarthrus,* and *E. vogeli*, of which *E. granulosus* is most often associated with disease (Nabavi et al., 2014).

The *E. granulosus* lifecycle requires both an intermediate and a definitive host. Intermediate hosts include herbivores such as sheep and cattle, while the final host is a definitive carnivore (Eckert et al., 2001; Eslami et al., 2016). Humans are aberrant dead-end hosts who become infected by contacting the feces of definitive hosts or after eating food contaminated by parasite eggs (Virginio et al., 2012). Since canines and domestic animals have the most contact in rural areas, domestic animals serve as the primary reservoir for human disease (Almalki et al., 2017).

In Saudi Arabia, the major meat-producing livestock includes sheep, goats, camels, and cattle. At a total of 13,444,435 heads, sheep are the primary source of meat (72%), with a high number being imported to fulfill the needs of the Saudi population (GASTAT, 2018). Echinococcosis affects both humans and their domestic animals in several countries in Saudi Arabia, mainly in the Western Region (Hayajneh et al., 2014).

Infection can lead to financial losses from the disposal of diseased organs and reduced milk, meat, and wool production (Singh et al., 2016). Moreover, helminth infections not only hinder productivity but also have detrimental effects on food quality (Toni et al., 2023). Infected animals can also be potential sources of co-contamination that can impact humans and other animals (Singh et al., 2016).

Since Echinococcosis is a significant zoonotic disease, it is critical to assess the prevalence and incidence of infection in potential intermediate hosts. This defines the role of each animal species in parasite conservation and disease movement (Cadavid Restrepo et al., 2016). Accurate disease diagnosis is required for the detection of the livestock source responsible for transmission (Cadavid Restrepo et al., 2016). In intermediate hosts, hydatidosis is usually

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asymptomatic and thus often overlooked by farmers (Elham et al., 2014). Inspection of meat from slaughtered animals is important for identifying cysts and determining disease prevalence. As a result, slaughterhouses remain the ideal location to study hydatidosis in livestock from different regions (Almalki et al., 2017). Numerous research studies have addressed the prevalence of Echinococcosis disease in Saudi Arabia. For instance, a study by Merst et al. (2023) explored the infection rates among stray dogs and slaughtered animals in the Al-Kharj region in Saudi Arabia, revealing a 14% infection rate in dogs and rates of 9.18%, 7.5%, and 10% for sheep, goats, and camels, respectively. Another study in the eastern region of Saudi Arabia identified three *Echinococcus* species in camels *E. granulosus*, *E. Canadensis*, and *E. ortleppi* (Al-Hizab et al., 2021). The prevalence of Echinococcosis, a zoonotic disease, is notable among livestock in the Arabian Peninsula countries (Al-Shaibani et al., 2021) and is a global concern (Alvi and Alsayeqh., 2022).

A deeper understanding of *Echinococcus* species will inform new methods of control, diagnosis, and treatment (McManus, 2010). Sequencing of the whole mitochondrial genome has proved extremely useful to the phylogenetic analyses of species of *Echinococcus* (Nakao et al., 2013). The current study aimed to determine the presence of *E. granulosus* over one year among sheep, goats, camels, and cattle and to assess the impact of season, region, and *Echinococcus* species on disease prevalence in Al-Jouf Province, Saudi Arabia. Hydatid cysts were evaluated by polymerase chain reaction (PCR), and DNA specific for the mitochondrial *cox1* gene was sequenced to compare with the genotype *E. granulosus* in slaughtered livestock in Saudi Arabia.

MATERIALS AND METHODS

Ethics approval

This study was approved by the Research Ethical Committee of Jouf University in Saudi Arabia (approval No: 40-08-48)

Study location

The study was conducted using slaughtered goats, sheep, camels, and cattle from four slaughterhouses in the Al-Jouf province located in the north of Saudi Arabia between longitudes (36° and 41°) east and latitudes (28° and 32°) north from January to December 2021. All slaughterhouses were operating in accordance with the Technical Regulations for Meat Inspection issued by the Ministry of Municipal and Rural Affairs in 2008.

Study procedure

A total of 156,754 slaughtered sheep, 36,337 goats, 8,590 camels, and 986 cattle were examined by veterinarians for cystic hydatidosis (Table 1). Region, season, and species information was obtained, and each slaughtered animal was comprehensively evaluated through meticulous visual inspection and manual exploration of the internal organs through palpation, according to Eckert et al. (1984). Ten hydatid protoscolices from each animal species (random selection) were dipped in Phosphate Buffer Saline PBS and transferred to the parasitology laboratory, Biology department, college of Science in Jouf University into sterile test tubes (each sample originating from a single cyst), fixed in 70% ethanol, and stored at -20°C for DNA extraction.

Table 1. Number and infection rate (%) of hydatidosis in slaughterhouses in the Al-Jouf Province, Saudi Arabia in 2021

Slaughterhouse location	Sheep	Goats	Camels	Cattle	Total
Sakaka	131/91620 (0.14%)	11/19830 (0.06%)	14/4673 (0.3%)	1/580 (0.17)	157/116703 (0.14%)
Dumat al-Jandal	39/16572 (0.24%)	7/3222 (0.22%)	7/1322 (0.53%)	0/161 (0%)	53/21277 (0.25%)
Al-Qurayat	349/37400 (0.93%)	33/11188 (0.3%)	16/1763 (0.91%)	4/202 (1.98%)	402/50553 (0.80%)
Tabarjal	160/11162 (1.43%)	17/2097 (0.81%)	9/832 (1.08%)	0/43 (0%)	186/14134 (1.32%)
Total	679/156754 (0.43%)	68/36337 (0.19%)	46/8590 (0.54%)	5/986 (0.51%)	798/202667 (0.39%)
P value	0.207	0.186	0.042*	0.334	0.295

Each box of this table includes information on the number of infected animals/number of animals examined and Infection rates (%). * $p < 0.05$ (significant)

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Examination of cysts and viability of protoscoleces

The cyst fluid was extracted using a needle and subsequently examined under a light microscope to determine the presence of protoscoleces (Eckert et al., 1984).

DNA extraction

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Hydatid cyst samples (25 mg) were processed, and DNA was dissolved in 50 µl elution buffer.

PCR amplification

According to a previous study by Bowles et al. (1992), the mitochondrial *cox1* gene was targeted with the forward primer 5'- TTTTTTGGGCATCCTGAGGTTTAT-3' and the reverse primer

5'-TAAAGAAAGAACATAATGAAAATG-3' (Vivantis Technologies Sdn. Bhd, Malaysia) in a 25 µl total reaction volume with 12.5 µl of COSMO-PCR-RED Master Mix (W1020300X, Willofort, UK), 0.5 µL (17 µM) of each primer, and 2 µL of target DNA (Bowles et al., 1992). The PCR program involved 5 minutes of denaturation at 95°C, 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 45 seconds, and a single extension step at 72°C for 10 minutes. The PCR products (450 bp) were analyzed using 1.2% agarose gel electrophoresis with a 100 bp Plus DNA ladder and analyzed using an InGenius3 gel documentation system (Syngene, UK).

Phylogenetic tree construction

Three cytochrome c oxidase subunit I (*cox1)* sequences from human isolates involved in this study (EEG1-EEG3) were registered in GenBank under the accession numbers MZ348904-MZ348906, and three nucleotides from camel isolates (EEG4-EEG6) were registered under the accession numbers, MZ348907-MZ348909. Positive PCR products for the *cox1* gene were sequenced (Macrogen, Korea) and analyzed using BioEdit 7.0.4 and MUSCLE. The resultant sequences were aligned with the *E. granulosus*. *cox1* reference sequences using a neighbor-joining method of the aligned sequences in the CLC Sequence Viewer 6 program, UK.

Statistical analysis

The findings were presented in the form of tables using descriptive analysis. The data obtained from the study were subjected to statistical analysis using SPSS software version 26. To assess the significance of the presence of hydatidosis in various locations and animal species, the Chi-square (X^2) test was employed, and the results were presented in Table 1. Additionally, the uniform distribution test was utilized to evaluate the significance of hydatidosis occurrence across different seasons and animal species, with the corresponding outcomes documented in Table 2. A p-value less than 0.05 shows a significant difference.

Table 2. Seasonal prevalence of hydatidosis in slaughtered animals in the Al-Jouf Province, Saudi Arabia in 2021

Animals / Season	Sheep No. (%)	Goats No. (%)	Camels No. (%)	Cattle No. (%)
Winter	173 (25.5)	15 (22.1)	11 (23.9)	1 (20)
Spring	189 (27.8)	21 (30.9)	9 (19.6)	0 (0)
Summer	169 (24.9)	15 (22.1)	19 (41.3)	4 (80)
Autumn	148 (21.8)	17 (25)	7 (15.2)	0 (0)
Total	679 (100)	68 (100)	46 (100)	5 (100)
P-value	0.524	0.687	0.048*	0.305

* $p < 0.05$ (significant), No: Number.

RESULTS

An overall hydatidosis prevalence of 0.43%, 0.19%, 0.54%, and 0.39% was detected among sheep, goats, camels, and cattle, respectively, obtained from four slaughterhouses in Al-Jouf Province, for a total prevalence of 0.39% (Table 1).

Of the four slaughterhouses in the Al-Jouf Province (Table 1), the Tabarjal slaughterhouse had the highest prevalence of hydatidosis in sheep, goats, and camels (1.43%, 0.81%, and 1.08%, respectively), while the Al-Qurayat slaughterhouse had the highest prevalence in cattle (1.98%). The Dumat al-Jandal slaughterhouse had the lowest prevalence of sheep (0.24%), the Sakaka slaughterhouse had the lowest in goats (0.06%) and camels (0.3%), and the Dumat al-Jandal and Tabarjal slaughterhouses had the lowest for cattle (0%). Statistically, the difference was not significant ($p > 0.05$) for all animals of the four slaughterhouses, barring for the camels, which had a significant difference ($p < 0.05$), as shown in Table 1.

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The highest prevalence of disease occurred in spring for sheep and goats (27.8% and 30.9%, respectively) and in summer for camels and cattle (41.3% and 80%, respectively), while the lowest prevalence occurred in autumn for sheep and camels (21.8% and 15.2%, respectively), in winter and summer for goats (22.1%), and in spring and autumn for cattle (0%, Table 2). Statistically, the difference was not significant ($p > 0.05$) for all animals of the four Seasons, except camels, which had a significant difference ($p < 0.05$), as shown in Table 2.

A 450 bp fragment was amplified from all cyst samples using *cox1* PCR (Figure 1). A phylogenetic tree of the isolates showed that they belonged to *E. granulosus.* Whole isolate sequences of the mtDNA revealed 98–100% homology with the *E. granulosus* reference sequence, MZ350810, isolated from sheep in Al-Taif, KSA, and MN720282 isolated from sheep in Al-Madinah, KSA (Figure 2).

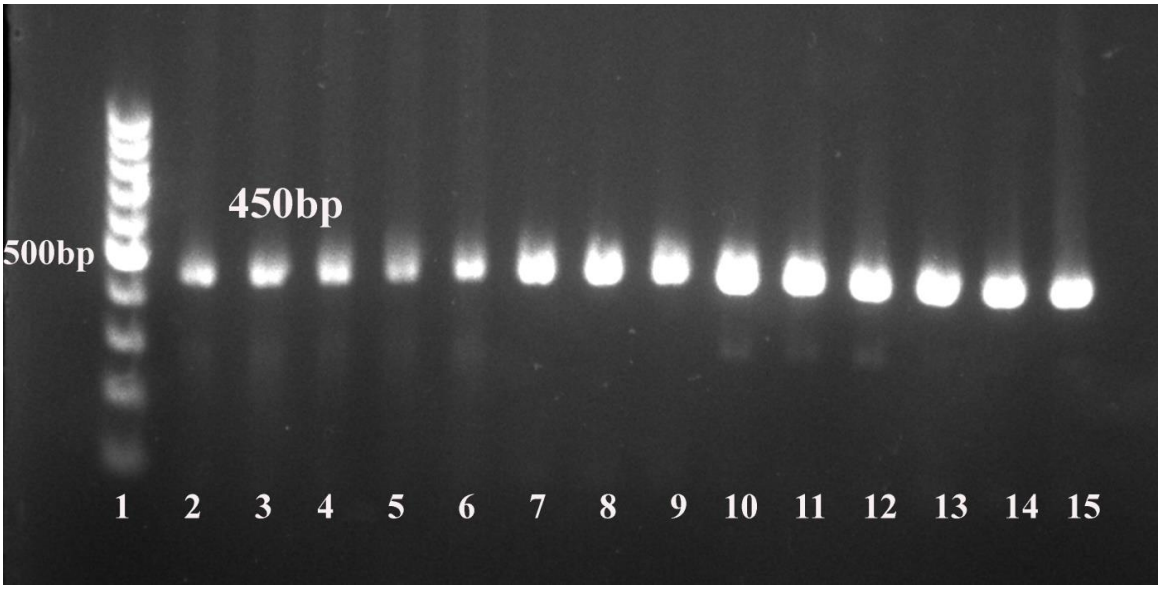

Figure 1. PCR analysis of the *cox1* gene for *Echinococcus granulosus* revealed a 450 bp band derived from representative sheep (lanes 2-5), goats (lanes 6-9), camels (Lanes 10-12), and cattle (Lanes 13-15)

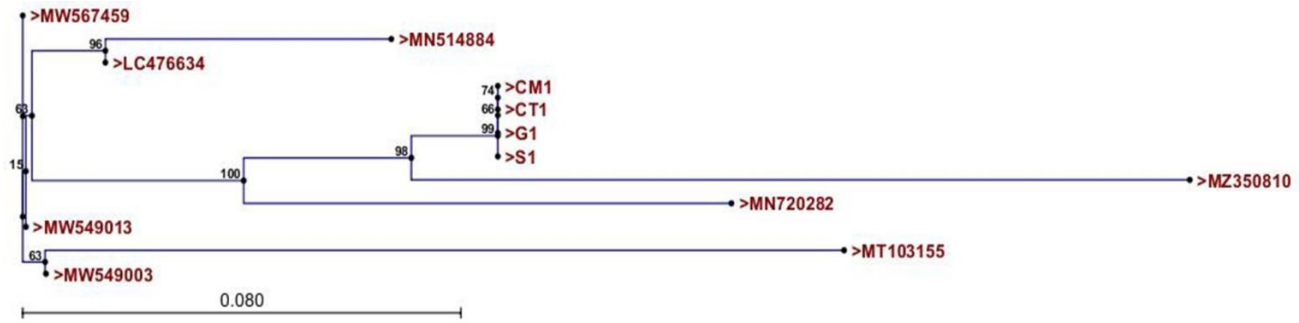

Figure 2. Phylogenetic tree of *Echinococcus granulosus* sequences isolated from sheep (S1), goats (G1), camels (CM1) and cattle (CT1) in Egypt and reference sequences from other *Echinococcus* species using the neighbor-joining method based on the *cox1* gene

DISCUSSION

Hydatid disease is one of the most transmissible and virulent zoonotic helminth infections worldwide. The disease has had a major health and economic impact on several countries (Raissi et al., 2021). In Saudi Arabia, hydatidosis is of particular concern to sheep farmers (Eckert et al., 2001; Ibrahim, 2010; Toulah et al., 2012; Almalki et al., 2017; Al-Shaibani et al., 2021; Al Malki and Ahmed, 2022), leading to great financial losses resulting from the disposal of diseased organs and a decline in meat production (Singh et al., 2014). Diseased animals also serve as possible sources of transmission to dogs, humans, and other animals (Singh et al., 2014). There is limited information on the rate of *E. granulosus* infection in farm animals in Saudi Arabia. Surveys of abattoirs are a vital data source on the prevalence of hydatidosis in different livestock since reliable disease diagnosis occurs using meat inspection (El-Ghareeb et al., 2017; Almalki et al., 2017). The reported Hydatid disease prevalence ranges from 0.4 to 41.6% in camels, 0.0 to 40.5% in cattle, 0.5 to 30.5% in goats, and 0.1 to 69.6% in sheep (Almalki et al., 2017). Most research has been conducted in the

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western and central regions of the country. The current study is the first to evaluate the prevalence of hydatidosis in sheep, goats, camel, and cattle meat from slaughterhouses in Al-Jouf, a province located in Northern Saudi Arabia. Although many studies indicate the spread of the Hydatid disease in other regions of the Kingdom of Saudi Arabia (Haroun et al., 2008; Ibrahim, 2010; Toulah et al., 2012; Fdaladdin et al., 2013; Hayajneh et al., 2014; Almalki et al., 2017; El-Ghareeb et al., 2017; Al-Hizab et al., 2018; Merst et al., 2023).

A total of 156,754 slaughtered sheep, 36,337 goats, 8,590 camels, and 986 cattle were inspected for cystic hydatidosis from January 2021 to December 2021. The prevalence of the disease was 0.43%, 0.54%, 0.39%, and 0.19% among the inspected sheep, camels, cattle, and goats, respectively, with a collective prevalence of 0.39%. A prior study found that the prevalence rate of Echinococcosis was 0.60%, 0.51%, 0.47%, and 0.38% in cattle, camels, goats, and sheep, respectively, with a total prevalence of 0.42% (Toulah et al., 2017). Unlike the current study, in which the highest disease prevalence was observed in camels, the highest prevalence rate was found in cattle, followed by camels, sheep, and goats. Former investigations conducted in Saudi Arabia and other countries have confirmed that sheep have a higher incidence of Echinococcosis than other animals, including goats and cattle (Ibrahim, 2010; Toulah et al., 2012; Hayajneh et al., 2014; Amer et al., 2018; Joanny et al., 2021).

In the current study, the incidence of cystic hydatidosis among sheep (0.43%) was the lowest among those reported in Saudi Arabia, including 1.06–2.33% in Riyadh (Almalki et al., 2017; Abdel-Baki et al., 2018), 12.61% in Al Baha (Ibrahim, 2010), 69.6% in Jeddah (Toulah et al., 2012), 9.6–13.5% in Al-Taif (Al-Malki and Degheidy, 2013; Hayajneh et al., 2014; Al Malki and Ahmed, 2022), 6.6.% in the Al-Ahsa region (El-Ghareeb et al., 2017), 9.6% in Al-Taif (Al-Malki and Degheidy, 2013), 6.8% in Najran (Almalki et al., 2017), 7.06% in Hail (Hasona et al., 2017) and 2.83% in Dammam (El-Ghareeb et al., 2017). There are several explanations for the differences in disease prevalence in different regions of Saudi Arabia, including different weather (rainfall and temperature) patterns, the timeframe in which the study was conducted, and the abundance of infected host dogs in each area (Hasona et al., 2017).

The prevalence of hydatidosis in camels (0.54%) was similar to that observed in Hail, Saudi Arabia (0.51%, Amer et al., 2018) but lower than that seen in Al Baha (37.5%, Ibrahim, 2010), Al-Madina Al-Munawwarah (34.6, Fdaladdin et al., 2013), Jeddah (41.6%, Toulah et al., 2017), and Egypt 5.4%, 7.4%, and 14.6% (Mousa et al., 2015; Dyab et al., 2018; Ahmed et al., 2021). The higher disease prevalence in camels than in other species may be related to several factors. First, camels may be infected with a strain that has adapted to be avirulent in cattle, goats, and sheep, or these other species may have developed immunity to strains that infect camels (Fdaladdin et al., 2013). Second, areas used to herd camels may have a high number of dogs, the final host responsible for transmitting the disease to camels (the intermediate hosts, Mirzaei and Fooladi, 2012). Third, the prevalence of hydatidosis may relate to camel herder-specific behaviors, including the way they raise and feed their camels and dogs (Mirzaei and Fooladi, 2012).

The prevalence of hydatidosis in goats (0.19%) was similar to that observed in Makkah, in Saudi Arabia(0.23%, Alsulami, 2019) but lower than that seen in Al Baha (9.58%, Ibrahim, 2010), Al-Madina Al-Munawwarah (15.11%, Fdaladdin et al., 2013), Jeddah (30.56%, Toulah et al., 2017) and Iran (14.5%, Daryani et al., 2007). Meanwhile, the prevalence of hydatidosis in cattle (0.51%) was similar to that observed in Egypt (0.64%, El-Alfy et al., 2017) but lower than that seen in Al-Madina Al-Munawwarah (28.7%, Fdaladdin et al., 2013), Al Baha (9.35%, Ibrahim, 2010), Jeddah (31.13%, Toulah et al., 2017), and Hail (2.76%, Amer et al., 2018).

The current study found that sheep and camels had a higher prevalence of hydatidosis, and were thus more likely to be infected than cattle and goats. Thus, it is probable that the prevalence of cystic echinococcosis in northern Saudi Arabia is lower than it is in other regions of the country. However, controlling and treating stray dogs against worms, and cautiously removing polluted viscera continue to be vital to managing the problem (Amer et al., 2018).

The prevalence of hydatidosis in different animal species found at four slaughterhouses in the Al-Jouf Province in Saudi Arabia was highest in the Tabarjal slaughterhouse (1.32%), followed by Al-Qurayat (0.8%), Dumat al-Jandal (0.25%), and Sakaka (0.14%, Table 1). Differences in disease prevalence rates by location may be due to differences in the virulence of parasite strains found in each region as well as cultural and regional variances in dog behavior (McManus, 2006; Haridy et al., 2006; Hayajneh et al., 2014). The variation in infection rates among the four slaughterhouses studied can be attributed to the distinct characteristics of these regions, including their urban, semi-urban, or rural nature. Notably, the Tabarjal region, which is predominantly rural and hosts many farms where many affected dogs reside, recorded the highest infection rates. This finding aligns with previous studies by Almalki et al. (2017) and Abdel-Baki et al. (2018). Torgerson (2006) revealed that infection rates vary by country. This study also showed that infection rates were significantly higher than those identified in the same region during the prior year.

Hydatidosis infection rates changed considerably during the study period, with a higher prevalence in spring for sheep (27.8%) and goats (30.9%) and a higher prevalence in summer for camels (41.3%) and cattle (80%, Table 2). Prevalence infection rates then gradually decreased to their lowest point in autumn for sheep (21.8%) and camels (15.2%), in winter and summer for goats (22.1%), and in spring and autumn for cattle (0%).

The highest prevalence of hydatidosis observed in this study was among sheep in spring, and it was similar to that observed in Al Baha in Saudi Arabia (Ibrahim, 2010) but differed from that seen by Abdel-Baki et al. (2018) in winter,

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Amer et al. (2018) in summer, and Dyab et al. (2018) in autumn. The lowest prevalence of hydatidosis in autumn was similar to that observed by prior studies conducted in Saudi Arabia (Al-Qureishy, 2008) and Egypt (Dyab et al., 2018) but differed from studies conducted in the winter (Ibrahim, 2010), spring (Amer et al., 2018), and summer (Abdel-Baki et al., 2018).

The current study found that hydatidosis infection in goats was highest in the spring, whereas Toulah et al. found that rates were highest in summer (Toulah et al., 2017). The lowest prevalence of infection in goats was observed in winter and summer. This was similar to rates observed by Ibrahim (2010) but differed from other studies that observed the lowest prevalence in spring (Daryani et al., 2007) and autumn (El-Ghareeb et al., 2017) for goat's infection.

The current study found that hydatidosis infection in camels was highest in summer. This differed from other studies that found the highest infection rates in winter (Amer et al., 2018; Ahmed et al., 2021), spring (Ibrahim, 2010), and autumn (Dyab et al., 2018). The lowest prevalence of infection in camels was observed in autumn and was similar to rates reported by Amer et al. (2018) but differed from other studies that observed the lowest prevalence in winter (Toulah et al., 2017), spring (Ahmed et al., 2021), and summer (Ibrahim, 2010). Meanwhile, the current study found that infection in cattle was highest in summer; a similar observation is reported by Daryani et al. (2007). However, Amer et al. (2018) and El-Alfy et al. (2017) reported the highest rates in spring and autumn, respectively. The lowest prevalence of infection in cattle occurred in spring and autumn in the current study, and it was similar to that observed by Daryani et al. (2007).

Seasonal variations in infection may be due to variations in the age of livestock during different seasons (Ibrahim, 2010). Changes in climate variables, including temperature, moisture, and rainfall, also impact the disease spread (Elmajdoub and Rahman, 2015), as does the condition of pastures in different seasons and the density and accessibility of infected final host populations (Ernest et al., 2009; Ibrahim, 2010).

Mitochondrial DNA sequences (mtDNA) are extensively used for molecular categorization and phylogenetic analyses of *E. granulosus*. The *cox1* gene is the most frequently used mtDNA gene for phylogenetic analysis of helminth parasites (Mirbadie et al., 2019; Al Malki and Hussien, 2021). PCR and phylogenetic analysis revealed that among the livestock tested in this study, sheep were the primary host of *E. granulosus* in Saudi Arabia. This aligns with prior studies suggesting that sheep serve as the primary source of hydatidosis transmission (Abdel-Baki et al., 2018; Amer et al., 2018). Additionally, the present study revealed a high sequence similarity between the *cox1* gene of hydatid cyst isolates from sheep and the genotype of *E. granulosus* isolated from sheep in Al-Madinah, referencing sequence (MN720282) (Al-Mutairi et al., 2020).

CONCLUSION

The findings highlight the need for a disease prevention plan to reduce *E. granulosus* infection in studied animals. Based on PCR and phylogenetic analysis of *E. granulosus* in the present study, more inclusive follow-up studies and surveys of abattoirs in different regions of Saudi Arabia are needed to verify the public health importance of hydatidosis. Additionally, it is recommended to improve monitoring of the slaughtering process and implement effective measures for managing stray dogs. Also, efforts should be intensified to control the transmission of cysts from slaughterhouses by properly disposing of the infected offal.

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Availability of data and materials
The data of the current study are available.

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Authors' contribution
Abdulsalam Alkhaldi collected and analyzed the data as well as wrote and revised the manuscript. Authors checked and confirmed the final draft of manuscript before submission to present journal.

Ethical consideration
The author declares that this manuscript is original and is not being considered for publication elsewhere. Other ethical issues, including consent to publish, misconduct, fabrication of data, and redundancy, have been checked by the authors.

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Competing interests

The author declared that they have no conflict of interest.

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Improving the Quality and Nutritional Value of a Mixture of Sago Pith and Indigofera Leaves Fermented with *Rhizopus oligosporus*

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ABSTRACT

The nutritional value of sago pith is limited due to its low protein content, making it less suitable for poultry feed. To increase the benefit values of the sago pith, it is necessary to process it through fermentation. The current study aimed to determine the effects of substrate composition and fermentation time of fermented sago (*Metroxylon sagu*) pith (SP) and *Indigofera* (*Indigofera zollingeriana*) leaves (IL) mixture using *Rhizopus oligosporus* as an inoculum on crude protein, crude fat and crude fiber content of fermented SP and IL, nitrogen retention, crude fiber digestibility, and energy metabolism in broiler chickens. The study was performed on 30 broiler chickens, average weighing ± 1.5 kg at 6 weeks of age, along with SP, IL, and *R. oligosporus*. This experiment was conducted using a randomized design in a 3×3 factorial with three replications. Substrate composition, or factor A, was made up of A1 (80% SP + 20% IL), A2 (60% SP + 40% IL), and A3 (50% SP + 50% IL). Fermentation time as Factor B entailed B1 (2 days), B2 (3 days), and B3 (4 days). The findings demonstrated a significant interaction between the time of fermentation and the composition of the substrate in relation to crude protein content, nitrogen retention, crude fat, crude fiber digestibility, and energy metabolism. It can be concluded that the composition of substrate 50% SP and 50% IL with 3 days of fermentation yielded the best result, with crude protein at 25.45%, nitrogen retention at 59.72%, crude fat at 0.020%, crude fiber at 6.40%, crude fiber digestibility at 57.34%, and metabolic energy at 2658.44 kcal/kg.

Keywords: Broiler chickens, Crude protein, *Indigofera*, Sago pith, *Rhizopus oligosporus*

INTRODUCTION

Sago pith extracted from the inner part of the sago stem after removing the outer skin is a readily accessible and economical source of carbohydrates (Syartiwidya, 2023). Indonesia, in particular, boasts a substantial supply, with sago pith covering 206,150 hectares in 2021 and producing 381,065 tons (Ministry of Agriculture, 2021). Despite its vast potential, only 15-20% of sago is consumed in the human diet. The pith is an abundant by-product in sago processing with great potential as a substitute poultry feed. The nutritional content of sago pith includes 4.45% crude protein, 1.83% crude fat, 8.22% crude fiber, 0.24% calcium, 0.65% phosphorus, and metabolic energy of 2,803 kcal/kg (Fajrona et al., 2023). Therefore, it can be inferred that the nutritional content of sago pith is of low quality because the protein content is too low, limiting its use for poultry feed. To increase the benefit values of the sago pith, it is necessary to process it by fermentation.

Fermentation is a process that can increase the protein content of feed through microorganisms that can convert starch into protein (Mirnawati et al., 2022). Fermented feeds are easier to digest and last longer without losing the nutritional value of the feed (Ciptaan et al., 2022). In the study conducted by Welvidani (2012), sago pith was subjected to fermentation using *Bacillus amyloliquefaciens* supplemented with Zinc (Zn), urea, and sulfur. The combination treatment of Zn (0.0025%), urea (3.0%), and sulfur (0.2%) yielded the best results. This treatment reduced crude fiber content from 18.61% to 12.425%, increased crude fiber digestibility from 29.74% to 53.336%, and increased metabolic energy from 1,777 kcal/kg to 2,525 kcal/kg.

In the current study, *Rhizopus oligosporus* (*R. oligosporus*) was used as the microorganism inoculum, a mold that is widely used in the production of fermented food, such as tempeh. Being saprophytic, it is widely found in nature. Protease, lipase, alpha-amylase, glutaminase, and alpha-galactosidase are all produced by *R. oligosporus* (Han et al.,

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2003). These enzymes play a pivotal role in facilitating the substrate hydrolysis, enhancing its digestibility in the poultry digestive tract. Wattiheluw (2012) used *R. oligosporus* to ferment a mixture of 90% chicken manure and 10% bran. The results showed an increase in crude protein content of 34.43%, a decrease in crude fiber content of 10.99%, and a decrease in crude fat content of 2.71%.

There are several factors to consider in the fermentation process, namely the composition of the substrate and the fermentation time. The nutrient-rich substrate is a growing medium for microorganisms. Availability of nutrients directly influences microbial growth; when ample nutrients are present, microorganisms proliferate accordingly. Moreover, the duration of fermentation plays a pivotal role. The amount of produced enzymes depends on the duration of fermentation. More microbes can proliferate and grow during longer fermentation times (Mirnawati et al., 2019a). More enzymes are produced when microbes grow, allowing more complex nutrients to be broken down into simpler forms and improving the quality and digestibility of the fermented product (Ciptaan et al., 2022).

Since the protein content of sago pith is low, it needs to be mixed with high-protein-content ingredients, such as Indigofera leaves. Indigofera are forages that are quite productive and have a high protein content. According to Sirait et al. (2012), *Indigofera zollingeriana* accounts for an average production of 63.57% of the total fresh yield, indicating its great potential as an alternative feed. Moreover, Indigofera also has relatively high nutritional content, including 21.75% crude protein, 0.19% crude fat, 26.99% crude fiber, 1.32% calcium, and 0.24% phosphorus. In addition, Indigofera leaves also contain carotenoids and xanthophylls that act as antioxidants in broiler rations (Akbarillah et al., 2008). The fermentation of a mixture of sago pith and Indigofera leaves is expected to compensate for the lack of nutritional content in sago pith, leading to the optimal use of this substrate mixture in poultry rations.

The current study aimed to obtain a combination of a mixture of sago pith and Indigofera leaves with the optimal period of fermentation with *R. oligosporus* on the nutritive value of fermented sago pith and Indigofera leaves, nitrogen retention, crude fiber digestibility and metabolic energy in broilers.

MATERIALS AND METHODS

Ethical approval

The broiler-rearing methods used in this study follow the regulation of the Minister of Agriculture of the Republic of Indonesia No. 31/Permentan/OT.140/2/2014 on guidelines for standard broiler and layer poultry farming.

Study materials

The current study was performed using sago pith, Indigofera leaves, *R. oligosporus* inoculant obtained through a mixture of rice bran and cassava leaf meal in a ratio of 9:1 (Annisa et al., 2020). Additionally, distilled water, NaOH, H_2SO_4, indicator methyl orange, Na_2CO_3, acetone, selenium, alcohol, and chemical substances for proximate analysis were employed. The study involved 30 female broiler Cobb 500 strain aged 6 weeks, with an average weight of 1.5 kg, consisting of 27 animals for treatment and 3 animals for correction factor. The sample used was a sago pith from a sago trunk sales company in Padang City, Indonesia. The sago pith was chopped and then sun-dried for 24 - 48 hours. The dried sago pith was then ground into fine granules. Indigofera leaves were obtained from the teaching farm, Faculty of Animal Science, Andalas University, Padang City, Indonesia.

Feed and substrate preparation

The substrate used was sago pith (SP) and Indigofera leaves (IL) in the proportions of 80% SP + 20% IL, 60% SP + 40% IL, and 50% SP + 50% IL. The experimental procedure involved weighing the substrate to 100 grams based on its composition and placing it in polypropylene plastic containers. Subsequently, 70 ml of distilled water was added. The mixture was then sterilized in an autoclave for 15 minutes at 1 atm pressure and 121°C and left to cool down. After the sterile substrate cooled, 0.02 grams of *R. oligosporus* inoculum was added, and it was incubated for 2 days for treatment B1, 3 days for treatment B2, and 4 days for treatment B3. At the end of the incubation period, the substrate was harvested and dried in an oven at 50-60°C until the weight remained constant. Then, the fermented product was analyzed for nutritional value following AOAC (1990) guidelines. Nitrogen retention, crude fiber digestibility, and metabolic energy according to Sibbald (1975) method.

Experimental design

The study was conducted using a completely randomized factorial design with three A factors as substrate composition (A1, A2, A3) × three B factors as fermentation period (B1, B2, B3), and three replications of each treatment. The A1 was 80% sago pith (SP) + 20% Indigofera leaves (IL), A2 was 60% SP + 40% IL, and A3 was 50% SP + 50% IL. Fermentation of the substrate contained B1 (2 days), B2 (3 days), and B3 (4 days).

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Statistical analysis

To determine the effect of treatment, data were statistically processed using analysis of variance (ANOVA) according to Steel and Torrie (1991). Differences between treatments were followed by the Duncan multiple range test. The p-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Table 1 shows that factor A (substrate composition) and factor B (fermentation time) interacted significantly, and each factor could significantly affect the crude protein content ($p < 0.05$). It can be seen that adding Indigofera leaves (IL) at different concentrations (20%, 40%, and 50%) on days 2 and 3 resulted in higher crude protein content. However, on day 4, the crude protein content decreased compared to days 2 and 3. The high crude protein content in A3 and B2 was due to increased mold growth, evident from the total number of colonies (1.4×10^{11} cfu/gr). More mold growth results in a higher protein contribution, supporting the findings of Ciptaan et al. (2022) and Mirnawati et al. (2022), indicating that mold bodies contain protein and an increase in crude protein content is correlated with an increase in mold growth. Enhanced expansion yields single-cell protein products or cell biomass with between 40 and 65% protein (Krishna et al., 2005). Moreover, the crude protein content can increase due to microbes producing enzymes, where the enzyme is a protein. Enzymes are produced in greater quantities as microbes proliferate. During the fermentation process, microbes, as a source of single-cell protein, produce enzymes that are proteins. The amount of crude protein can rise following fermentation. To explain, *R. oligosporus* is a multienzyme organism that includes lipase, protease, a-amylase, glutaminase, and a-galactosidase (Han et al., 2003).

Table 1. The results of the nutritional content of a mixture of sago pith and Indigofera leaves fermented with *Rhizopus oligosporus* included in broiler chicken diet

Parameters	Factor A (Composition substrate)	Factor B (Fermentation time)			Average
		B1 (2 days)	B2 (3 days)	B3 (4 days)	
Crude protein (%)	A1 (80% SP + 20% IL)	14.66 ^{cC}	18.33 ^{aC}	16.10 ^{bC}	16.36
	A2 (60% SP + 40% IL)	18.30 ^{cB}	22.84 ^{aB}	19.46 ^{bB}	20.20
	A3 (50% SP + 50% IL)	22.39 ^{cA}	25.45 ^{aA}	23.98 ^{bA}	23.94
Crude fat (%)	A1 (80% SP + 20% IL)	0.034 ^{aA}	0.031 ^{aA}	0.033 ^{abA}	0.0331
	A2 (60% SP + 40% IL)	0.033 ^{aA}	0.024 ^{bB}	0.030 ^{cB}	0.0291
	A3 (50% SP + 50% IL)	0.030 ^{aB}	0.020 ^{bC}	0.022 ^{bC}	0.0244
Nitrogen retention (%)	A1 (80% SP + 20% IL)	35.77 ^{cC}	41.88 ^{aC}	39.63 ^{bC}	39.63
	A2 (60% SP + 40% IL)	46.31 ^{cB}	54.04 ^{aB}	52.25 ^{bB}	50.87
	A3 (50% SP + 50% IL)	56.42 ^{bA}	59.72 ^{aA}	57.86 ^{cA}	58.00
Crude fiber (%)	A1 (80% SP + 20% IL)	11.56 ^{aA}	9.41 ^{cA}	10.42 ^{bA}	10.46
	A2 (60% SP + 40% IL)	10.47 ^{aB}	7.64 ^{cB}	9.92 ^{bB}	9.35
	A3 (50% SP + 50% IL)	8.74 ^{aC}	6.40 ^{cC}	7.51 ^{bC}	7.55
Crude fiber digestibility (%)	A1 (80% SP + 20% IL)	45.32 ^{cC}	51.84 ^{aC}	47.32 ^{bC}	48.16
	A2 (60% SP + 40% IL)	47.43 ^{cB}	54.84 ^{aB}	48.64 ^{bB}	50.19
	A3 (50% SP + 50% IL)	51.60 ^{cA}	57.34 ^{aA}	55.26 ^{bA}	54.73
Energy metabolism (kcal/kg)	A1 (80% SP + 20% IL)	1455.22 ^{bB}	1829.41 ^{aB}	1537.77 ^{bC}	1607.46
	A2 (60% SP + 40% IL)	1624.89 ^{bB}	1956.51 ^{aB}	1778.97 ^{aB}	1786.79
	A3 (50% SP + 50% IL)	1831.32 ^{cA}	2658.44 ^{aA}	22 54.56 ^{bA}	2248.11

Different lowercases in rows and uppercases in the same column are significantly different ($p < 0.05$). A1: 80% sago pith + 20% Indigofera leaves, A2: 60% sago pith + 40% Indigofera leaves, A3: 50% sago pith + 50% Indigofera leaves. B1: 2 days of fermentation, B2: 3 days of fermentation, B3: 4 days of fermentation.

The longer fermentation period was the reason for the low crude protein content on day 4. Enzymes break down more material during extended fermentation, leading to reduced crude protein content. However, as fermentation duration increases, the nutrients in the media become less available, the microbes eventually die, and the amount of crude protein declines (Agustina et al., 2015). The A3 and B2 group treatments in this study yielded the best crude protein, that is, 25.45% (93% increase). This outcome is greater than that of Annisa et al. (2020), reporting a 28.47% increase in cassava leaves and tofu dregs fermented with *R. oligosporus* for 3 days.

Crude fat content throughout treatments indicated a highly significant interaction between factors A (length of fermentation) and B (substrate composition, $p < 0.01$). Factors A and B both demonstrate a highly significant difference ($p < 0.05$). The results indicated a decrease in crude fat content with increasing additions of Indigofera leaves (20%, 40%, and 50%), attributed to significant mold growth during fermentation, as evidenced by the proliferation of mold colonies (1.4×10^{11} cfu/gr). With increased mold growth, more lipase enzymes were produced, breaking down fat into glycerin and fatty acids. Microbes utilized these fatty acids as an energy source, leading to a reduction in crude fat content post-fermentation. On day 4, the high crude lipid resulted from the prolonged fermentation. Lengthening the fermentation time decreased nutrient availability in the substrate, hindering microbial growth and ultimately resulting in

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increased crude lipid content. In accordance with the present findings, Agustiana et al. (2021) reported that longer fermentation time could decrease nutrient availability in the substrate, leading to a decrease in microbial growth and an increase in fat content.

Fermentation time (factor B) and substrate composition (factor A) had a significantly different interaction ($p < 0.05$) regarding their influence on nitrogen retention ($p < 0.05$) as presented in Table 1. It can be seen that increasing the addition of Indigofera leaves (20%, 40%, and 50%) resulted in higher nitrogen retention. This high nitrogen retention can be attributed to the increased protein availability in the substrate for microbial growth during the 2-3 day period. This phenomenon is facilitated by several enzymes produced by mold that can improve the quality of sago pith protein, and Indigofera leaves after fermentation. This aligns with the findings of Mahfudz et al. (2004) and Mirnawati et al. (2019b), revealing that the ability of microbes to break down proteins into amino acids for easier digestion can increase nitrogen retention. The increased growth of microbes leads to an increase in enzyme activity. The enzyme converts proteins into amino acids, improving the quality of the final product. In addition, the high nitrogen retention is caused by the protein content in the diet. This supports the findings of McDonald et al. (2002) and Ciptaan et al. (2022), indicating that the amount of protein in the ration impacts the quality of nitrogen retention. The nitrogen retention value increases with the ration's higher protein content. The protein quality in the ration was the reason for the low nitrogen retention on day 4. According to Corzo et al. (2005), ration consumption, particularly protein consumption and digestibility, balances nitrogen consumption and ration metabolic energy. These factors also affect the percentage of nitrogen retention.

Factor A (substrate composition) and Factor B (fermentation time) indicated a highly significant difference in their interaction ($p < 0.05$), considering their influence on crude fiber content ($p < 0.05$). It is evident from the above data that the crude fiber content decreased when more Indigofera leaves were added to the substrate. The decrease occurred in A3 and B2, namely with a substrate composition of 50% sago pith and 50% Indigofera leaves. The decrease in crude fiber content was due to the addition of Indigofera leaves, which have a high protein content, and thus satisfy the nutrients in the substrate composition. When the nutrient content of the substrate was available, the microbes grew more (1.4×10^{11} cfu/gr). More cellulase can be generated by accelerating microbial growth. Cellulase can lower the amount of crude fiber in a substrate by breaking it down into glucose to produce energy (Mirnawati et al., 2019b). Similarly, Sudarmono et al. (2016) claimed that when microbes proliferated, more cellulase was generated to convert cellulose into simple sugars, lowering the amount of crude fiber during fermentation. The longer the fermentation time, the lower the crude fiber content observed until day 3. However, on day 4, the crude fiber content was high for all substrate compositions (80% SP + 20% IL, 60% SP + 40% IL, and 50% SP + 50% IL). The extended fermentation period (4 days) in the B3 treatment decreased the substrate's nutrient content and microbial growth, which produced fewer enzymes. This extended fermentation period was the cause of the treatment's high crude fiber content. On day 3, the microbes were still growing well and producing the cellulase enzyme to break down cellulose into glucose, so the crude fiber increased at the end of fermentation. However, by day 4, the microbes decreased, and the cellulase enzyme was no longer produced, so the crude fiber content increased after fermentation. In accordance with the opinion of Fardiaz (1992), fermentation time that exceeds the optimum limit causes a lower availability of nutrients for microbial growth.

For substrate compositions of 80% SP + 20% IL, 60% SP + 40% IL, and 50% SP + 50% IL, the crude fiber content decreased with increasing fermentation time up to day 3 but increased on day 4. The extended 4-day fermentation period reduced substrate nutrient content and microbial growth, resulting in minimal enzyme production and causing high crude fiber content in the B3 treatment. On day 3, the microbes were still growing well and producing the cellulase enzyme to break down cellulose into glucose, so the crude fiber increased at the end of fermentation. However, by day 4, the microbes decreased and the cellulase enzyme was no longer produced, so the crude fiber content increased after fermentation. This aligns with Fardiaz's (1992) observation that fermentation duration beyond the optimum limit could restrict nutrient availability for microbial growth, decreasing microbial growth. The present results indicated that the lowest crude fiber content was found in the A3 and B2 treatment with a substrate composition of 50% SP + 50% IL and a fermentation of 3 days that was 6.40%. This result indicated a lower crude fiber content value, compared to the results of a study by Nensih (2006), where fermentation was performed with a substrate composition of 80% SP + 20% tofu dregs with the mold *Neurospora* sp. The crude fiber content obtained was 7.31%. This difference is due to the different composition and fermentation times.

The interaction between factors B (fermentation time) and A (substrate composition) was significantly different with regard to the crude fiber digestibility parameter ($p < 0.05$; Table 1). Since enough microbes were growing, there was an increase in the digestibility of crude fiber in A3 and B2, specifically with a substrate composition of 50% SP and 50% IL and a fermentation time of 3 days. In other words, adding 50% Indigofera leaves provided sufficient nutrient availability in the substrate for the mold to grow in large numbers (1.4×10^{11} cfu/gr). After fermentation, less crude fiber was left because as microbes multiplied, more cellulase enzymes were created to convert cellulose to glucose. This aligns with the findings of Ciptaan et al. (2022), who observed a negative correlation between crude fiber and digestibility, indicating that higher crude fiber content leads to decreased digestibility. The lower crude fiber digestibility on day 4 was due to the prolonged fermentation time, resulting in high crude fiber content. With reduced microbial

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growth due to extended fermentation, enzyme production was limited, leading to minimal cellulose degradation. Present results indicated the highest crude fiber digestibility in the A3 and B2 treatment with a substrate composition of 50% SP + 50% IL and a fermentation time of 3 days (57.34%).

Regarding energy metabolism, there was a significant between factor A (substrate composition) and factor B (fermentation time, $p < 0.05$). Each factor displayed a highly significant difference. An increase in metabolic energy content could be associated with a decrease in crude fiber content and an increase in the digestibility of crude fiber, resulting in an increased energy level used by the animal's body. The crude fiber in fermented sago pith and Indogefera leaves was converted to glucose by cellulase, and this glucose was counted as an energy source, so it produced metabolic energy in fermentation products. According to Sembiring (2006), it facilitates the conversion of challenging-to-digest components like cellulose and hemicellulose into simpler sugars, thereby increasing metabolic energy. This energy boost results from the substrate's rich nutrient content, supporting robust microbial growth. However, an excessively long fermentation period can hinder microbial growth due to decreased substrate nutrients, leading to a decline in metabolic energy content. Yunus and Zubaidah (2015) state that a shorter fermentation period results in less microbial growth and the production of fewer enzymes, whereas a longer fermentation period causes the microbial population to decline due to the substrate's nutrient depletion. The results of this study showed that after 3 days of fermentation and a substrate composition of 50% SP and 50% IL, the A3 and B2 treatment had the maximum metabolic energy of fermentation of a mixture of SP and IL with *R. oligosporus*, 2658.44 kcal/kg.

CONCLUSION

Based on the current findings, it can be determined that the best results in terms of crude protein (25.45%), crude fat (0.02%), nitrogen retention (59.72%), crude fiber (6.4%), crude fiber digestibility (57.34%), and metabolic energy (2658.44 kcal/kg) could be obtained from fermented sago pith and Indigofera leaves with *R. oligosporus* at substrate composition level of 50 + 50 with fermentation time of 3 days. Additional study is required to determine the effects of fermenting 50% sago pith and 50% Indigofera leaves with *R. oligosporus* on the productivity and product quality of broilers and other poultry as a sustainable alternative feed source.

DECLARATIONS

Availability of data and materials

The study data and materials are accessible by request.

Ethical consideration

Before being published in this journal, all of the authors have reviewed ethical concerns such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct.

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Authors' contribution

The final draft of the manuscript was written and revised with assistance from Ade Djulardi, Lovita Adriani, Mirnawati, and Malik Makmur. Anifah Srifani, Ridho Kurnia, and Gita Ciptaan were involved in designing the manuscript, experimental procedure, and data analysis. Every author confirmed the final revised manuscript.

Competing interests

No conflicts of interest have been disclosed by the authors.

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The Modulation of *In Vitro* Differentiation of Monocyte-derived Macrophage by *Trypanosoma evansi* Antigens in the Dromedary Camel

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ABSTRACT

Studies on the camel immune response to *Trypanosoma (T.) evansi*, the causative agent of Surra, are very limited. In the present study, flow cytometry was employed to investigate the modulatory effects of different *T. evansi* antigens on the *in vitro* differentiation of camel blood monocytes into macrophages. For this, *in vitro*, separated camel monocytes were differentiated into monocyte-derived macrophages (MDM) in the presence or absence (control) of formalin-fixed (inactivated) *T. evansi* whole parasite (*T. evansi* group) or the purified Ro Tat 1.2 antigen (Ro Tat 1.2 group). The analysis of the antimicrobial functions of MDM (phagocytosis and reactive oxygen species (ROS) production) revealed reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens. In addition, a lack of ROS-response was observed in camel MDM generated in the presence of *T. evansi* antigens after stimulation with PMA. These results indicated a compromising effect of *T. evansi* on the innate defense mechanisms in camels. Phenotypic analysis revealed the upregulation of major histocompatibility complex (MHC) class II molecules together with the lower abundance of the scavenger receptor for haptoglobin–hemoglobin complexes (CD163) on MDM generated in the presence of whole *T. evansi* parasites, indicating a polarizing effect of *T. evansi* on the differentiation of camel monocytes into an M1 phenotype. However, the reduced antimicrobial functions of these cells argue against their pro-inflammatory nature. Although both MDM generated in the presence of whole *T. evansi* antigens or their purified Ro Tat 1.2 proteins indicated similar expression levels of CD14 and MHCII molecules, the different abundance of the cell surface molecules CD172a, CD163, CD45, and CD44 indicated different phenotypes of the two MDMs. The results of the present study revealed compromising effects of *T. evansi* antigens on camel macrophages differentiated *in vitro* from blood monocytes. Whether these effects contribute to the *in vivo* pathogenesis of *T. evansi* in camels remains to be determined in future studies.

Keywords: Camel, Flow cytometry, Immunity, Macrophage, Monocyte, *Trypanosoma evansi*

INTRODUCTION

Surra is an arthropod-borne disease caused by *Trypanosoma* (*T.*) *evansi*, and a blood parasite derived from *T. brucei* (Diall et al., 2022; Birhanu et al., 2016) and affects several animal species, including dromedary and Bactrian camels with significant economic losses due to reduced fertility and high mortality rates (Birhanu et al., 2016). *T. evansi*, *T. brucei*, and *T. equiperdum* are currently classified under the subgenus *Trypanozoon* (Desquesnes et al., 2022). Several recent phylogenetic and immunological studies reported high genetic and antigenic similarities between *T. evansi*, *T. equiperdum*, and *T. brucei* (Lai et al., 2008; Carnes et al., 2015; Kocher et al., 2015) suggesting close relationship between the three parasites and leading to the assumption that *T. evansi* and *T. equiperdum* could be considered as subspecies of *T. brucei* (Desquesnes et al., 2022).

Upon leaving the bloodstream, blood monocytes can differentiate into different subsets of macrophages based on the tissue-specific modulatory effects of the microenvironment (Chen et al., 2023). Pro-inflammatory monocyte-derived macrophages (MDM), also known as classically-activated M1 macrophages, are usually differentiated under microbial stimuli like lipopolysaccharide or inflammatory cytokines like interferon-gamma (Muller et al., 2017). On the other hand, anti-inflammatory macrophages, also known as alternatively-activated M2 macrophages, are differentiated under the effect of helminthic antigens, nonsteroidal drugs, or the dominance of T helper 2 cytokines like interleukin (IL) 4 or IL13 (Chen et al., 2023). While M1 macrophages are inflammatory cells with enhanced antimicrobial functions like bacterial phagocytosis and killing capacity and production of inflammatory cytokines, M2 macrophages are anti-inflammatory cells with functions related to wound healing and resolution of inflammation (Eming et al., 2021). The killing of trypanosome is associated with the development of M1 macrophages that produce inflammatory mediators including tumor necrosis factor α (TNF-α), reactive oxygen species (ROS), and nitric oxide (NO; Stijlemans et al.,

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2007). Although these mediators were induced in a *T. evansi* mouse model, there was no correlation between their levels, the control of the parasitemia, and animal survival (Baral et al., 2007).

The development of effective *T. evansi* vaccines requires a better understanding of the pathological and immunological mechanisms involved in Surra. Studies on the immune response of camels to *T. evansi* are still limited to serological detection of specific antibodies in the serum of infected animals (Al-Harrasi et al., 2023; Habeeba et al., 2022), while the role of the cellular immune response has not been investigated so far. The main objective of the present study was, therefore, to investigate the modulatory effects of *T. evansi* antigens on the differentiation of monocytes into monocyte-derived macrophages in the dromedary camel.

MATERIALS AND METHODS

Ethical approval

Animals ethical approval was obtained from the Ethics Committee of King Faisal University, Saudi Arabia, with an approval number (KFU-REC-2021- DEC -EA000326).

The study was conducted in September 2023. Blood samples (5 mL) were collected from four (two male and two female) adult (9, 11, 11, 13 years old) healthy dromedary camels (Camelus dromedarius) reared at the farm of the Camel Research Center of King Faisal University, Al-Ahsa, Saudi Arabia. Blood samples were obtained by venipuncture of the jugular vein (vena jugularis externa) into vacutainer tubes containing EDTA (Becton Dickinson, Heidelberg, Germany).

Separation of mononuclear cells from camel blood

Camel peripheral blood mononuclear cells (PBMCs) were separated from buffy-coat blood by density gradient centrifugation over *Lymphoprep*™ (STEMCELL Technologies, Vancouver, Canada). For this, 5 mL of blood was diluted 1:2 with phosphate-buffered saline (PBS), and the mixture was layered on 5 mL of *Lymphoprep*™ in a 15 mL sterile falcon tube. After centrifugation at 4°C for 30 minutes at 800×g without a break, the PBMC-containing interphase was collected carefully using a 10 mL pipette. After three washes in cold PBS (400×g, 200×g, 100×g for 10 minutes at 4°C), the cells were analyzed for cell purity and vitality (Supplementary Figure 1), counted, and suspended in RPMI culture medium at 2×10^6 cell / mL (Duvel et al., 2014).

Monocyte isolation and *in vitro* differentiation into macrophages

Camel monocytes were separated by allowing camel PBMCs (1×10^6 cells / well) to adhere in 24-well culture plates (Nunc, NY, Rochester, USA) for 24 h at 37◦C and 5% CO_2 in serum-free RPMI 1640 culture media (MOLEQULE-ON, New Lynn, Auckland, New Zealand) followed by the removal of non-adherent lymphocytes by washing with fresh culture medium (Rao Muvva et al., 2019). A freeze-dried suspension of inactivated, purified, and fixed trypanosomes of the Variable Antigen Type (VAT) Rode Trypanozoon antigen type (Ro Tat) 1.2 (OIE-Reference Laboratory for Surra, Institute of Tropical Medicine, Antwerp, Belgium) was reconstituted with 1 mL RPMI medium (4×10^6 parasite / mL). Adherent monocytes were incubated for 4 days at 37°C and 5% CO_2 in culture medium containing whole *T. evansi* parasites (1×10^4 cells) or their purified Ro Tat 1.2 antigen (1 µg/mL) purchased from the OIE-Reference Laboratory for Surra (Institute of Tropical Medicine, Antwerp, Belgium). Day 4 macrophages were detached by incubation in cold PBS containing 5mmol/L EDTA on ice for 30 minutes, followed by several mixings of the well content using the pipette. Harvested cells were counted on the Accuri C6 flow cytometer (BD Biosciences) after the acquisition of 50 µl of the cell suspension (Eger et al., 2016).

Phagocytosis and reactive oxygen species assays

The bacterial phagocytosis by camel MDM was performed by incubating MDM with heat-killed *Staphylococcus aureus* (*S. aureus)* bacteria (Calbiochem, Nottingham, UK) labeled with a FITC labeling kit (Sigma-Aldrich, Missouri, USA) according to the manufacturer instructions (Silva et al., 2021). Camel MDM (1×10^3 cells in 100 µL RPMI medium) were incubated with *S. aureus-FITC* (20 bacteria/cell) for 30 minutes at 37°C and 5% CO2. After washing the plate with RPMI medium (300×g for 3 minutes), the cells were resuspended in 100 µL of PBS and analyzed by flow cytometry (Hussen et al., 2023).

Generation of ROS by MDM was measured in 96-well round-bottom microtiter plates (Corning, NY, USA) as previously described (Hussen et al., 2023). Camel MDMs (1×10^4/100 µL/well) were incubated in 50 µL RPMI culture medium alone or in medium containing 10 ng/mL phorbol-myristate-acetate (PMA; Invivogen Darmstadt, Germany) for 30 minutes (37°C, 5% CO2). After 15 minutes of incubation, dihydrorhodamine (DHR) 123 (Mobitec, Goettingen, Germany) was added to the cells at a final concentration of 750 ng/ml. The cells were washed in RPMI medium and ROS production was analyzed by flow cytometry.

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Phenotypic properties of monocytes-derived macrophages

The expression level of cell surface markers was evaluated by flow cytometry (Elnaggar et al., 2019) after cell labeling with monoclonal antibodies. For this, 1×10^3 MDM were incubated in 96 well plates with monoclonal antibodies to the cell surface molecules CD45, CD44, CD14, CD163, MHCII, and CD172a for 15 min at 4◦C on an ice pack. After two washings (3 min at 300×g) in PBS, secondary FITC-conjugated goat anti-mouse IgG1 and PE-conjugated goat anti-mouse IgG2a were added to the cells for 15 minutes at 4°C on ice in the dark. Finally, the cells were washed with PBS, resuspended in 100 µl PBS, and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed using the Prism software (GraphPad). Means and standard deviation (SD) were calculated using the column statistic function Prism. Differences between means were tested with the One-way analysis of variance (ANOVA) and Bonferroni correction. P values less than 0.05 indicate that significant differences exist between the groups.

RESULTS AND DISCUSSION

The blood parasite *T. evansi*, the causative agent of Surra, is a World Organisation for Animal Health (WOAH) listed pathogen. However, surra is still a neglected disease in terms of research into improved prevention and control strategies (Desquesnes et al., 2022). Especially in camel *T. evansi* infection, studies on the immune response, the host-pathogen interaction mechanisms, and immune evasion strategies of the parasite are very limited (Birhanu et al., 2016; Diall et al., 2022). In the present study, flow cytometry was employed to investigate the modulatory effects of different *T. evansi* antigens on the *in vitro* differentiation of camel blood monocytes into macrophages. For this, *in vitro* differentiation of separated camel monocytes into monocyte-derived macrophages (MDM) was performed in the presence or absence of inactivated *T. evansi* whole parasite or the purified Ro Tat 1.2 antigen (Ro Tat 1.2 antigen is native Variable Surface Glycoproteins of bloodstream-form *T. evansi* trypanosomes).

For the analysis of the phagocytosis activity of MDM generated under *in vitro* stimulation with *T. evansi* antigens, day 4 macrophages were incubated with FITC-labeled *S. aureus* and their phagocytosis activity was analyzed by flow cytometry (Figure 1 A, B, C).

For MDM generated in medium control alone, the percentage of phagocytic cells was 15% of total cells with a mean fluorescence intensity (MFI, indicating the number of bacteria ingested by each cell) of 110182. The percentage of phagocytic cells within MDM generated in the presence of *T. evansi* (11.0% of total cells) or the purified Ro Tat 1.2 antigen (11.5% of total cells) was significantly lower than control MDM ($p < 0.05$). Similarly, the MFI values were significantly lower for *T. evansi* MDM (98718) and the purified Ro Tat 1.2 MDM (95171) compared to the control MDM (Figure 1 D and E, $p < 0.05$).

Stimulation of day-4 MDM generated without any trypanosomal antigens with the phorbol ester phorbol-12-myristate-13-acetate (PMA) induced a significant increase in the MFI signals of the ROS indicator Dehydrorohdamine-123 (DHR-123), indicating ROS generation in these cells ($p < 0.05$). In contrast, MDM generated in the presence of whole *T. evansi* parasites or their purified Ro Tat 1.2 antigen failed to respond to PMA stimulation with no increase in their ROS generation. For MDM without PMA stimulation, the baseline ROS amount was higher in MDM generated in the presence of the purified Ro Tat 1.2 antigen ($p < 0.05$, Figure 2).

Phagocytosis and the production of reactive oxygen species (ROS) are essential antimicrobial functions of macrophages (Fu and Harrison, 2021). Although it has not been proven in animals with Surra infection, the reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens indicates a negative effect of the *T. evansi* parasite on the antimicrobial capacity of camel macrophages (Neaga et al., 2013). This is also supported by the lack of ROS-response in camel MDM generated in the presence of *T. evansi* antigens after stimulation with PMA. These results indicated a compromising effect of *T. evansi* on the innate defense mechanisms in camels, which may pave the way for secondary bacterial infections.

Upon leaving the bloodstream, monocytes can differentiate into different subsets of macrophages based on the local tissue-specific stimuli in their microenvironment (Hussen et al., 2014; Helft et al., 2015). The expression pattern of several cell surface molecules was analyzed to identify the functional phenotype of MDM generated under in vitro stimulation with T. evansi antigens. In comparison to control MDM generated in medium control alone, the presence of the whole *T. evansi* parasites during the *in vitro* differentiation of monocytes into macrophages resulted in MDMs with lower ($p < 0.05$) abundance of the cell surface molecules CD14, CD172a, CD163, CD45, and CD44, while the abundance of MHCII molecules was higher on *T. evansi* MDM ($p < 0.05$; Figure 5A-F). For MDM generated in the presence of the purified Ro Tat 1.2 antigen, only a reduced ($p < 0.05$) abundance of CD14 with enhanced ($p < 0.05$) MHCII expression was observed, compared to control MDM (Figure 3A-F).

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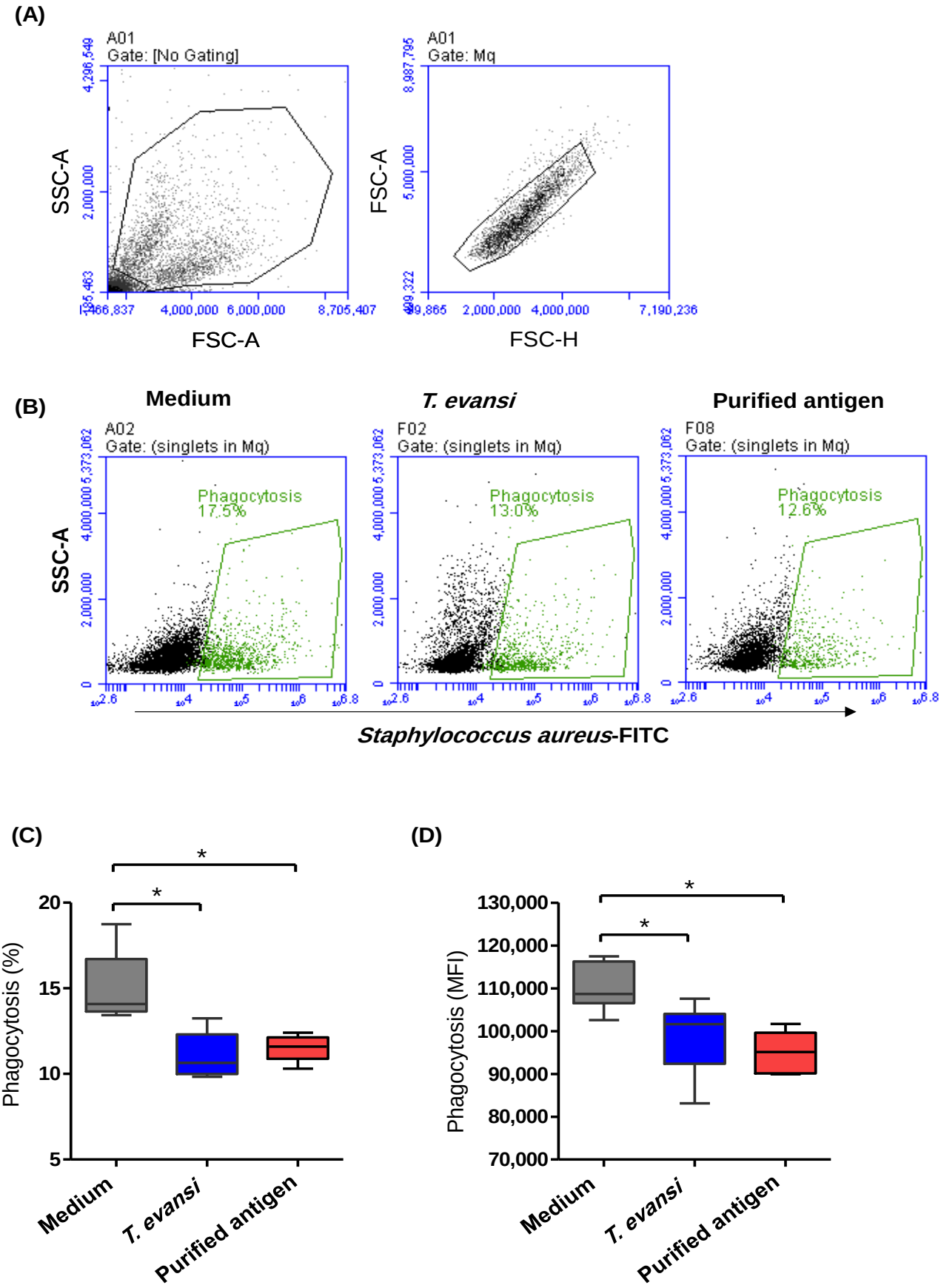

Figure 1. Phagocytosis of *Staphylococcus aureus* by camel monocyte-derived macrophages (MDM). Camel monocytes were allowed to differentiate into macrophages in the presence of whole inactivated *T. evansi* parasites or with purified Ro Tat 1.2 antigen or in culture medium alone for 4 days. **A:** Day-4 macrophages were gated based on their forward scatter (FSC) and side scatter (SSC) properties after the exclusion of cell duplets. **B:** The phagocytosis was analyzed by flow cytometry after incubation of MDM with FITC-labeled *Staphylococcus aureus*. The percentage of phagocytic cells (**C**) and the mean fluorescence intensity (MFI) of phagocytic cells (**D**) were calculated and presented as boxplots. * Indicates significant differences with a p-value less than 0.5.

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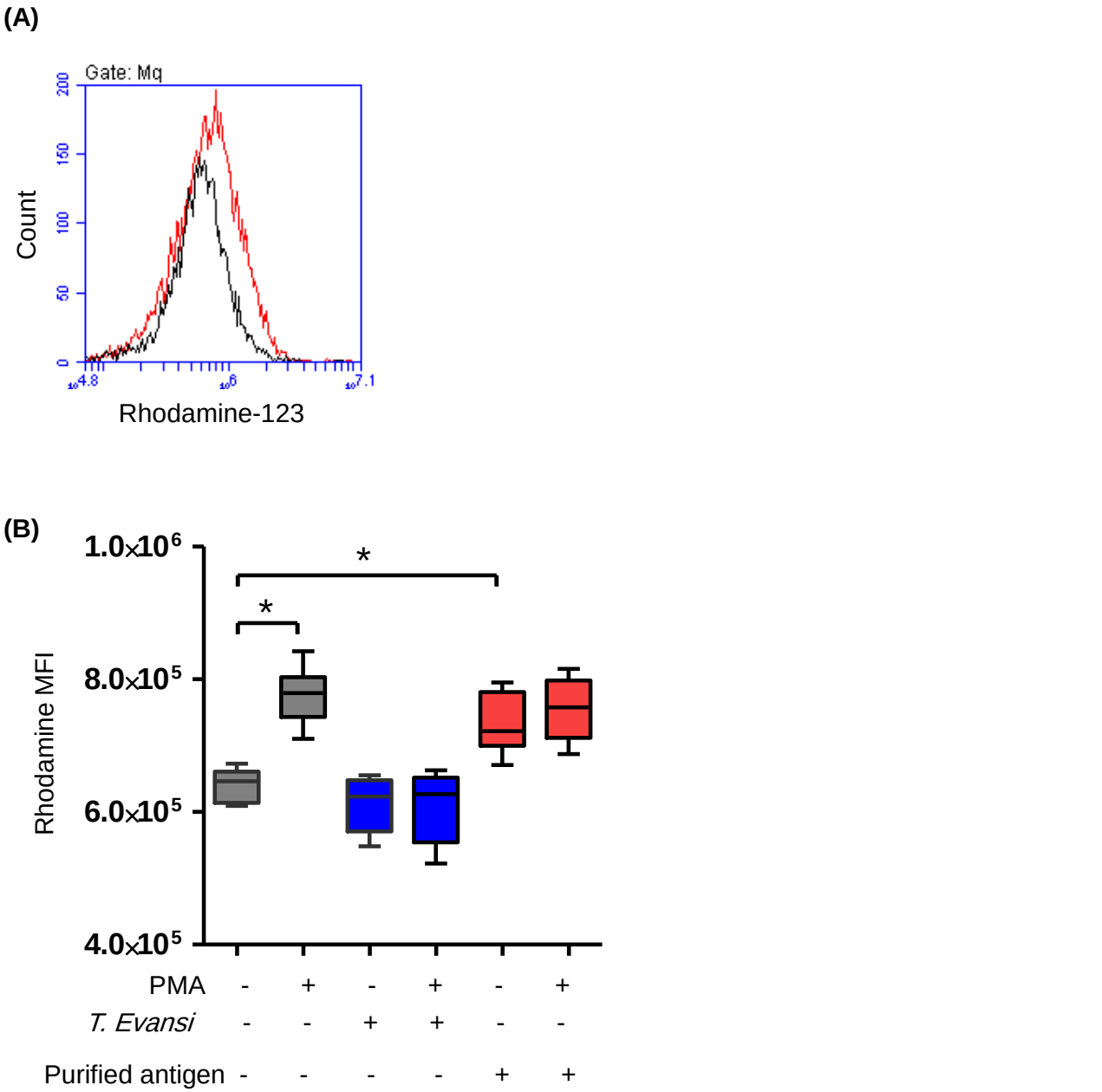

Figure 2. Reactive oxygen species (ROS) generation by monocyte-derived macrophages (MDM). Camel monocytes were allowed to differentiate into macrophages in the presence of whole inactivated *T. evansi* parasites or with purified Ro Tat 1.2 antigen or in culture medium alone for 4 days. **A:** Flow cytometric measurement of ROS production by day-4 macrophages stimulated with PMA (red line) or left without stimulation (black line) was performed after labeling the cells with dehydrorhodamine-123. **B:** Rhodamine-123 MFI values were calculated and presented for stimulated and non-stimulated cells as boxplots. * Indicates significant differences with a p value less than 0.5.

The major histocompatibility complex (MHC) class II molecules and the scavenger receptor for haptoglobin–hemoglobin complexes CD163 are recognized as markers of classically-activated inflammatory M1 MDM and alternatively-activated anti-inflammatory M2 MDM, respectively (Chavez-Galan et al., 2015; Hu et al., 2017). In the present study, the upregulation of MHCII molecules together with the lower abundance of CD163 molecules on MDM generated in the presence of whole *T. evansi* parasites indicates a polarizing effect of *T. evansi* on the differentiation of camel monocytes into an M1 inflammatory phenotype. The reduced antimicrobial functions of these cells, however, argues against their pro-inflammatory nature. The dominance of pro-inflammatory IFN-gamma-induced M1 macrophages has been found essential for the early control of parasitemia in *Trypanosoma*-infected animals (Magez et al., 2020). However, the reduced phagocytosis and ROS production capacities of camel MDM generated in the presence of *T. evansi* parasite may represent an immune escape mechanism that may contribute to the early establishment of the infection. Although both MDM generated in the presence of whole *T. evansi* antigens or their purified RoTat 1.2 proteins showed similar expression levels of CD14 and MHCII molecules, the different abundance of the cell surface molecules CD172a, CD163, CD45, and CD44 indicates different phenotypes of the two MDMs.

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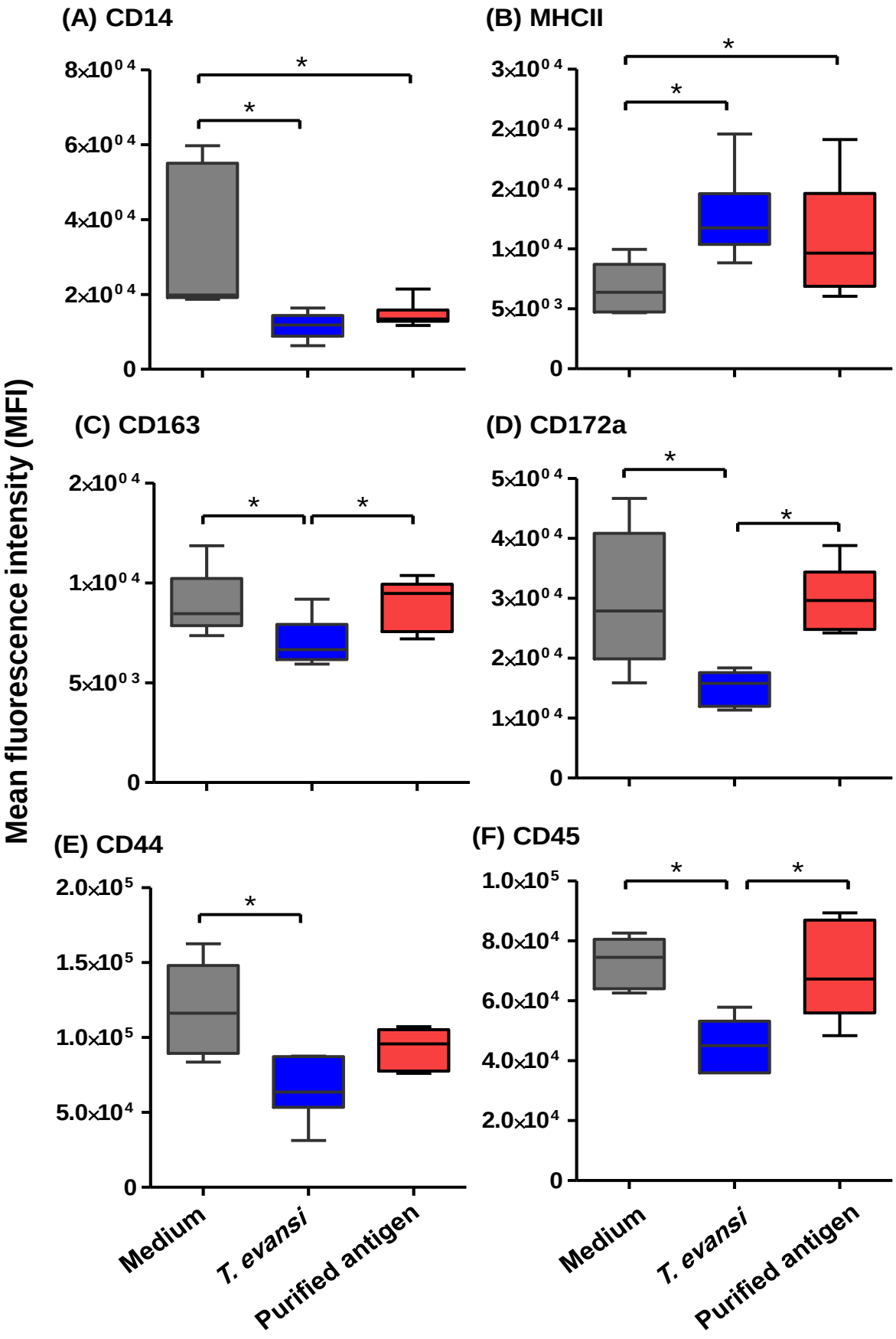

Figure 3. Phenotype of monocyte-derived macrophages (MDM) generated in the presence of whole inactivated *T. evansi* parasites or with purified Ro Tat 1.2 antigen or in culture medium alone. Day-4 macrophages were labeled with mAbs to several cell markers and analyzed by flow cytometry. MFI values indicating the expression levels of CD14 (**A**), MHCII (**B**), CD163 (**C**), CD172a (**D**), CD44 (**E**), and CD45 (**F**) were calculated and presented for the three setups of macrophages. * indicates significant differences with p value less than 0.5.

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CONCLUSION

The results of the present study indicated compromising effects of *T. evansi* antigens on the phenotype and function of camel macrophages differentiated *in vitro* from blood monocytes. The reduced phagocytosis activity of camel MDM generated in the presence of *T. evansi* antigens in addition to the lack of their ROS-response to stimulation with PMA indicates a compromising effect of *T. evansi* on the innate defense mechanisms in camels. Whether this could be related to the modulatory effect of other *T. evansi* antigens that are only present in the whole parasite but not in the purified RoTat 1.2 protein, it needs to be investigated in future studies. Future research may focus on the functional characterization of MDM generated from *T. evansi-infected* camels. For this, the analysis of key M1 cytokines like IFN-gamma or M2 cytokines like IL-10 may uncover the role of these cells during *T. evansi* infection.

DECLARATIONS

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Availability of data and material

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Not applicable

Authors' contribution

Essa Ali Alhilal did sample collection and manuscript revision; Mayyadah Abdullah Alkuwayti did supervision and manuscript preparation; Noof Abdulrahman Alrabiah did manuscript preparation and revision. Omar Al-Jabr did manuscript preparation and revision; Jamal Hussen did Analysis, funding acquisition, and writing of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

There is no competing interests to declare.

Ethical consideration

Ethical issues have been checked by all the authors.

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Microfeed Incorporated with Probiotic for Aquaculture: A Review

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ABSTRACT

Ensuring the availability of high-quality larvae in sufficient quantities remains a significant bottleneck for the grow-out phase of aquaculture. Over the past century, various alternative dietary solutions for larval stages have been explored, encompassing bacteria, microalgal pastes, yeasts, and various inert microparticles, though with inconsistent outcomes. This review aimed to discuss the innovative integration of probiotics into microfeeds, highlighting encapsulation, coating, and fermentation techniques to propel aquaculture productivity. Microfeeds, which are often nutrient-rich and easily assimilated in powdered or liquid form, play a crucial role in larval fish nutrition. These can be classified into microencapsulated, dry, liquid, and live feeds. The choice of microfeed is pivotal, ensuring appeal, digestibility, and water stability tailored to each larval stage. As probiotics gain popularity in aquaculture for their potential to enhance growth, bolster disease resistance, and improve water quality, their administration methods have diversified. The probiotics can be administered through direct immersion and bath treatments to biofloc systems and feed additives. The results indicated that microfeed incorporated with probiotics showed a positive result impact on the aquaculture industry.

Keywords: Alternative diets, Aquaculture, Microfeed, Probiotics

INTRODUCTION

The aquaculture sector is in a solid foundation to address the escalating need for protein, a demand fuelled by the upward trajectory of user incomes, evolving lifestyles, and the expanding population. However, challenges in the aquaculture industry (such as regulatory framework, water quality management, and food production) of larval fish rearing limit the supply of sufficient numbers and high-quality larvae for grow-out production (Vadstein et al., 2018).

Feed is the most crucial yet often underutilized aspect of fish production, constituting over half of the expenses due to its high manufacturing costs and extra care (Iliyasu et al., 2016). The cost of producing live feed accounts for between 20% and 50% of a hatchery's overall operating expenditures. To find alternate or supplemental meals that might successfully reduce the dependency on live feeds for raising postlarvae, ongoing research remains active in this field (Nagappan et al., 2021). Over the past century, there has been much research on the usefulness of many alternative diets as food for larval stages, including bacteria, microalgal pastes, yeasts, and particular types of inert microparticles, with differing degrees of success (Albentosa et al., 2002; Enes et al., 2003; Ponis et al., 2003; Espinosa et al., 2006).

Micro-encapsulation is a method of enclosing liquids and particle food components within a finely designed wall (Nagappan et al., 2021). The biological system of the target fish or prawn larvae absorbs the internal nutrients in active locations. In the fish or prawn larval body, the wall or shell disintegrates by bacterial action, enzymatic action, pH shift, or rupture (Langdon, 2003). The capsule's wall could be made of a bio-degradable polymer, such as modified gelatine, with the nutrients inside being released by the animal's enzymatic processes or microflora in its gut. Capsules of the microfeed with a diameter of less than 20 microns have been reported, giving the culturist a wide variety of sizes of nutritionally diverse capsules to meet the different growth phases of the fish species (Vadstein et al., 2018).

Probiotics or beneficial microorganisms are believed to improve the fish immune system, especially in stressful environments, by influencing the gut colonization of probiotic bacterial strains and the generation of antibodies, acid phosphatase, lysozyme, and antimicrobial peptides (Salminen et al., 1999; Taoka et al., 2006; Panigrahi, 2007; Mohapathra et al., 2012a). Probiotics have direct immunostimulant properties, which can improve disease resistance, reduce stress response, and enhance gastrointestinal morphology (Ige, 2013). Benefits to fish producers and consumers include increased fish appetite, growth performance, feed utilization, carcass quality, meat quality, and fewer deformities

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(Ige, 2013). This review discussed the integration of microfeeds and probiotics, a practice increasingly adopted in the aquaculture industry to enhance growth index and health parameters.

MICROFEED

Microfeed is defined as a specific kind of feed designed to suit the particular nutritional needs (protein and lipid) of larval fish throughout their developing phases (Hamre et al., 2013). The type of fish being cultured and the particular nutritional needs of the larvae at various stages of development will determine the type of microfeed needed (Langdon, 2003). The nutritional needs of larvae during development would help enhance the quality of larvae and juveniles by optimizing diets and feeding procedures (Hamre et al., 2013). In 2017, fish and seafood intake accounted for 17% of protein altogether obtained from animals, and this fraction has been growing continuously (FAO, 2020).

Microfeeds for fish larvae commonly consist of very nutrient-dense, readily assimilated powdered or liquid diets (Langdon, 2003). Rotifers, artemia, copepods, and other kinds of phytoplankton are a few typical microfeeds for fish larvae (Dhont et al., 2013). For early-stage larvae, dry feeds like microparticles and powders are commonly used (Powell et al., 2017). Mid-stage larvae are usually fed with liquid feeds like emulsions and suspensions (Hua et al., 2019). For late-stage larvae, live feeds like algae and zooplankton are widely used (Murugesan et al., 2010).

The term "microencapsulated feed" refers to the sort of feed that has been specially formulated for the tiny mouths of fish larvae. Microencapsulated feeds offer a rich blend of easily digestible proteins, fats (lipids), and carbohydrates, complemented by vital minerals, vitamins, and micronutrients. Typically ranging from 50 to 500 mm in diameter, these feeds are sized optimally to ensure efficient consumption and digestion by larvae (Kolkovski, 2008). The feed components are encapsulated under a protective covering throughout production to increase stability and avoid nutrient loss (Temiz et al., 2018). This coating is often made of gelatine or other polymers (nylon and aramide, Temiz et al., 2018).

Dry feeds come in the form of microparticles and powders, designed especially in the early stages of larvae development (Jafari et al., 2008). For freshwater species like tilapia or catfish, the dry feeds can range from around 50 to 200 microns in size, while for marine fish larvae, which are often smaller than their freshwater counterparts, the dry feed size ranges from 20 to 100 microns in size, depending on the specific species, developmental stage and mouth size of the fish (Portella et al., 2008). Microparticles and powders (dry feed) for fish larvae can be fed directly into the larval rearing tanks or mixed into a solution to guarantee optimal distribution and consumption. To meet the larvae's particular dietary needs, they consist of a well-balanced blend of proteins, lipids, carbs, vitamins, minerals, and other vital components. Dry feeds may be produced using various methods, including grinding, milling, extrusion, and spray drying (Jobling et al., 2001). The final product's required physical qualities, target particle size, feed components, and processing technique are among the crucial considerations (Sørensen, 2012).

Liquid feed is another form of microfeed widely used for feeding mid-stage larvae in various applications, including aquaculture and insect rearing (Hua et al., 2019). In larval rearing, the emulsions technique is frequently produced by mixing lipid-based oils (fish oil, vegetable oil) with water-based solutions, including nutrients and other ingredients, compared to the suspensions technique that needs dispersing finely ground or micronized solid feed materials (fishmeal, microalgae, yeast) in water or another liquid media. Microalgae, which include *Chlorella* and *Nannochloropsis*, are small photosynthetic organisms that provide fish larvae with a suitable liquid diet. Large amounts of microalgae could possibly grow in specialized cultures. When compared to dry feeds, liquid feed is a more readily digestible source of nutrition, ensuring effective nutrient absorption.

Live feed is considered a microfeed for fish larvae. It includes algae and zooplankton that are commonly used as food sources for late-stage larvae in aquaculture and marine hatcheries (Das et al., 2007; Abbas et al., 2015). Omega-3 fatty acids, which are necessary for the proper growth of marine species, can be found in some microalgae (Adarme-Vega et al., 2012). Microalgae not only offer a natural food that closely reflects the creatures present in the larvae's natural habitat (Mau et al., 2017), but it can also be maintained at different growth stages to fit the nutritional requirements of varied larval species. Another live feed source for late-stage larvae is zooplankton, which are tiny floating organisms commonly found in aquatic ecosystems (Dumont et al., 2013). Depending on the particular needs of the larvae being raised, zooplankton consists of a variety of species, such as copepods, rotifers, and brine shrimp (Das et al., 2012). As the primary food supply for most marine species in the larval stages, they (algae and zooplankton) play an important part in the marine food web to ensure that the larvae receive a nutritionally balanced diet (Rasdi et al., 2020a; Rasdi et al., 2020b).

SELECTION OF MICROFEED

There are some essential elements for selecting a microfeed for fish larvae. Depending on the type of fish being cultured, the particle size might change. Feed particle size varies depending on the species, age, and feeding behavior of the fish,

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as well as the aquaculture operation's output goals productions (Jobling et al., 2001). To ensure that fish larvae can effortlessly consume the feed particles, the particles need to be suitable for their small mouth.

To promote their growth (fish larvae), immune system, and general health, the microfeed should include high-quality proteins, necessary amino acids, vitamins, minerals, and fatty acids (Arney et al., 2015). The FAO reported that carnivorous species had dietary protein needs of 40–55 percent, whereas freshwater omnivore and herbivorous species have levels of 30–40 percent (FAO, 2010). For example, juvenile tilapia may need up to 40% protein daily for good growth (Luz et al., 2012). Lipids, contributing between 10% and 20%, provide the larvae with vital energy essential for their overall growth and optimal organ function (Lee et al., 2002). The development of the neurological system and visual abilities in fish larvae depends on omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Pilecky et al., 2021a). A potential strategy for improving dietary protein availability in compound feeds would be to prevent leaching by efficient encapsulation of feed particles that are supplemented with high levels of free amino acids (FAA), peptides, and soluble protein to mimic live feeds (Tonheim et al., 2007).

The term "digestibility" pertains to the extent to which something can be assimilated and broken down by the digestive system of fish (Tonheim et al., 2007). The higher digestibility indicates that more nutrients are accessible for the fish's growth and development (Tonheim et al., 2007). Fish larvae are categorized according to whether they have stomachs; a gastric fish lacks a stomach even as an adult (Rønnestad et al., 2013). The solubility of proteins, the source and level of lipids, the manufacturing processes used to make the feed, the presence of anti-nutritional factors that can reduce digestibility, and the feed's particle size each have an influence on the feed's digestibility for fish larvae.

Due to their frequent feeding demands and relatively high metabolic rates, fish larvae must be fed often (at least twice per day). The number of larvae per unit volume of water or the amount of food transferred per unit area or volume are the two common ways to represent the feeding density (Blancheton, 2000). The number of larvae per litter or cubic meter of water is generally utilized to represent the appropriate feeding density for fish larvae. To maintain the larvae's proper growth and survival, it is compulsory to develop an ideal feeding environment as well as temperature (warm water temperature), which affects metabolism with respect to protein and energy (Luz et al., 2012). Microfeeds that remain stable in water for an extended length of time are selected to give an assurance that the feed is accessible to fish larvae while not disintegrating or polluting the water excessively (Jobling et al., 2010). While the microfeed must be stable in water, it must also possess the right solubility properties. Dissolved nutrients from the microfeed can help the water become more nutrient-loaded, which might cause excessive ammonia levels, oxygen depletion, or algal blooms (NOAA, 2023). Less frequent feeding intervals are possible if the feed stays stable for a longer period of time, which reduces the labour and operational expenses linked to regular feed replenishment (Vivas et al., 2006).

Biosecurity is a vital part of fish larval rearing, including the feed used in the process (Kumar et al., 2022). One of the processes that may be employed in the creation of microfeed is to implement quarantine measures for a freshly obtained feed before introducing it into the larval rearing system. In order to maintain its quality and prevent contamination, fish larval feed must be handled and stored properly, for example, by selecting feed producers who follow good manufacturing procedures (GMP) and have strict quality control procedures to maintain the quality of production for the microfeed (Padilla, 2009).

FORMULATION OF MICROFEED

The properties of the feed should be appealing, stable in the water for a long period, and continued moving until the first feeding larvae can catch it. The standard diet size spans from 5 to 300 µm, however, the diet's particle size and specific gravity depend on the species being produced and its embryonic stage (Amirkolaie et al., 2006). Due to its increasing cost and erratic availability, fish meal must be replaced in fish feed with less expensive materials of plant origin (Bhosale et al., 2010). The basic ingredients processing the microfeed consist of soybean meal, milk powder, egg, corn flour, cod liver oil, agar powder, and vitamin mixture (vitamin B complex and vitamin E). The nutritional value for each of the mentioned ingredients is beyond doubt as it is far-reaching among manufacturers. To maximize the development and preservation of the larvae, the microfeed composition and feeding regimens must be optimized for each type of fish larvae. 4% vitamin premix is suggested to maintain ideal feed solubility and the best possible feed quality (Mau et al., 2017).

It is hard to formulate a compound diet suitable for fish larvae because standard nutritional techniques cannot be used to estimate the nutritional requirements of fish larvae and because the current available commercially prepared diets do not sustain larval development (Cahu et al., 2001). Improving comprehension of the digestive processes in larvae has resulted in suggested food compositions that satisfy their nutritional needs.

Alternative feed component formulations usually fulfill the nutritional needs of a specific species while staying economically competitive and efficient (Sørensen, 2012). Low molecular weight, water-soluble chemicals can be delivered via lipid coatings with little leaching into the surrounding water (Langdon, 2003). Recent research has found that larvae have distinct dietary needs (Cahu et al., 2001). However, sufficient proteins must be provided to maintain

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optimum growth because development is primarily protein deposition. The use of a ready-made diet allowed researchers to look into larvae's protein requirements, collect the most definitive data on lipid requirements, and examine glucidic and vitaminic nutrition.

Feed attractants should be included to induce a feeding response (Kolkovski et al., 2009). Physical and chemical characteristics, such as color, shape, size, movement, and smell stimulation at the molecular level, primarily influence the seeking, identification (of feed attractants), and ingesting processes. Some production of feeds fortified with astaxanthin, a carotenoid pigment, enhances the development of red coloration in the fillet of farmed charr in the weeks prior to harvest (Jobling et al., 2010). Using sustainable sources or naturally grown macroalgae in powder or meal form is a low-cost alternative to integrating feed attractants and stimulants (Kolkovski et al., 2009).

ADVANTAGES OF MICROFEED

The nutritionist puts forth considerable effort to reduce feed costs as part of an environmentally friendly aquaculture strategy. The reason is that they can spare protein and thus increase the cost-effectiveness of the diet, feedstuffs with relatively high quantities of carbohydrates are favored when formulating fish feed (Bhosale et al., 2010). The lipidic component of live prey significantly impacts the performance of larvae because it is more changeable and manageable. As a result, lipids and the essential fatty acids of fish larvae have been given more emphasis (Izquierdo et al., 2000).

The digestibility of a particular feed ingredient reflects the fish growth. The choice of dietary protein to be used in practical rations is an economic decision, which depends on the protein source as well as on the expected returns from fish growth and value (Bhosale et al., 2010). To obtain the best growth and development of fish larvae, DHA, EPA, and ARA should also be included in their (fish larvae) diet (Mejri et al., 2021). According to several studies, these fatty acids (DHA, EPA, and ARA) serve as a precursor for certain prostaglandins and other biologically active substances that control growth (de Mello et al., 2022). Therefore, protein and fatty acids contribute to satisfactory larvae fish growth.

Early-stage aquatic species have small mouths and limited feeding abilities, making it difficult for them to swallow normal feed particles. For their quick development and growth, it often contains high concentrations of proteins, and lipids, along with essential nutrients like vitamins and minerals (Siddik et al., 2023). Microfeed can assist optimum growth rates and general development by providing adequate nutrition in an accessible approach. Therefore, microfeeding is essential for their effective growth. In order to ensure that larvae and fry receive a sufficient amount of nutrients, microfeed is made to be small enough so that it can be easily digested. Besides, it will also increase the survival rates of the fish larvae indirectly as it has the right amount of feed consumed (Sardi et al., 2023)

Microfeed, which has been processed or ground to a smaller size than regular feed particles, has less of an impact on the environment (such as biodiversity loss and resource depletion). By giving animals small amounts of food on a regular schedule, microfeeding reduces feed wastage. It generates less trash and suspended particles, which can enhance water quality and reduce the danger of nutrient imbalances and decreased oxygen levels. For aquaculture systems to be healthy and productive, good water quality (pH from 6 to 9) must be maintained. It can enhance the overall economic effectiveness of animal production systems by reducing feed waste (Henriksson et al., 2021).

Besides, microfeeding has an improving effect on animal (fish larvae) welfare and wellness. Early-stage aquatic organisms fed on microfeed are more resistant to stressors and diseases because they deliver sufficient nutrition and sustain optimal health (Siddik et al., 2023). The particle size of microfeed also helps in lowering the possibility of feed loss, which can result in problems with the water supply and disease outbreaks in intensive culture systems (Siddik et al., 2023). It also contributes to a healthier gut microbiota and lowers the risk of digestive diseases by supplying an ongoing supply of nutrients, which helps maintain a stable gastrointestinal environment. Additionally, by fulfilling an animal's natural needs, regular feedings can reduce stress and improve performance (Siddik et al., 2023).

APPLICATION OF PROBIOTICS IN AQUACULTURE

In aquaculture, the application of probiotics has become increasingly prevalent (Table 1). These beneficial microorganisms serve as a powerful tool to protect aquatic organisms from diseases, foster growth, and enhance their immune capabilities, thus contributing significantly to the overall health and productivity of aquatic ecosystems. Generally, probiotics refer to the application of beneficial microorganisms as supplements for feed or water to improve the health and growth performance of aquatic organisms, such as fish, shrimp, and shellfish, in aquaculture systems. The most widely utilized probiotics are bacteria (such as *Bacillus*, *Lactobacillus*, and *Enterococcus* species) and yeast (such as *Saccharomyces cerevisiae*, Kurniawati et al., 2021). For instance, in carps, probiotics such as *Lactobacillus* spp., *Bacillus* spp., *Saccharomyces cerevisiae*, and *Lactococcus* spp. are among the most common species that are currently being utilized (Feng et al., 2019). Probiotics offer several potential benefits in aquaculture, including enhancement of digestion and nutrient absorption (Samat et al., 2021).

Probiotics can be administered to aquatic life in a variety of methods, including as feeding additives. Usually, probiotics are added to the meal in liquid or dry form. It can be included in the formulation of commercial aquafeed or

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prepared as a supplement to be manually added to the feed during the manufacturing process. To achieve optimum efficiency without having negative side effects, the amount of probiotics administered to the fish diet should be calculated based on the specific requirements of the aquatic organisms. In a study on keureling, *Tor tambra* (*Cyprinidae*) fish fry, the amount of probiotics that should be consumed is determined to be 10 ml kg^{-1} of feed (Muchlisin et al., 2017). Nevertheless, it is vital to comprehend that the recommended dose of probiotics for different species might differ (Muchlisin et al., 2017).

Another way to administer probiotics to aquatic organisms is by direct immersion in water containing probiotics. A concentrated solution of probiotics is prepared, typically by culturing the desired probiotic strains in a suitable medium range from a few minutes to several hours, depending on the species, size, and health condition of the aquatic organisms. The frequency of direct immersion might be a one-time treatment or repeated at regular intervals, such as daily or weekly, to maintain a consistent presence of beneficial microorganisms. A study found that submerging larvae of Nile tilapia in probiotic (*Lactobacillus plantarum)* led to considerably greater development and survival rates than the control group (Sherif et al., 2020).

Bath treatment is one of the applications of probiotic. The aquatic organisms are placed in a bath containing a concentrated solution of probiotics. The organisms come into direct contact with the probiotic solution, allowing the beneficial microorganisms to colonize their external surfaces and provide potential benefits, especially in the early stages of larval development, such as *Lactobacillus acidophilus* on the growth and survival of zebrafish (*Danio rerio*) larvae. Depending on the intended application, the particular probiotic strains utilized, and the tolerance of the aquatic organisms, bath treatments can last from 1 to 4 hours. It improves disease resistance and fish growth (Song-Lin et al., 2012). Wang et al. (2020) also stated that *Lactobacillus acidophilus* bath treatment had a positive effect on growth performance, digestive enzyme activity, and disease resistance in juvenile grass carp that persisted for at least 4 weeks after the treatment.

Biofloc systems are defined as a form of aquaculture that uses microbial communities by converting waste into protein-rich food for fish (Crab et al., 2012). Probiotics in biofloc systems can support effective nutrient cycling and limit the spread of harmful microorganisms. Enzymes and other substances produced by the biofloc system will help break down feed elements and increase the bioavailability of nutrients (Kumar et al.,2021). A study on the evaluation of the biofloctechnology system showed improvement in mean final weights, specific growth rates, feed conversion ratios, and total biomass in Nile tilapia (Mohammadi et al., 2020). Recently, He et al. (2023) conducted a study showing that the addition of *Bacillus* (*B.*) *subtilis* to biofloc systems enhanced the water quality, growth performance, and immune enzymes of *Litopenaeus vannamei*.

PROBIOTICS MODE OF ACTION

The mode of action or mechanisms of probiotics refers to the specific ways these live microorganisms exert good impacts on the host's health. It is crucial the particular mechanisms of probiotics can vary depending on the strains used and the individual's initial characteristics (Plaza-Diaz et al., 2019).

In the first place, probiotics compete with dangerous microbes for resources and space, assisting in retaining microbial communities in the gut. Physical barriers that stop dangerous bacteria from adhering to the intestinal wall are created by probiotics' adhesion to the intestinal lining and occupation of surface regions. By occupying this area, fewer dangerous bacteria have a chance to grow and establish a presence (Amara et al., 2015).

Besides, short-chain fatty acids and other metabolites that promote gut health and general well-being are created when probiotics ferment food fibers. During the fermentation process, probiotics break down these dietary fibers into various by-products, among the most essential being short-chain fatty acids (SCFAs). Probiotics can produce a variety of additional metabolites from the fermentation of dietary fibers in addition to SCFAs, including bioactive substances with potential health advantages and gases like carbon dioxide and hydrogen (Bamigbade et al., 2022).

Some probiotics are thought to influence the metabolism of certain nutrients, such as B vitamins and fatty acids. They may also aid in the absorption of minerals like calcium. It has been shown that some probiotic bacteria may ferment food substrates to create B vitamins, including folate, riboflavin (B2), and cobalamin (B12, Kaprasob et al., 2018). These vitamins are crucial for several metabolic activities, such as DNA synthesis, cell development, and energy generation (Indira et al., 2019).

To conclude, some probiotic strains have been proven to aid in the detoxification of toxic substances, possibly lowering the chance of contracting specific illnesses. Antioxidant qualities in some probiotics may help fight off damaging free radicals and lessen oxidative stress. Heavy metals may be chelated or bound to by certain probiotics, aiding in the removal of these potentially hazardous chemicals from the body (Chen et al., 2022).

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Table 1. The application of probiotics incorporated with microfeed using different methods toward different fish (larvae) species

Fish species	Probiotics	Technique	Outcomes	References
Nile tilapia (*Oreochromis* niloticus)	*Bacillus subtilis* and *Lactobacillus plantarum* (10^7 CFU/g respectively)	Fermentation (*Saccharomyces cerevisiae*)	higher amylase activity than the fish-fed control diet	Essa et al. (2010)
Gilthead sea bream (*Sparus aurata)*	*Bacillus subtilis, B. licheniformis and B. cereus* (solid form, 10 ppm per m^3)	Direct addition	Specific alkaline and acid protease activity was greatly boosted (for improved growth parameters and nutrient conditions).	Nihan et al. (2013)
European sea bass (*Dicentrarchus labrax L.)*	Lactic acid bacteria (*Pediococcus acidilactici*) (3.0g per kg, twice a day)	Direct addition	increases fish survivability and blood chemistry	Eissa et al. (2022)
Pike-perch larvae	*Lb. paracasei* subsp. *paracasei* BGHN14 (1.3g saline × volume and glycerol at 0.3 × volume per wet pellet weight)	Coating	supports skeleton development and improves fish growth.	Ljubobratović et al. (2021)
Nile tilapia (*Oreochromis niloticus)*	*Bacillus amyloliquefaciens* (10^6 CFU/ml) and *Bacillus subtilis* (10^6 CFU/ml)	Microencapsulated	enhances fish health and zootechnical aspects by showing how probiotics function as an immunomodulator.	de Moraes et al. (2022)
Seabream (*Sparus aurata)*	*Bacillus subtilis* (10^7cell/g)	Microencapsulated	improves the survival rates and length gain	El-Dakar et al. (2020)

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PROBIOTIC IN MICROFEED

Probiotic feed in aquaculture is an alternate protein source with a highly digestible protein and energy content as well as a healthy amino acid profile (Gomes et al., 2009; Nayak, 2010). A number of investigations have been conducted to evaluate the nutritional content of various foods (Gomes et al., 2009; Nayak, 2010). The incorporation of probiotics increases hemopoesis and produces nonspecific immunity in fish, according to the latest studies (Marzouk et al., 2008; Lazado et al., 2014). The lack of understanding of larval feeding contributes to the inability of microfeed development.

Foremost, probiotics can be incorporated into microfeed by direct addition. The probiotics are usually in the form of freeze-dried or lyophilized bacteria, which are mixed with the other ingredients of the microfeed before it is given to the aquatic organisms (Jalali et al., 2012). The type and amount of probiotics added must be examined to reduce the risk of probiotic degradation during processing or storage, which causes the probiotics to not survive the digestive process and limits their effectiveness. For instance, the microfeed is made by adding the probiotic bacteria containing 10^6 colony-forming units (CFU) of *B. subtilis* per gram of feed to the feed of juvenile Nile tilapia. It is proven that the microfeed incorporated with probiotics improved their growth performance, feed utilization, and immune response (Chen et al., 2014).

Another method is encapsulation allows disease-control drugs, such as antibiotics or probiotics, to be delivered to filter-feeding bivalves, improving disease management while reducing expenses, environmental harm, and threats to human health (Luzardo-Alvarez et al., 2010). It will create a capsule by enclosing the probiotics' core elements inside a wall material. In the process known as microencapsulation, the probiotics can be shielded from damaging environmental factors while being processed, stored, and transported through the gastrointestinal system (Rajam et al., 2022). Spray drying, lyophilization, emulsion, and extrusion are techniques used for encapsulation (Rajam et al., 2022). Spray drying is widely used in the aquaculture industry. It is cost-effective as it involves atomizing a solution containing the probiotics into a stream of hot air or nitrogen gas, forming small droplets that dry to form powder particles (Vivek et al., 2023).

Another approach is to coat the surface of the microfeed particles with probiotics. Although simple and cost-effective, the probiotics are not protected from harsh environmental conditions during digestion, which reduces their survival rate and effectiveness. Bahrami et al. (2023) proved the effectiveness of microencapsulated *Lactobacillus acidophilus* using an alginate/starch coating in Nile tilapia fry. The alginate-chitosan-probiotic-inulin layer is then applied to the microfeed particles after they have been submerged in this solution (Bahrami et al., 2023). This technique ensures that the probiotics can reach into the intestine, where they can exert their beneficial impacts while also helping to protect them from severe conditions (gut failure) of the gut (Bahrami et al., 2023).

Lastly, there is a fermentation technique process by utilizing microbial fermentation to produce a mixture of probiotics and microfeed. The probiotics are added to the feed, and the microorganisms break down the feed components and produce metabolites, which enhance the nutritional value of the feed and encourage the development of advantageous microorganisms (Soemarie et al.,2021). A study has been carried out on the Nile tilapia (*Oreochromis niloticus*) by analyzing their responses to supplemental probiotic brewer's yeast (*Saccharomyces* cerevisiae), which affects their development, feed consumption, and intestinal morphology (Islam et al., 2021). The fermentation that had been applied gave a positive result which improved feed conversion ratios and increased nutrient utilization (Islam et al., 2021). Another study is the utilization of lactide bacteria and yeast (for fermentation) with cellulase enzymes in the conversion of seaweed powder to seaweed silage, which was enriched with probiotics as food for fish larvae and rotifers showed better feed conversion and growth rates in Asian seabass after 56 days (Santhanaraju Vairappan, 2021).

CHALLENGE IN THE PRODUCTION OF PROBIOTIC-INCORPORATED MICRO FEED

Probiotics enhance digestion and nutrient absorption in the gut through the inclusion of beneficial bacteria into the feed diet increases feed utilization efficiency. While probiotics have been shown to have several benefits (on the digestive processes of aquatic animals) in aquaculture, incorporating them into microfeed can be challenging as it needs to be addressed to ensure their effectiveness and stability (El-Saadony et al.,2021).

It is needed to optimize the production process (preparation and preservation) to minimize the negative effect on probiotic viability. Probiotics may be subjected to a range of stressors during the microfeed manufacturing process, including heat, pressure, and mechanical forces (Fenster., 2019). Probiotics can be exposed to oxygen throughout the microfeed production and storage procedures, such as drying and packing, which may decrease their viability. It is necessary to find strains that are durable and resistant to the processing conditions, such as *B. subtilis*, *B. licheniformis*, and *Lactobacillus acidophilus* (Elshaghabee et al., 2017).

Probiotics have a limited shelf life, particularly when they are subjected to unfavorable factors like high temperatures, humidity, or light (Sun et al., 2023). An essential factor to take into consideration is extending the shelf life of probiotic-containing microfeed without affecting its viability. The effectiveness of probiotics in enhancing animal health, growth, and disease resistance will be impacted by their shelf life (Al-Shawi et al., 2020). Microfeed in fish feed

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has a shelf life ranging from a few months to a year. In order to maintain the shelf life of microfeed, packing and storage conditions are very important.

The cost-effectiveness of using probiotics as specialized additives in microfeed is being examined in light of the advantages of probiotics in terms of fish performance improvement (Al-Shawi et al., 2020). Since formulating and producing tiny particles requires additional effort, microfeed is often more expensive than standard feed. High-quality proteins, specialized components, marine-based components, and medicinal additives are all incorporated into the microfeed. The reason the microfeed is effective is that the nutrients required serve specific nutritional needs or treat particular medical issues for the fish larvae (Beski et al., 2015).

Developing suitable techniques or technologies, such as the entrapment of probiotic bacteria in a gel matrix of gellan, alginate, xanthan, and k-carrageenan for delivering and distributing probiotics in microfeed can be one of the challenging issues in microfeed production. To protect against moisture, light, and oxygen, proper packaging materials must be selected to keep the probiotic incorporated with microfeed from contamination (Fenster,2019). It helps to keep probiotics alive and extends their shelf life. However, it is undeniable that the microfeed depends on the region and the particular probiotic strains used; thus, there may be legal requirements and limitations on the use of probiotics in aquaculture (Fenster, 2019).

CONCLUSION

When microfeed combines with probiotics, it increases its use in aquaculture. It appears that it will offer additional benefits (improving fish growth and reproduction), especially when the live stream is replaced. It is without debate that probiotics are widely used in the aquaculture sector. Further study of microfeed incorporated with probiotics must be done for the sustainability of aquaculture, especially for microencapsulated feed, as the live feed is limited and high-cost.

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Authors' contributions

Nor Mala Yaslikan conducted literature reviews and wrote the original manuscript. Nur Jasmin assisted in manuscript preparation and revision. Nadiah Rasdi contributed to the manuscript revision. Murni Karim supervised the content, revision, and proofreading of the manuscript. All authors confirmed the final draft of the manuscript.

Ethical considerations

The authors declare and confirm that the manuscript is original, has no misconduct, has never been published in another journal, and is confirmed to be published in this journal.

Availability of data and materials

All data generated during the research are relevant and included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Using Helminths to Fight Cancer: An Innovative Approach

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ABSTRACT

As an alternative treatment in cancer therapy, there has been a growing interest in using helminths, such as *Trichinella spiralis* (*T. spiralis*), *Echinococcus granulosus* (*E. granulosus*), *Toxocara canis* (*T. canis*), and *Taenia solium* (*T. solium*). This study aimed to investigate the antigens and mechanisms that contribute to the anticancer properties of helminths, providing insights into how helminths may be used as a new and innovative treatment modality for cancer. The current review analyzed preclinical and clinical studies published between 2000 and 2023. The present study sought to obtain information on helminths, such as *E. granulosus*, *T. spiralis*, *T. canis*, and *T. solium*, to treat cancers of the breast, pancreas, melanoma, and leukemia by exploring databases, such as PubMed, Google Scholar, and Scopus. Studies focusing on helminth therapy against particular cancer types for *in vitro* and animal models were included. Several studies have shown the possibility of inhibiting breast, colon, melanoma, and leukemia tumor growth, inducing apoptosis, and modulating the tumor microenvironment with *E. granulosus*, *T. spiralis*, *T. canis*, and *T. solium* based on *in vitro* and animal models studies. Some studies have indicated that helminth therapy can improve survival rates, reduce tumor growth, and stimulate the immune system in cancer patients. A potential improvement in treatment outcomes can be used for combination therapies, such as antigen selection, immune profiling, and individualized approaches based on helminth therapy. Helminth therapy is an additional option for cancer treatment, emphasizing *T. spiralis*, *E. granulosus*, *T. canis*, and *T. solium*. These helminth antigens could modulate immune responses and directly cause cytotoxicity in cancer cells.

Keywords: Cancer, *Echinococcus granulosus*, *Taenia solium*, *Toxocara canis*, *Trichinella spiralis*

INTRODUCTION

Modern medicine must deal with the daunting challenge of treating cancer, a disease that affects millions of people worldwide. Researchers are still seeking more efficient and less harmful cancer treatment options despite impressive advances in conventional methods such as radiation therapy and chemotherapy (Mansouri et al., 2021; Hajjafari et al., 2022; Saeed et al., 2022). Alternative approaches to dealing with this complicated condition have recently gained considerable popularity (Sadr et al., 2023a). One promising approach is to utilize parasitic worms, which have co-evolved with humans for thousands of years (Asouli et al., 2023; Sadr et al., 2023b).

Flukes, roundworms, and tapeworms are all examples of helminths (Lotfalizadeh et al., 2022). Historically, most of them are responsible for human diseases, but scientific evidence in recent years indicates that they may also provide therapeutic benefits, especially for cancer treatment (Oikonomopoulou et al., 2014; Scholte et al., 2018). Parasitic helminths such as *Trichinella spiralis* (*T. spiralis*), *Echinococcus granulosus* (*E. granulosus*), *Toxocara canis* (*T. canis*), and *Taenia solium* (*T. solium*) have offered a novel perspective on cancer treatment due to their antigen similarity with cancer (Ditgen et al., 2014; Asghari et al., 2022). It has been discovered that some of these molecules can modulate the immune system at a fundamental level, which is a critical factor in the development and progression of cancer (Callejas et al., 2019).

To avoid the host's immune response, helminths can induce an immune state of immunomodulation, achieved by switching from a pro-inflammatory and inflammatory environment to a regulatory one, thus evading the host's defense (Bruschi and Chiumiento, 2012; Bruschi et al., 2022). Several studies have demonstrated that helminth-induced immune modulation has profound implications for cancer, suppressing tumor-promoting inflammation, enhancing antitumor immune responses, and restoring immune balance in tumor microenvironments (Guan et al., 2019; Raisnia et al., 2022).

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There is significant evidence that helminth infestation improves survival rates, inhibits tumor growth, and minimizes metastasis in animal infestation models (Daneshpour et al., 2016; Berriel et al., 2021). Some preliminary clinical trials conducted with helminths showed promising results, such as tumor regression, immune activation, and improved overall survival in patients treated with helminths (Aref et al., 2013; Daneshpour et al., 2019).

While helminth therapy has many benefits, it has many challenges as well. The development of protocols must consider several important factors, including ethical considerations, safety concerns, and standardization. To identify patient selection criteria and optimize treatment strategies, more research is necessary to identify the individuals' responses to helminth therapy. The present study aimed to understand the intricate relationship between helminths and cancer by analyzing how helminths interact with the immune system. By doing so, researchers can better understand how helminths exert their anticancer properties.

METHODOLOGY

This systematic review aimed to investigate the potential therapeutic benefits of *T. spiralis*, *E. granulosus*, *T. canis*, and *T. solium* in treating breast, pancreatic, leukemia, and melanoma cancers by applying an integrated methodology. The studies published between 2000 and 2023 were reviewed for the present review.

To begin the review process, several databases were searched in the current study, including PubMed, Embase, Scopus, and Web of Science. Several relevant keywords and their variations were included to ensure comprehensive coverage. These keywords include "helminths," "*Echinococcus granulosus*," "*Trichinella spiralis*," "breast cancer," "pancreatic cancer," "leukemia", and "melanoma".

In the scope of this systematic review, the inclusion criteria included studies that employed an integrated methodology. These studies explored the therapeutic potential of *T. spiralis*, *E. granulosus*, *T. canis*, and *T. solium* in various cancers, specifically breast, pancreatic, leukemia, and melanoma. As a result, exclusion criteria include studies that do not pertain to the therapeutic aspects of these helminths in cancer, those that do not use an integrated methodology, publications that do not coincide with the specified timeframe, studies that are not focused on cancer, non-clinical or non-preclinical research, and studies that do not follow a primary objective to assess whether helminth therapy can be beneficial for cancer. English-language publications are the only ones considered, whether performed *in vitro*, *in vivo*, or in clinical tests. The current study selected 43 studies out of 427 studies published between 2000 and 2023 to ensure that contemporary research is represented.

ANTICANCER PROPERTIES

Animals and humans both suffer from helminth infections as parasitic organisms. A variety of parasites live in the animal and human body, including roundworms (nematodes), tapeworms (cestodes), and flukes (trematodes). Recent research shows that helminths' Excretory-Secretory Proteins (ESP) may have therapeutic potential against cancer despite their mysterious anticancer properties (Kang et al., 2013; Liao et al., 2018).

Helminths anticancer properties must be better understood by unraveling the mechanisms underlying them. Helminth has multifaceted strategies to interact with the immune system, such as creating an environment that is uniquely anticancer. By activating the immune system, helminths can modify the host's immune response (Ding et al., 2020; Hu et al., 2021). It is important to note that immunomodulation plays a significant role in suppressing tumor growth and metastasis (Vasilev et al., 2015).

Bioactive molecules produced by helminths may directly target cancer cells or may be influenced by their microenvironment (Gutierrez-Millan et al., 2021). Various molecules, including glycoproteins and ESP, have anticancer properties (Osinaga, 2007; Bahadory et al., 2022). When ES proteins are secreted, apoptosis can be induced by helminth-derived proteins in animal-induced cancer cells, and angiogenesis and metastasis can be inhibited (Mu et al., 2021). Excretory and secretory proteins of helminths possess exceptional immunomodulatory characteristics and a direct effect on cancerous tissues (Murphy et al., 2020). By creating anti-inflammatory and immune-regulating environments, they have adapted tactics to undermine the host's immune system reaction and counteract inflammation that reduces tumor development (Kahl et al., 2018). The tumor microenvironment influences the immune system by interacting with various regulatory molecules and immunological cells like CD8+ T cells and natural killer (NK) cells (Sotillo et al., 2020).

Helminths can include enhancing the activity of NK cells by stimulating a type 2 immune response (Nutman, 2015). Moreover, immune modulation by helminths can trigger the production of anti-inflammatory cytokines and the recruitment of regulatory T cells (Tregs), which suppress excessive immune responses (White et al., 2020). Helminths can inhibit tumor growth by dampening chronic inflammation, which is a known risk factor for cancer. In addition to controlling chronic inflammation, helminths help regulate the immune system by inhibiting tumor growth (Vennervald and Polman, 2009).

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Through the interaction of helminth-derived molecules with host immune cells, the immune system alters the function of immune cells, produces cytokines, and creates immune signaling pathways in response to inflammation (Elliott and Weinstock, 2012; Motran et al., 2018; Maizels, 2020). To harness the anticancer potential of helminths effectively, it is imperative that researchers fully comprehend the details of immunomodulation. Helminths exert their anticancer effects by modulating the immune system and directly targeting cancer cells (Reens et al., 2021; Asghari et al., 2022; Lee et al., 2023). Helminth's capabilities could be harnessed to enhance or complement anticancer therapies.

IMMUNE SYSTEM MODULATION

How helminths interact with the immune system

Interfacing with the host's immune system can be accomplished by helminths in several different ways. In addition to direct interactions with immune cells, immunomodulatory molecules are secreted, and immune signaling pathways are modulated during these interactions (Hewitson et al., 2009; Zheng et al., 2020). Helminths influence the immune system in a variety of ways, including pattern recognition receptors (PRRs). Lipids, glycoproteins, and other molecules derived from helminths can bind to PRRs within immune cells (Abou-El-Naga and Mogahed, 2022). Immune responses are activated by PRRs when they recognize pathogen-associated patterns (Tsubokawa, 2023). Immune cells become activated after helminth molecules bind to PRRs, which affects downstream processes.

Helminths can modify Antigen-Presenting Cells (APCs) such as dendritic cells and macrophages, also modifying how antigens are presented to T cells (Harn et al., 2009; Nutman, 2015). Consequently, distinct immunological responses that are less favorable to tumor growth can be developed.

Helminths influence immune responses by influencing cytokines, which are significant for signaling (Kaur and Ghorai, 2022). Parasites can induce anti-inflammatory cytokines, such as IL-10 and TGF-β, while suppressing pro-inflammatory cytokines, like IL-6 and TNF-α (Yeo et al., 2021; Silva et al., 2023). This cytokine modulation contributes to an immunosuppressive environment. By recruiting Tregs and promoting an anti-inflammatory environment, helminths may facilitate immune tolerance (Ryan et al., 2020).

Some helminths are capable of directly interacting with immune cells, including eosinophils, macrophages, and Natural Killer (NK) cells (Maggi et al., 2020; Zhou et al., 2020; Varadé et al., 2021). This results in their activation and recruitment to infection or inflammation sites. Antitumor action can be exerted by activated immune cells by directly interacting with tumor cells or enhancing immune surveillance (Gong et al., 2020; Peng and Fadeel, 2022).

Impact of helminth-induced immunomodulation

Aside from altering the immune system by activating DCs and T cells, helminth infections have profound implications for cancer development and progression. Helminths have the capability to alleviate chronic inflammation associated with the growth of cancer (Arabpour et al., 2021). By reducing pro-inflammatory cytokine production in the immune system and increasing anti-inflammatory cytokine production, helminths prevent tumor development (Shi et al., 2022). Helminth infection increases the activity of immune cells, such as cytotoxic T cells and NK cells, that promote the immune system's ability to fight cancer (Dyck and Mills, 2017; Roe, 2022). The cancerous cells can be detected and destroyed by these immune cells. Activating the immune system induced by helminths can improve its ability to identify and eliminate cancerous lesions by enhancing their effector activities (Maizels and McSorley, 2016; Callejas et al., 2018). Helminths appear to influence angiogenesis, which involves the formation of blood vessels that supply oxygen and nutrients to tumors (Williams et al., 2016). Helminths prevent angiogenesis by inhibiting angiogenic factor secretion, which prevents tumor development and metastasis (Dehne et al., 2017). Further, helminth-induced immunomodulation can restrict the invasion of cancer cells and their migration, thereby suppressing metastatic spread (Chakraborty et al., 2023).

T cells in helminth therapy

Regulatory T cells (Tregs) play a crucial role in maintaining immune homeostasis and limiting excessive immune responses. In helminth infections and treatments, Tregs play an important role in modulating immune responses (Babu and Nutman, 2019). Immunosuppressive properties can be induced by helminths, which can expand and activate Tregs (McManus and Maizels, 2023). To suppress immune responses against tumors, Tregs suppress the function of cytotoxic T cells and natural killer cells by blocking their operations. As a result, Treg suppression diminishes the immune responses against tumors (Belkaid and Tarbell, 2009).

There is much to learn about Tregs and their role in cancer helminth therapy. Through their ability to reduce inflammation and minimize immune-mediated damage, helminths facilitate the formation of Tregs and support antitumor immunity (Toomer and Chen, 2014). Previously, studies have been conducted on the interaction between helminths, Tregs, and antitumor immune responses (Grazia Roncarolo et al., 2006; Li et al., 2015). Determining how helminths modulate Treg activity is imperative to optimize helminth-based therapeutic strategies (Vahidian et al., 2019; Figure 1).

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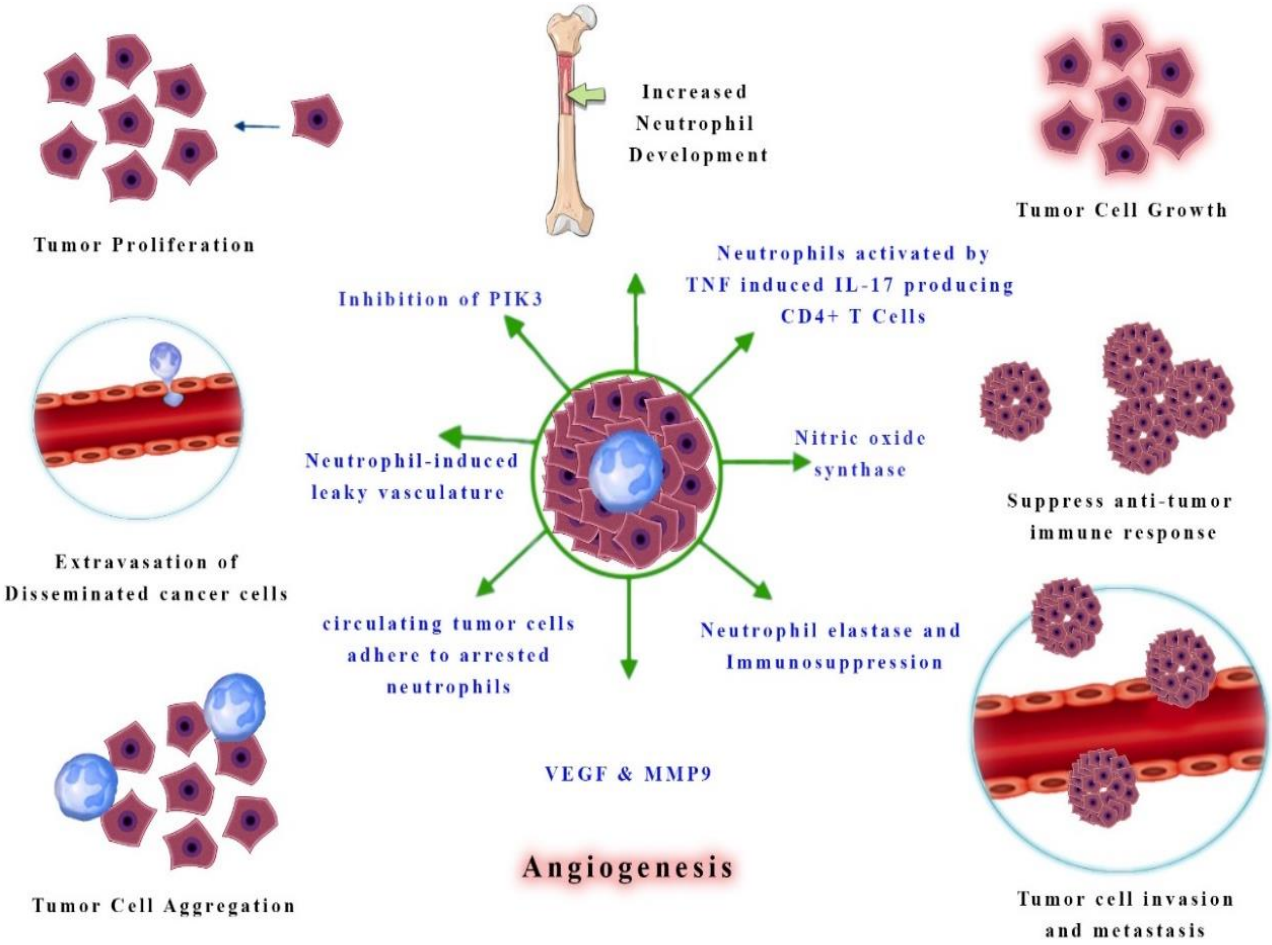

Figure 1. The mechanisms of helminths interact with the immune system. Helminth-induced immunomodulation on cancer development and progression, PIK3: Phosphoinositide 3-kinases, VEGF: Vascular endothelial growth factor, TNF: Tumor necrosis factor, IL: Interleukin, MMP9: Matrix metalloproteinase 9, CD 4+: Cluster of differentiation 4

HELMINTH AND CANCER TREATMENT

Echinococcus granulosus

Echinococcus granulosus is a tapeworm primarily found in canids such as dogs and wolves, and herbivores such as sheep and cattle serve as intermediate hosts (Devadharshini et al., 2022; Shams et al., 2022). To survive, *E. granulosus* goes through various stages in its life cycle. During adulthood, worms live in definitive hosts' small intestines, producing eggs expelled in their feces (Heidari et al., 2019). Infected canids pass the tapeworm eggs in their feces, which are then eaten by intermediate hosts (usually herbivores) where they develop into cysts, which are then consumed by the canids, releasing the tapeworm's adult stage into their intestines (Tamarozzi et al., 2020).

Intermediate hosts swallow eggs and hatch them in the small intestine, releasing oncospheres. The oncospheres enter the bloodstream through the intestinal wall, which causes a hydatid cyst to form in an organ like the liver or lungs (Pal et al., 2022; Thompson, 2023). The protoscoleces that develop within hydatid cysts can produce adult worms if eaten by the appropriate definitive host (Borhani et al., 2021).

Several antigens obtained from *E. granulosus* have been investigated as possible cancer treatment options (Chookami et al., 2016; Sharafi et al., 2016; Asouli et al., 2023). Numerous studies have been conducted on the immunogenicity of antigen B (AgB) and its ability to induce immune reactions in response to tumor cells (Darani and Yousefi, 2012). Evidence shows that the multi-subunit antigen AgB triggers both humoral and cellular immune responses (Rigano et al., 2007). Cancer cells can be induced to undergo apoptosis by AgB, the growth of tumors can be slowed down, and immune cells attack tumors more effectively by AgB (Noya et al., 2013; Zheng, 2013). Antigen 1 (Ag1) and Antigen 5 (Ag5) from *E. granulosus* have also been investigated concerning their anticancer capabilities (Daneshpour et al., 2016; Darani et al., 2016; Sharafi et al., 2018; Bo et al., 2020).

Trichinella spiralis

Several types of mammals carry *T. spiralis*, including carnivorous and omnivorous mammals such as humans (Zarlenga et al., 2020). There are different stages in the life cycle of *T. spiralis*. Adult worms live in the small intestine, where they reproduce sexually and produce larvae (Tang et al., 2022). By injecting the larvae into the bloodstream, the

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larvae penetrate the intestinal wall and migrate to different tissues, including skeletal muscles. As the larvae penetrate muscle tissue, they get encapsulated, producing nurse cells (Barlow et al., 2021). When an animal feeds on infected muscle tissue, larvae are released, which can grow into adult parasites.

Many antigens derived from *T. spiralis* benefit cancer treatment (Eissa et al., 2016; Bruschi et al., 2022; Yousefi et al., 2023). Glycoprotein *T. spiralis* is known to possess anticancer properties based on preclinical trials. Several studies have shown that *T. spiralis* triggers apoptosis in tumor cells, prevents tumor growth, and enhances immune reactions against tumors (Callejas et al., 2018; Ding et al., 2021; Sadr et al., 2023c). It has also been found that TSL-ES, an excretory-secretory antigen of muscle larvae of *T. spiralis*, suppresses tumor proliferation by modulating tumor microenvironments (Ding et al., 2021; Ding et al., 2022). The ability of *T. spiralis* antigens to activate dendritic cells and trigger immune responses against tumor cells has also been explored (Saad and Ghanem, 2020; Bruschi et al., 2022) (Figure 2). The dendritic cells can then present tumor-associated antigens to T cells, ultimately enhancing the immune response against tumor cells and reducing the risk of malignancies.

Toxocara canis

Toxocara canis is an intestinal parasite found mainly in dogs (Macpherson, 2013). However, it can also infect humans, leading to the development of a disease known as human toxocariasis (Jahanmahin and Borji, 2023). This occurs when someone accidentally ingests the parasite's eggs through contact with contaminated soil or objects. *T. canis* has been investigated for its potential anticancer effects (Oikonomopoulou et al., 2013; Jahanmahin and Borji, 2023;). Despite *T. canis* infections most commonly affecting the liver, lungs, and eyes in humans, Garn et al. (2021) indicated that exposure to *T. canis* antigens may reduce the risk of certain types of cancer, such as breast and colon cancers (Garn et al., 2021). There is still uncertainty about the mechanisms underlying these effects, but it is believed that they include multifaceted interactions between *Toxocara*'s antigens and the host's immune system. According to studies, *Toxocara* antigens have the potential to modulate the immune reaction, resulting in an increase in immune cell activity, such as macrophages, DCs, and NK cells. Macrophages and NK cells can recognize and target cancer cells, leading to their demise. Aside from the stimulation of chemokines and cytokines, *Toxocara* antigens also contribute to the regulation of the immune response and the development of tumors. The infection of *Toxocara* may also trigger the production of T2 immune responses related to the production of specific antibodies and cytokines that inhibit tumor growth (Menon et al., 2021). Research is needed to understand how *Toxocara* antigens generate anticancer effects and determine their effectiveness as cancer therapeutics.

Recent studies reported the effect of *T. gondii* and *T. canis* egg antigens on transplanted WEHI-164 fibrosarcoma in BALB/c mice (Darani et al., 2009; Darani and Yousefi, 2012). Researchers found that parasites *T. gondii* and eggs of *T. canis* inhibit tumor proliferation in the fibrosarcoma mouse model. However, the underlying mechanisms responsible for these effects require further investigation.

Based on the *T. canis* ESP, Bahadory et al. (2022) assessed the potential anticancer activity of this parasite (Bahadory et al., 2022). The expression of cancer-related genes and tumor cell viability was studied in liver and gastrointestinal cancer cell lines treated with synthesized peptide components obtained from *T. canis*. More than 32 µg/ml concentrations showed efficacy in other cancer cell lines.

Taenia solium

According to studies, certain constituents of *Taenia solium* can potentially interact with cancer cells and possibly exert anticancer effects (Arora et al., 2020; García-Gutiérrez et al., 2020; Plata and Castañeda, 2020). Schcolnik-Cabrera 2020 evaluated the anticancer activities of recombinant *T. solium* calreticulin (rTsCRT) by treating tumor cells at various concentrations (Schcolnik-Cabrera et al., 2020). Tests were also undertaken by employing cancer cell lines with rTsCRT and 5-fluorouracil, a chemotherapy medication. It was demonstrated that rTsCRT acted as an antitumor agent dose-dependently in SKOV3 and MCF7 cell lines. The study found that cells had lower viability and colony-forming ability in combination with 5-fluorouracil. In addition, cancer stem-like cells showed greater sensitivity to the treatment of rTsCRT. In addition, rTsCRT's involvement in scavenger receptor interactions profoundly affected its antitumor properties since blocking these receptors reversed rTsCRT's viability reduction characteristics. The rTsCRT may be beneficial as a therapy for breast and ovary cancers because it interacts with scavenger receptors.

CHALLENGES AND FUTURE DIRECTIONS

Helminth therapy can be a valuable treatment option for cancer, but several challenges and limitations must be addressed to implement it in clinical settings successfully. Future advancements in the field can be facilitated by improving treatment methods based on helminths and surmounting these obstacles.

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CURRENT LIMITATIONS AND CHALLENGES

As promising as helminth therapy has been in preclinical studies and limited clinical trials, safety concerns remain paramount. When administered live helminths, individuals with impaired immune systems may experience uncontrolled infections, migration to unintended organs, or adverse reactions (Sobotková et al., 2019). Applying comprehensive evaluations and maintaining standardized processes should make helminth therapy safe (Weinstock and Elliott, 2009).

Ethical questions must be addressed if individuals are intentionally infected with parasitic organisms to undergo helminth therapy (Lukeš et al., 2014). The debate regarding the deliberate administration of parasites into patients is going on, bearing in mind possible therapeutic advantages and ethical considerations. A framework and guidelines that protect patient well-being and autonomy are imperative to navigate these complicated issues (Sobotková et al., 2019).

No standardized treatment protocol exists for helminth therapy, including terms such as duration of therapy, dosage, and timing (Cheng et al., 2015). Guidelines must be designed to ensure consistent and reproducible helminth administration, monitoring, and patient follow-up (Fleming and Weinstock, 2015).

Different types of cancer, stages, and genetic profiles exist among individuals with cancer, depending on the type and location of the disease (Zheng, 2013). There may be some individual differences in response to helminth therapy, which necessitates the identification of patient selection criteria as well as predictive biomarkers for optimizing treatment outcomes. Developing personalized approaches to helminth-based cancer therapy requires knowledge of the factors responsible for heterogeneity in response (Douglas et al., 2021).

ONGOING RESEARCH AND POTENTIAL IMPROVEMENTS

Researchers are currently investigating the development of antigens derived from helminths capable of delivering robust anticancer activity with the least adverse effects possible (Muzzarelli, 2010; Noya et al., 2013). Researchers need to aim to formulate standardized and controlled antigen compounds by pinpointing and isolating essential immunogenic elements. Using recombinant DNA technology facilitates the preparation of helminth antigens at high levels in a reproducible and reliable way. By manipulating antigen structure and composition precisely, improving their effectiveness and safety may be possible (Bolhassani et al., 2011). Some of these properties are increasing immunity, reducing side effects, or improving tissue targeting. Additionally, engineering recombinant antigens permit various antigen fusions and modifications for maximum anticancer efficacy (Saylor et al., 2020). It is possible to increase the potency of helminth therapy when combined with immunotherapy adjuvants or other cancer treatments (Li et al., 2019). Helminth antigens stimulate an immune response, strengthening their anticancer effects when used with immune adjuvants (Liu et al., 2019). Helminth therapy may synergize with traditional treatments, such as chemotherapy and immunotherapy, and improve the overall efficiency of such therapies (Lim, 2015).

Combination therapies

It may be possible to enhance treatment results when helminth therapy is used alongside conventional cancer treatments, for example, radiotherapy, chemotherapy, and immunotherapy. Treatments that target the immune system, such as adoptive cell therapies or immune checkpoint inhibitors, may be boosted by immune modulation caused by helminths (Elsegood et al., 2017; Thuru et al., 2022). In many ways, helminth therapy can improve the immune response against tumors by enhancing the immune reaction triggered by regulatory and anti-inflammatory environments induced by helminth therapy (Crinier et al., 2019). Helminth therapy might make cancerous cells more susceptible to chemotherapy, making them more vulnerable to its cytotoxic effects. By modulating the tumor microenvironment and strengthening immune responses to chemotherapy, helminth therapy can improve chemotherapy efficacy and minimize the duration and dosage of treatment (Wei et al., 2021). Colliding helminth therapy with radiation therapy can sensitize tumor cells more prone to radiation. Helminths may modulate the immune system, and the tumor microenvironment may be altered, leading to a better tumor response to radiation.

CONCLUSION

Helminth therapy is proving to be a promising new treatment frontier for cancer, offering improved outcomes, fewer side effects, and a personalized approach. Using helminth therapy in combination with traditional cancer treatments may lead to better treatment outcomes. As a result of coupling helminth therapy's unique mechanism of action with chemotherapy, radiotherapy, or immunotherapy, more complete and efficient cancer treatment plans can be developed. Combining chemotherapy with radiotherapy for precision or pairing immunotherapy with radiation therapy for immunomodulation can achieve more comprehensive and highly successful treatments for breast, colon, lung, and melanoma cancers. Moreover, antigen selection and immune profiling allow helminth-based treatments to be customized individually to the patient's requirements, ensuring optimized treatment responses. Future advancements in helminth

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therapy can be achieved by designing creative therapies that use helminths and gaining a better understanding of their mechanisms.

DECLARATIONS

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Authors' contributions

The conceptualization of the study was led by Melika Zamanian, with all authors contributing to the methodology. Formal analysis and investigation were carried out by all authors, and the original draft of the manuscript was collectively prepared by them. Additionally, all authors participated in the review and editing process, with Melika Zamanian overseeing the project as the supervisor. All authors checked and approved the final version of the manuscript for publication in the present journal.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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The Current Status and Potential Development of Genetic Resources of Indigenous Toraya Spotted Buffalo in Indonesia: A Systematic Review

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ABSTRACT

Buffaloes are integral to the Asiatic market as they are crucial for agricultural work and transportation and provide a significant source of dairy and meat, contributing to various industries, local economies, and cultural practices across the region. Indonesia is a mega biodiversity country abundant in livestock genetic resources, including indigenous, local, and introduced breeds that play a crucial role in agriculture and the livestock industry. These genetic resources offer the potential for selective breeding and improving the quality of livestock populations through well-designed breeding programs, ensuring sustainable livestock production for the future. The current study was performed using the "Publish or Perish" software, and the data obtained was analyzed using the CADIMA web tool. The Toraya buffalo population is the largest in the Tana Toraja and North Toraja regencies and is widely distributed within South Sulawesi Province, Indonesia. The population of Toraya buffalo in this region reached 43674 heads. Toraya buffaloes exhibit diverse body color characteristics, such as black, gray, white, and mixed. Moreover, Toraya buffaloes possess distinct quantitative traits that set them apart from other swamp buffalo breeds. Although there are limited studies on Toraya buffaloes, the potential for broader and more comprehensive studies offers opportunities to uncover new information on the characteristics, genetics, reproduction, health, and management of Toraya buffaloes. The development of Toraya buffalo farms also holds significant economic promise, as it can lead to increased agricultural productivity and improved livelihoods for local communities by enhancing the quality by implementing well-planned breeding programs and leveraging reproductive technology, and genetics-based selection, growth, and productivity can be produced. To increase the population and productivity of Toraya buffaloes, a well-structured breeding program integrating reproductive technology and selection based on quantitative and molecular genetics is essential. The development potential of Toraya buffalo is vast, not only due to its high cultural value but also its superior quantitative traits compared to common swamp buffalo, positioning it as a potential national meat provider. The present review article aimed to discuss the characteristics and development potential of Toraya buffaloes, along with the implementation of reproductive biotechnology and molecular genetics to enhance the population, productivity, and quality of Toraya buffaloes in Indonesia.

Keywords: Genetic resource, Spotted buffalo, Swamp buffalo, Toraya buffalo

INTRODUCTION

As a mega biodiversity country, Indonesia possesses a diverse array of livestock genetic resources, encompassing indigenous, local, and introduced livestock. These genetic resources hold significant importance for agriculture and the livestock industry, as they facilitate the selection and enhancement of livestock population quality through well-planned breeding programs, thereby ensuring sustainable livestock production in the future. Among the valuable livestock genetic resources, proper management and preservation of buffalo populations are crucial. Buffalo plays a pivotal role in overall social development contributions of draft power to meat, milk, and hide production (Pineda et al., 2021).

Buffaloes are a prominent livestock species that continue to be widely distributed and traditionally raised in Southeast Asia, particularly Indonesia. There are two subspecies of buffalo, namely the river buffalo (*Bubalus bubalis*) from South Asia and the swamp buffalo (*Bubalus bubalis carabanensis*) from Southeast Asia. Buffaloes play a vital role in rural communities and significantly contribute to the national supply of meat and milk. According to the 2021 data from the Indonesian Statistics Agency (Badan Pusat Statistik [BPS]), the national buffalo population reached 1.189.260 heads, distributed across Indonesia with varying population densities and agroecosystems. However, the general buffalo population has experienced a drastic decline in several regions over the past decades (BPS, 2021).

Buffaloes play a substantial role in the livestock economy, providing essential contributions to milk, meat, and labor. The potential for buffalo development as a meat source holds promising prospects, particularly in line with population growth, directly influencing meat demand in Indonesia. One such buffalo breed is the Toraya buffalo or

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striped buffalo, which represents a significant part of Indonesia's biodiversity and belongs to the swamp buffalo (*Bubalus bubalis carabanensis*).

Swamp buffaloes are frequently used in traditional and religious ceremonies across various regions of Indonesia and indicate high social status within the community. Toraya buffalo is native to South Sulawesi, Indonesia, and has been officially designated as a local buffalo breed through Minister of Agriculture Decree No. 2845/Kpts/LB430/8/2012. The development of buffalo farming can be influenced by the presence of natural resources, social factors, and economic opportunities, potentially leading to changes in their geographical distribution (Rozaq and Sugiarto, 2020). Notably, the Toraja region exhibits a distinct and unique culture and belief system, with the Rambu Solo funeral procession being one of its famous traditions closely associated with the Aluk To Dolo belief. The reliance on buffaloes during the Rambu Solo ceremony has economic, social, and ecological implications (Syamsuddin et al., 2023).

The utilization of buffaloes in traditional ceremonies as sacrificial animals necessitates careful attention to the buffalo population in Toraja. The low birth rate resulting from traditions discouraging natural mating and the limited application of artificial insemination technology in male Toraya buffaloes have contributed to the decline in the Toraya buffalo population. As an essential measure, adopting reproductive technology is paramount to addressing the challenges facing Toraya buffalo. The present review article aims to discuss the characteristics and development potential of Toraya buffaloes, along with the utilization of reproductive biotechnology and molecular genetics to enhance the population, productivity, and quality of Toraya buffaloes in Indonesia.

DATA COLLECTION

The literature searches were carried out using the publish or perish (POP) software version 8. The keywords Toraya buffalo and striped buffalo were used in searching the Google Scholar and Scopus databases published from 2005 to 2023. The literature obtained was then stored in RIS format and processed using the CADIMA web tool (CADIMA, 2020). The reference list, which combined 44 unique records according to CADIMA (with no duplicates), was then randomized, and full-text analysis was conducted for the final review. In total, six publications were selected from the field of reproductive biotechnology and molecular genetics. This study was approved by the Animal Ethics Commission of the National Research and Innovation Agency, Indonesia, with the license number 050/KE.02/SK/03/2023.

POPULATION STATUS

Swamp buffaloes were domesticated in Thailand and subsequently spread to various regions (Wang et al., 2017; Zhang et al., 2020). They migrated northwards to China and southwards to Sumatera. Buffaloes from China also extended their distribution to the Philippines, while those from Sumatera spread to Indonesia, Sulawesi, and Nusa Tenggara islands. Sumatran buffaloes possess relatively superior genetic quality, compared to buffaloes from other Indonesian islands due to direct genetic inheritance from Thai buffaloes (Yusnizar et al., 2015). Zhang et al. (2020) reported that after being domesticated in Thailand, swamp buffaloes migrated in a southerly direction, traversing the Malay Peninsula and reaching Indonesian islands, including Sumatera, Java, Sulawesi, as well as the northern and northeastern regions of central China. They subsequently continued their expansion using the eastern island route, passing through Taiwan and eventually reaching the Philippines and Borneo.

Saputra et al. (2020) conducted a study based on microsatellite markers and identified two groups of swamp buffalo in Indonesia. The initial cluster consists of Aceh, North Sumatera, and Riau, while the subsequent group encompasses Central Java, West Nusa Tenggara, South Sulawesi, and ten other regions in Indonesia. Similarly, in a study by Rusdin et al. (2020), two distinct clusters were identified among eight Indonesian swine breeds, distinguished through cytochrome b gene markers. The primary cluster includes Aceh, North Toraja, West Nusa Tenggara, Banten, Kolaka, and Konawe, while the secondary cluster comprises Bombana Island, Bombana mainland, Kolaka, and Konawe, totaling eight distinct breeds.

Geographically, Toraja is located between 2-3 degrees south and 119-120 degrees east longitude, bordered by Enrekang district to the south, Luwu district to the east, Polewali district to the west, and Central Sulawesi to the north (Figure 1). The largest distribution of buffalo populations is found in the Toraja region, specifically the Tana Toraja and North Toraja districts, and the Toraya buffalo is an indigenous livestock breed prevalent in South Sulawesi Province, Indonesia. Toraya buffaloes have been raised for generations due to their role in religious rituals and close ties with local culture, including the rambu tuka (wedding ceremony) and rambu solo (death ceremony). The designation of Toraya buffalo as a local breed was confirmed by the Minister of Agriculture Decree Number 2845/ Kpts/ LB430/8/2012.

The buffalo population in South Sulawesi Province reached 122.012 heads in 2021 (BPS, 2021), with the highest population found in Tana Toraja and North Toraja districts, accounting for 43.674 heads or 40% of the buffalo population in South Sulawesi. According to FAO (2022), the Toraya buffalo population was recorded as 19.239 heads, but detailed classification information was not provided. The distribution of Toraya buffalo populations is not limited to

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Toraja but also extends to other areas, including Enrekang Regency, Pinrang Regency, Maros Regency, and Takalar Regency.

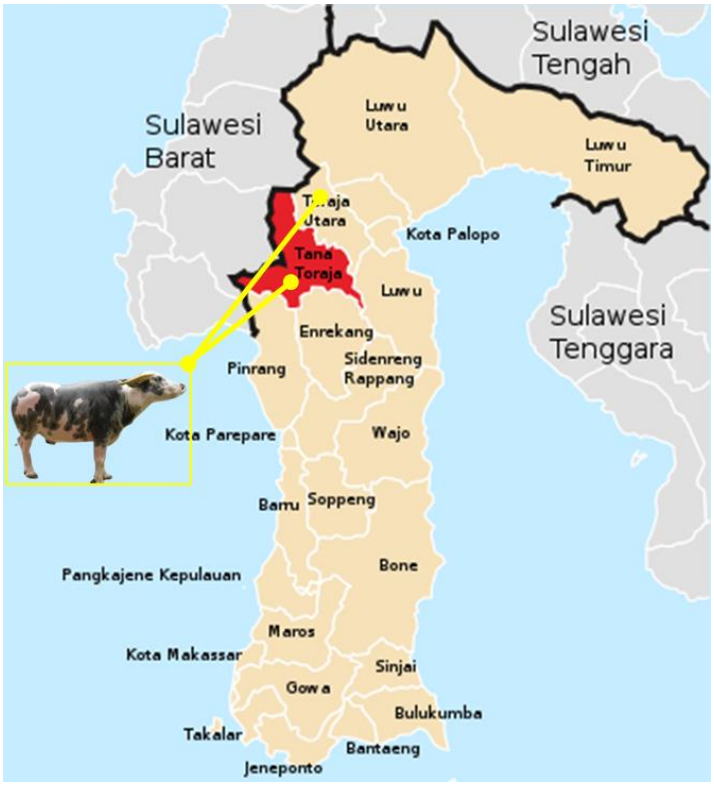

Figure 1. The map depicts the distribution of Toraya buffalo populations in the Tana Toraja and North Toraja districts of South Sulawesi Province, Indonesia.

Figure 2. Spotted color pattern types on Toraya buffaloes at Toraja Utara and Tana Toraja, South Sulawesi Province, Indonesia. **a**: Saleko, **b**: Lotong boko, **c**: Bonga, **d**: Sambo batu, **e**: Bulan (Albino), **f**: Toddi, **g**: Sorri, and **h**: White eyes (marbles). Source: The images were taken by the authors of this study.

CHARACTERISTICS

Toraya buffaloes exhibit a range of body color characteristics, including variations of black, gray, white, and mixed patterns, setting them apart morphologically from other swamp buffaloes (Windusari et al., 2019). Generally, Toraya buffaloes have a distinctive reddish-white and black color pattern with white eyes. Their head color varies, encompassing shades of gray, black, and white, while their horns are curved from the side toward the back, and their ears are directed toward the sides (Figure 2). Based on Minister of Agriculture Decree Number 2845/Kpts/LB430/8/2012 and SNI 8292.4:2016, Toraya buffalo possesses its own unique characteristics (Table 1). In addition to having a uniform physical form, Toraya buffaloes exhibit distinctive features that distinguish them from swamp buffaloes, indigenous buffaloes, and other local breeds.

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Nooy-Palm (2003) reported several variations in terms of color combinations and markings among Toraya buffaloes, commonly referred to as "bonga". The variations include Bonga saleko characterized by an almost equal combination of black and white with spots scattered throughout the body, Bonga sanga'daran distinguished by the dominance of black in the mouth area, Bonga randan dali' displaying black eyebrows, Bonga takinan gayang featuring a black pattern resembling a long machete on the back, Bonga ulu exhibiting white color exclusively on the head, while the neck and body remain black, Bonga lotong boko' showcasing a black color on the back, Bonga bulan having the entire body in white, Bonga sori presenting a white color only on the head.

There are variations in the qualitative traits of Toraya buffaloes, such as skin color, horn shape, dorsal line, white chevrons, and number of whorls. The skin color, horn shape, back line, and white chevrons on the neck are distinctive features that distinguish Toraya buffaloes from swamp buffaloes in general (Wang et al., 2017). However, the number of *unyeng-unyeng* and the white color of the feet tend to be uniform among Toraya buffaloes so that they can be used as consistent characteristics (Table 2). Furthermore, in studying the quantitative traits of Toraya buffaloes, focus is given to the body size and body weight of adult males and females to understand their stable growth patterns and consistent quantitative traits. As can be seen in Table 3, although Toraya buffaloes are categorized as swamp buffaloes, they (both males and females) have a larger body size and body weight than swamp buffaloes. Reproductive traits in female livestock refer to characteristics associated with the ability of females to reproduce. Some important female reproductive traits include sexual maturity, reproductive cycle, fertility, mating success, and gestation period.

Reproduction traits of Toraya buffalo are presented in Table 4. These reproductive characteristics encompass the male-to-female ratio, parent fertility, birth rate, age at puberty, oestrus cycle, and length of pregnancy (Perera, 2008; Komariah et al., 2012; Kep Mentan No.2845/ Kpts/ LB430/8/2012; Komariah et al., 2015; Nardi et al., 2017). Reproductive traits are economically important for sustainable food production, especially for monotocous livestock, such as buffalo (Shao et al., 2021). The reproduction characteristics are related to breeding and feeding management (Komariah et al., 2012). Komariah et al. (2012) reported that the first estrus cycle in female spotted buffaloes occurred at the age of 2.48 years. These buffaloes demonstrated 86.5% conception and 89% calving rates. The initial birthing event for spotted buffaloes typically happened at an average age of 3.74 years, with the earliest recorded at 2.87 years. The calving period extended over 12 months, leading to a calving interval of 2.04 years or 24 months. The onset of puberty in male buffalo exhibits variability similar to that in females and is primarily influenced by body weight rather than age. In optimal circumstances, the process of testicular spermatogenic cell divisions initiates around 12 months of age, and active spermatogenesis becomes evident when male buffaloes reach 15 months (Perera et al., 2008).

Table 1. The classification of color pattern in Toraya buffalo in South sulawesi, Indonesia

Classification	Color patterns	Eye color
Saleko	Spreading black color patterns with a dominant reddish-white skin base color	White
Lotong boko	Black color patterns are found on the back with a dominant reddish-white base color	White
Bonga	Reddish white color patterns are found on the entire face, neck, legs, and tail	White
Lambo Batu	Predominantly white and black on the head	White
Bulan	White dominant albino buffalo	White
Toddi'	Predominantly black with white spots on the upper face up to the nose	White
Sorri	Predominantly black with white spots on the face	White

Table 2. Qualitative traits of Toraya buffalo in South Sulawesi, Indonesia

References / Qualitatif traits	Toraya buffalo[1]	Toraya buffalo[2]
Body color	A wide variety of colors, including black, gray, white, and a mix of other colors. Color patterns on buffalo can be plain, large patches, small patches, or plain black spots.	Plain black to gray, predominantly black with white spots, predominantly white with black spots, and white stripes all over the body
Body shape	-	Large, compact, and rectangular body with sturdy legs
Head	Varies from gray, black, and white	Varies from white to black with a neckline of 1-2 stripes
Horns	Curved from side to back	Leading sideways and backwards
Ear shape	Upright towards the side	Leading sideways
Legs	-	Black and gray-white from knee to topline

[1]Kep Mentan No.2845/ Kpts/ LB430/8/2012, [2]SNI 8292.4: 2016

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Table 3. Quantitative traits of Toraya buffalo in South Sulawesi, Indonesia

Quantitative traits	Toraya Buffalo[1]		Toraya Buffalo[2]		Swamp Buffalo[3]	
	Male	Female	Male	Female	Male	Female
Shoulder height (cm)	127.1 ± 2.6	125.1 ± 2.6	112-128	110-124	110-120	105-115
Body length (cm)	140.1 ± 8.7	121.3 ± 2.3	118-149	114-136	110-125	105-120
Chest circumference (cm)	200.3 ± 5.4	190.3 ± 5.4	167-199	158-183	180-190	160-170
Scrotal circumference (cm)	-	-	22-26	-	20	-
Body weight (kg)	350-685	337-547	-	-	300-350	200-250

[1]KepMentan No. 2845/ Kpts/ LB430/8/2012, [2]SNI 8292.4:2016, [3]SNI 7706.1:2011

Table 4. Reproduction traits of Toraya buffalo in South Sulawesi, Indonesia

References / Reproduction traits	Toraya Buffalo[1]	Toraya Buffalo[2]	Swamp Buffalo[3]	Swamp Buffalo[4]	Swamp Buffalo[5]
Male to female ratio	-	3:2	-	-	-
Parent fertility (%)	78.9	86.5 ± 0.07	-	-	-
Birth rate (%)	77.8	89 ± 0.05	-	-	-
Age at puberty (years)	2.8	2.48 ± 0.37	2.5-3.0	2.13	1.3-3.8
Oestrus cycle (days)	21-23	19.5 ± 7.48	-	-	17-26
Length of pregnancy (months)	10	11.8±0.79	10.15 – 10.85	11.8±0.79	10.52-11.8

[1]Kep Mentan No.2845/ Kpts/ LB430/8/2012, [2]Komariah et al. (2012), [3]Nardi et al. (2017), [4]Komariah et al. (2015), [5]Perera (2008)

REPRODUCTIVE BIOTECHNOLOGY AND MOLECULAR GENETICS

The application of reproductive biotechnology and molecular genetics in Toraya buffalo holds significant potential to enhance population size, quality, and productivity. Several studies have been conducted to determine the molecular characteristics of Toraya buffalo, which identified phylogenetic analysis of Toraya buffalo through mitochondrial D-loop region (Sari et al., 2013) and genetic variation of Toraya buffalo based on cytochrome b gene marker (Rusdin et al., 2020). Reproductive technologies, such as artificial insemination (AI) and embryo transfer (ET), offer efficient and selective means to improve Toraya buffalo reproduction. Compared to cattle, artificial insemination is more difficult in buffaloes due to variable estrus cycles, reduced estrus behavior, and reproductive seasonality (Devkota et al., 2022). Gunawan et al. (2015) highlighted the positive impact of livestock biotechnology, particularly AI and ET, on society. These reproductive biotechnologies increase reproductive efficiency and shorten generation intervals (Visscher et al., 2000). Additionally, molecular genetics plays a crucial role in the development of Toraya buffalo.

According to Spötter and Distl (2006), molecular genetics provides genetic marker information that can aid in livestock selection. By utilizing molecular genetics techniques, breeders can produce offspring with desired characteristics, such as stripe patterns, eye color, horn shape, and coat patterns. Furthermore, molecular genetics can identify and map genes responsible for essential traits in breeding. Microsatellites, found in various parts of the genome, including coding and non-coding regions, have successfully been used to detect quantitative trait loci (QTL; Sellner et al., 2007). Methods like QTL mapping and molecular marker analysis allow researchers to establish connections between genetic patterns and phenotypic traits in Toraya buffalo, facilitating more effective and accurate parent selection based on genetic information. Additionally, genetic selection can be performed on candidate genes responsible for specific production traits, aiding in the identification and collection of genes associated with desired traits in the genome or chromosomes (Yuan et al., 2013).

The application of reproductive biotechnology and molecular genetics also significantly contributes to preserving genetic diversity. Through the identification of molecular markers, an analysis of the genetic diversity of the Toraya buffalo population can be conducted, aiding in sustainable breeding program planning and preventing the decline in genetic diversity resulting from intensive selection. Factors, such as the natural nature of buffalo with a challenging-to-detect breeding cycle, decreased genetic quality due to inbreeding, a shortage of males, and traditional husbandry practices slow down buffalo population growth. Notably, large-scale Rambu Solo events, particularly death ceremonies, involve the slaughter of multiple male buffaloes of the Saleko, Bonga, Lotong Boko, and Toddi breeds, along with some female buffaloes. Indonesian Statistics Agency reveals a high number of buffalo slaughters in the Toraja region, particularly in Tana Toraja and North Toraja districts (Indonesia), posing a threat to the future of the striped buffalo population if conservation efforts are not undertaken (BPS, 2021).

ROLE OF REPRODUCTIVE BIOTECHNOLOGY

Reproductive biotechnology plays a crucial role in managing mating for genetic improvement, particularly through the utilization of artificial insemination (AI) and embryo transfer (ET) techniques in domestic animals (Srirattana et al., 2022). The

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combined use of AI and ET can effectively accelerate the dissemination of genetic superiority in both male and female animals. A notable impact of this approach is the increase in time and cost efficiency when implementing genetic improvement programs (breeding schemes, van Arendonk, 2011). However, research in the field of reproductive biotechnology for Toraya buffalo is still relatively limited, compared to other ruminants, which can be attributed to challenges in accessing research materials and resources related to Toraya buffalo. Currently, research on Toraya buffalo is limited in terms of scope and the number of conducted studies. Although some research has been conducted, further studies are needed to expand the understanding of Toraya buffalo. A more comprehensive and broader study would offer opportunities to explore new information concerning the characteristics, genetics, reproduction, health, and management of Toraya buffaloes. Such efforts would significantly contribute to the more effective and sustainable development and utilization of the potential of Toraya buffalo.

In the domain of reproductive biotechnology research for Toraya buffalo, some studies have been conducted on the preservation and cryopreservation of sperm from the epididymis and the application of artificial insemination using frozen epididymal semen (Surachman et al., 2009; Yulnawati et al., 2010; Yulnawati et al., 2013b). A summary of these studies can be found in Table 5. For instance, Kaiin et al. (2017) reported on the comparison of sperm X (female) and sperm Y (male) characteristics of spotted buffalo from Toraja, South Sulawesi (Indonesia). When an oocyte is fertilized, an X sperm will form a female embryo, while a Y sperm will form a male embryo. The Y sperm carries the SRY (sex-determining region Y) gene, responsible for initiating testes development in male animals, and the gene is absent in X sperm (Susilawati, 2011). The study revealed that sperm X has an area of 29.86 ± 1.56 µm2 with a head length of 8.65 ± 0.33 µm and head width of 4.31 ± 0.28 µm, while sperm Y has an area of 27.16 ± 1.57 µm2, head length of 7.78 ± 0.51 µm, and head width of 4.14 ± 0.19 µm. Consequently, it was found that male sperm has a smaller size than female sperm. It is evident that further research in the field of reproductive biotechnology for Toraya buffalo holds significant potential and should be pursued to harness the genetic improvement and development of this valuable livestock breed.

Based on the data presented in Table 6, it is indicated that sperm derived from ejaculate and epididymis exhibit a similar level of quality in Toraya buffalo. Spermatozoa obtained from the epididymis, whether from slaughtered animals or livestock, represent a viable alternative sperm source for various reproductive technologies. The cauda epididymis serves as a storage site for sperm before its release during the ejaculation process (James et al., 2020). Chaveiro et al. (2015) reported that spermatozoa from the cauda epididymis demonstrate comparable motility and fertilization capacity to ejaculated spermatozoa. Studies on African buffaloes have also demonstrated that epididymal spermatozoa possess similar qualities to ejaculated spermatozoa (Herold et al., 2004; Herold et al., 2006).

The application of molecular genetics in livestock breeding, particularly for obtaining QTL and gene markers, presents a novel approach enabling the early selection of superior traits. This approach allows livestock breeding to focus on maintaining individuals with detected superior genes, consequently reducing the required resources and costs compared to breeding programs without applying molecular genetics. By utilizing molecular genetics techniques, livestock breeding programs can be more efficient and targeted, developing superior livestock with desired traits. Information on molecular genetics research on Toraya buffalo is limited and still in its early stages. In a recent molecular study focusing on non-functional traits related to coat color in Toraya buffalo, Yusnizar et al. (2015) reported Two distinct loss-of-function genetic mutations, including an early stop codon and a truncation site donor mutation in the microphthalmia-associated transcription factor (MITF), were found to be significantly linked to the presence of mottled coat color. The MITF gene controls the stripe color pattern in Toraya buffaloes, but the occurrence of stripe levels may be due to other additive/quantitative genes.

Table 5. Biotechnology reproduction research in Toraya buffalo, Indonesia

Reproductive technology	Research topic	References
Artificial insemination applications	Frozen-Thawed Epididymal Sperm Quality and the Success Rate of Artificial Insemination in Spotted Buffaloes (*Bubalus bubalis carabanensis*)	Yulnawati et al. (2013a)
Epididymal sperm	Epididymal Sperm Quality of Buffaloes with Different Spotted Types	Yulnawati et al. (2013b)
Sperm cryopreservation	Quality of Epididymal and Ejaculated Sperms of Spotted Buffalo in Dextrose Supplemented Extender	Yulnawati et al. (2010)
Sperm preservation	Quality of Liquid Semen from Striped Buffalo Epididymis in Andromed Diluent with Sucrose Addition	Surachman et al. (2009)
Sperm sexing	Separation of X and Y sperm using the bovine serum albumin (BSA) column method	Kaiin et al. (2017)

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Table 6. Sperm quality of Toraya buffalo, Indonesia

Sperm quality / Parameters	Ejaculate[1]	Ejaculate[2]	Epydidimis[3]	Epydidimis[4]	Epydidimis[5]
Volume (mL)	4	-	-	-	-
Color	Creamy	-	-	-	-
pH	6-7	-	-	-	-
Consistency	Moderate	-	-	-	-
Mass movement	++	-	-	-	-
Motility (%)	75	70.0 ±0.0	65.00±0.0	74.17±1.86	65.0±0.0
Concentration (x106)	1.790	2695+ 1045	10.533±47	3578.33±740	10710±49
Viability (%)	79.4	-	76.00±2.83	85.02±2.35	-
Abnormality (%)	17	6.5 + 1.5	15.33±2.49	7.10±1.16	15.0±3.0
IM (%)	70.9	77.5±1.5	80.80±0.40	86.22±1.94	79.0±0.0

IM: Integrity membrane. Sources of table: [1]Kaiin et al. (2017); [2,5]Yulnawati et al. (2010); [3]Surachman et al. (2009); [4]Yulnawati et al. (2013b)

DEVELOPMENT POTENTIAL TO INCREASE PRODUCTIVITY

The development of livestock resources, particularly buffaloes, aims to enhance the regional economy while considering the sustainability of natural resources. Besides fostering economic growth and improving community welfare, this development objective also entails preserving the environment and reducing social inequalities (Devkota et al., 2022). This approach is in accordance with the principles of sustainable development, which prioritize long-term environmental and economic viability. In the framework of sustainable development, livestock farming economic sustainability demonstrates the capacity to maintain a growing livestock population and positively impacts the welfare of livestock owners (Suyitman et al., 2016).

Toraya buffalo, as one of the swamp buffalo breeds, exhibits significant potential for development. This potential is tied to the cultural customs of the people of South Sulawesi, particularly the people of Tana Toraja, where buffaloes play a crucial role and symbolize cultural ceremonies and social status (Mangopang et al., 2018; Saputra et al., 2020; Warman et al., 2022). The number of buffaloes sacrificed in a traditional feast directly correlates with a person's social status, increasing demand for buffaloes during such ceremonies, especially the Rambu Solo (death ceremony). The high demand for Toraya buffaloes often exceeds their availability, necessitating buffalo imports from areas outside Tana Toraja, North Toraja, Takalar District, and other regions in West Sulawesi Province, Indonesia (Anshar, 2013). In the past, traditional ceremonies in Toraja typically demanded only two buffaloes as a standard requirement (Sapu Randanan). However, modern practices now vary based on an individual's financial capacity, with some ceremonies involving hundreds of buffaloes as a gesture of respect for deceased relatives (Rombe, 2010), contributing to a decline in the buffalo population.

In Toraja society, a specific valuation scheme is utilized to determine the value and price of Toraya buffaloes. Research conducted by Saleh and Asnawi (2014) stated that the selling price of Toraya buffaloes in Bolu Animal Market, North Toraja Regency, is determined based on certain characteristics, such as striped motifs, eye color, horn model, tail condition, and spotted coat color. Socio-cultural factors heavily influence the pricing of Toraya buffalo. Despite being considered livestock, their value falls under the art category, making the price of buffaloes incomparable to other livestock commodities. The more the buffalo fulfills the socio-cultural criteria, the higher its price will be.

The potential for the development of Toraya buffalo livestock is immense in Indonesia. Toraya buffaloes are distinct from swamp buffaloes, offering more specific and focused development opportunities (Rohaeni et al., 2023). One of the primary assets is the high artistic value associated with the Toraya buffalo and its close ties to Toraja traditional ceremonies. This creates a consistent and sustainable demand for Toraya buffalo, deeply rooted in local culture and tradition. Moreover, Toraya buffalo development holds significant economic potential. By increasing the population and productivity of Toraya buffalo through planned breeding programs and the application of reproductive technology and genetic-based selection, superior animals can be produced in terms of quality, growth, and productivity. This opens up opportunities for farmers to increase their income by selling Toraya buffaloes, which have a high market value. Comprehensive support is required to optimize this development potential, including further extensive research, breeder development, access to reproductive and genetic technologies, and supportive policies. With a holistic and sustainable approach, Toraya buffalo development can positively impact local communities, the environment, and the regional economy.

CONCLUSION

To increase the population and productivity of Toraya buffalo, the implementation of a planned breeding program combining reproductive technology and selection based on quantitative and molecular genetics is necessary. The development potential of Toraya buffalo is immense; it not only possesses high cultural value but also exhibits superior

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quantitative characteristics, such as growth rates and increased meat yield, compared to swamp buffalo in general, positioning it as a potential national meat provider.

DECLARATIONS

Availability of data and materials

All data are presented in the published manuscript.

Authors' contributions

This study was carried out with the contribution of all authors. Tulus Maulana collected information and drafted the manuscript. Hikmayani Iskandar collected information and contributed to writing the manuscript. Syahruddin Said and Asep Gunawan revised the manuscript. All authors read and approved the final version of the manuscript.

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Ethical consideration

The author checked plagiarism, misconduct, data fabrication and/or falsification.

Competing interests

The author declares no conflicts of interest.

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A Pathologic Case of Gout Tophi Deposition with a Concurrent Systemic Bacterial Infection in a Leopard Gecko (*Eublepharis macularius*)

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ABSTRACT

Gout is caused by excessive uric acid in the blood deposited in tissues (visceral gout) or joints (articular gout), leading to severe inflammation and pain. A female leopard gecko was presented to the University Veterinary Hospital, University of Putra, Malaysia, with a history of swelling at the left caudal mandible, inappetence, and weight loss. An oral examination indicated a swollen mouth with scabs on the upper right mandible and multiple whitish deposits inside the mouth. The preliminary diagnosis was mouth rot, and the treatment included metronidazole and a multivitamin supplement. The leopard gecko died 12 days after treatment since there was no improvement. Post-mortem examination revealed that the liver was slightly enlarged with generalized moderate congestion and the presence of whitish deposits, as well as noticeable whitish deposits on the pleural surface of the lungs. Histopathological examination of the lungs revealed a granuloma with an inflammatory reaction predominantly by abundant mononuclear cells and fibrin deposition. An irregular collection of amorphous materials in the visceral pleura suggested gout tophi. The liver was infiltrated with amorphous material and fibrinous tissue, and it had mild congestion, indicating visceral gout and bacterial infection. *Klebsiella pneumoniae* and *Proteus mirabilis* were isolated from the lungs and liver samples, respectively. In conclusion, gout tophi is common in reptiles, but visceral involvement is rare, and early detection is critical to avoid secondary bacterial infection, as demonstrated in this case.

Keywords: Amorphous material, Bacterial infection, Gout tophi, Histopathology, Leopard gecko

INTRODUCTION

Leopard geckos belong to the *Eublepharidae* family, which has six genera and 38 species worldwide (Uetz et al., 2023). Leopard gecko, a popular exotic pet, can live up to 22 years and are native to central and south Asia, including Afghanistan, Iraq, Iran, Pakistan, and India (Woods, 2001). In the wild, they eat invertebrates, but in captivity, they eat a variety of invertebrates, such as crickets, worms, and locusts (RSPCA, 2019). The ability to manage uric acids is determined by the amount of protein consumed, the type and frequency of feeding, and the state of hydration in all reptiles, including geckos (Rich and Axelson, 2023).

Gout is a metabolic disorder resulting from either the overproduction of uric acid or the body's inability to eliminate it, leading to excessive amounts of uric acid in the blood, which eventually deposited in tissues (visceral gout) or joints (articular gout), resulting in severe pain and inflammation (Lock, 2017; Doneley et al., 2018). High-protein diets, dehydration, low temperatures, hunger, nephrotoxic substances, and inactivity are possible risk factors (Mader, 2005; Rich and Axelson, 2023).

Several clinical signs of gout have been reported in various species of reptiles. Lethargy, weakness, painful joints, and swollen feet are symptoms of articular gout (Mader, 2005; Lock, 2017). In visceral gout, a swollen mouth due to stomatitis can be misdiagnosed as infectious stomatitis in snakes, in which the whitish lesions in the gingiva were thought to progress from the infectious stomatitis, as it were the depositions of gout tophi and anorexia (Mader, 2005). The present study reported pathological findings of gout tophi in a pet leopard gecko in Malaysia.

CASE REPORT

A one-year-old female leopard gecko weighing 35 gm was presented to the Avian and Exotic Animal Unit, University Veterinary Hospital UPM, with complaints of weight loss and a swollen mouth. The leopard gecko was the only one in a

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vivarium with moist and warm hiding places. It was occasionally fed mealworms with calcium powder and had a drinking water source available 24 hours a day. As mentioned by the owner, no abnormalities were observed during the most recent shedding. The gecko began to eat less and gradually lost tail thickness, as well as developing left-side mouth swelling. Physical examination revealed that the gecko was quiet but alert and responsive, with a lower body condition score assessed by the amount of fat storage in the tail (Figure 1A). It had a swollen mouth at the left caudal mandibular area (Figure 1B) with scabs visible on the upper right side, multiple whitish deposits inside the mouth when opened, and retained shed skin on the toe. At this point, a preliminary diagnosis of stomatitis was made. The prescribed treatment plan included oral administration of metronidazole (Metrogyl® suspension, Unique Pharmaceutical Laboratories, India) at a dosage of 30 mg/kg every 48 hours for two treatments. Additionally, 1 mL of multivitamins (Uphavit syrup, Duopharma, Malaysia) was prescribed daily until recovery. In addition, it was advised to increase the humidity from 20% to 40% and monitor the vivarium using a hygrometer.

However, 12 days post-treatment, the gecko was found dead and was immediately sent to the Post-mortem Laboratory, Veterinary Laboratory Service Unit (VLSU), Faculty of Veterinary Medicine, Universiti Putra Malaysia, for further investigation. The post-mortem examination revealed that the carcass was in fair condition, with minimal visceral and subcutaneous fat stores, incomplete skin shedding and sunken eyes indicating severe dehydration. The liver was slightly enlarged with generalized moderate congestion, and whitish deposits were also present on the pleural surface of the lungs. Histopathological findings by hematoxylin-eosin (H&E) staining of the lungs included dilated capillaries in the alveolar walls, thickened alveolar walls and moderate vascular congestion with a morphological diagnosis of bacterial infection, lung granuloma with inflammatory reaction predominantly by abundant mononuclear cells and fibrin deposition, apart from the presence of irregular accumulation of amorphous materials (gout tophi) in the lungs suggestive of bacterial infection and visceral gout, respectively (Figure 2 A-C). The visceral pleura was covered by abundant fibrin, necrotic cellular debris, and it was expanded by increased amounts of fibrous connective tissue and the infiltration of amorphous materials, indicating bacterial infection and visceral gout, respectively (Figure 2B). The liver was moderately congested with degenerating hepatocytes containing numerous cytoplasmic lipid droplets, and the infiltration of amorphous materials and fibrinous tissue is suggestive of bacterial infection and visceral gout (Figure 2C). Bacterial culture of the lungs and liver samples revealed *Klebsiella pneumonia* and *Proteus mirabilis* were isolated with degrees of bacteria isolation of 2+ and 4+, respectively. Based on the findings, the final diagnosis was visceral gout with a systemic bacterial infection.

DISCUSSION

Gout can be caused by uric acid overproduction, such as long-term dehydration and a high-protein diet, or by a failure to excrete uric acids due to renal failure (Lock, 2017). Environmental changes, particularly temperature and humidity, may disrupt reptiles' physiological functions and homeostasis, deteriorate their general health, and cause dehydration. In this case, the gecko refused to eat for more than 2 weeks, while force-feeding, including the water, was deemed crucial. Improper care may result in dehydration. A high-protein diet may increase the chances of hyperuricemia (Hong et al., 2020). According to Liu et al. (2020), mealworms have approximately 17.6% protein and 49.1% dry matter, so regular consumption of mealworms may contribute significantly to the high uric acid production. Some of the clinical symptoms associated with visceral gout in reptiles, including depression, weakness, and dehydration, as well as reluctance to move and eat, were noted in this gecko (Lock, 2017). In cases of articular gout, swollen joints in the legs and feet can be seen, along with or without nodules on the ribs, but this was not the case in this instance. In leopard geckos, hyperuricemia is defined as uric acid levels greater than 8 mg/L (Mader, 2005). A radiograph is another diagnostic test that is less invasive than blood collection and can be used to diagnose gout in reptiles and leopard geckos (Lock, 2017).

There is little research on the treatment of gout in reptiles. According to Martinez-Silvestre (1997), allopurinol therapy at the dosage of 20 mg/kg is effective in treating tortoises with gout. Allopurinol dosages are based on human prescription, and while they have no side effects, they should be used cautiously in reptiles. In that study, 97.3% of tortoises were given 50 mg/kg orally once a day for 30 days, leading to reduced uric acid by day 7 post-treatment. Furthermore, the medication was continued for 3 years, and administered once every 3 days. The urate tophi disappeared 2 to 4 months after treatment with concentrations less than 2 mg/L of uric acid. Thus, the use of allopurinol for the treatment of gout in all reptile species should be investigated further. Allopurinol works by inhibiting the actions of xanthine oxidase to promote urate excretion and manage acute gouty arthritis attacks with anti-inflammatory drugs such as colchicine and corticosteroids (Mader, 2005).

In this case, the main cause of visceral gout would be dehydration due to low humidity, which impaired the kidney's ability to remove uric acid properly, causing uric acid to accumulate in the blood and cause hyperuricemia (Mader, 2005; Boyer et al., 2014). This caused uric acid crystallization, leading to deposits in internal tissues and histopathologically observed in the visceral pleura, lungs, and liver, as seen in this case. The likely pathogenesis for this case included injury to the mouth, leading to the loss of the skin barrier. Subsequently, this loss facilitated the

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development of stomatitis (mouth rot) by opportunistic pathogens of the normal flora from the oral cavity. The resulting pain further contributed to reduced feed and water intake, resulting in dehydration. Next, the leopard gecko became uncomfortable eating or drinking, resulting in inappetence and dehydration, and consequently, problems, such as dysecdysis arose. The cause of death for the gecko could be attributed to circulatory failure resulting from a decrease in cardiac output caused by a decrease in venous return. This decline can be a consequence of vasodilation caused by bacteremia or septicemia from the proliferation of bacteria in the lung. Concurrently, there was bacterial proliferation in the lungs, along with the presence of visceral gout due to hyperuricemia. The hyperuricemia could have resulted from the invasion of pathogenic bacteria through the respiratory tract from the oral cavity either through ingestion or ascending infection of stomatitis (mouth rot) caused by opportunistic pathogens of the normal flora, such as *Proteus mirabilis* and *Klebsiella pneumonia*. *Proteus mirabilis* and *Klebsiella pneumonia* are classified as normal flora in many reptiles (Gigani et al., 1986; Ghosh et al., 2018). The presence of these bacteria in the studied tissues indicated bacteremia or septicemia, originating from the normal flora becoming opportunistic pathogens.

CONCLUSION

Visceral gout can be misdiagnosed since such an occurrence is rare in leopard geckos, compared to articular gout. The prognosis is poor with secondary bacterial infection. A proper diagnosis is required to determine the definitive diagnosis and formulate effective treatment.

DECLARATIONS

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Authors' contribution

Mazlina Mazlan, Muhamad Alif Zakaria, and Mohd Asrul Syafiq performed the necropsy and diagnosis. Mohd Asrul Syafiq, Mazlina Mazlan, Luqman Abdul Samad, and Azlan Che-Amat contributed to the case analysis and drafted a manuscript. Azlan Che-Amat and Mazlina Mazlan revised the final edition of the manuscript. All authors approved the article before publication in the present journal.

Competing interests

There is no conflict of interest.

Ethical consideration

The owner of the gecko has given consent and signed the permission for data publication. The authors have checked and compiled ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy).

Availability of data and materials

The data of the current case report are available.

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Hematological and Biochemical Parameters of Macropod Progressive Periodontal Disease in Wild Western Gray Kangaroos

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ABSTRACT

Macropod progressive periodontal disease (MPPD), known as *Lumpy Jaw*, poses a persistent and potentially fatal threat in Western gray kangaroos when they are kept in captivity. Such a condition leads to the development of osteomyelitis and sepsis in Western gray kangaroos (*Macropus fuliginosus*). This case study presented the inaugural examination of hematological and biochemical aspects of MPPD with a progression toward sepsis in a captive environment. The primary objective of this research was to pinpoint hematological and biochemical indicators associated with severe MPPD in a Western gray kangaroo held in captivity. The study employed various methods, including clinical, radiographical, hematological, and biochemical analyses, as well as microbiological study methods. The case was a 2.5-year-old male wild Western gray kangaroo with fever (39.7 °C), dehydration, dyspnea, tachycardia, and involuntary jaw clenching due to stress and agitation. The kangaroo had a history of lethargy, anorexia, swelling of the soft tissues of the lower jaw on the left side, and tenderness during palpation. A radiograph of the head revealed mandible proliferative lesions. The hematological and biochemical examinations indicated an increase in the total count of leucocytes, level of neutrophils, number of erythrocytes, hematocrit level, and lymphopenia. Increased activity of alkaline phosphatase, amylase, and creatinine elevated azotemia. There was a decrease in the content of albumin, glucose, and total bilirubin. The bacteria, consisting of *Fusobacteriaceae* spp., *Porphyromonadaceae* spp., and *Bacteroidaceae* spp., were found and identified in all samples. However, this comprehensive diagnosis of MPPD based on clinical signs, radiography, and especially hematological and biochemical parameters of the septic process can be helpful in diagnosis and treatment.

Keywords: Macropod Progressive Periodontal Disease, *Macropus fuliginosus*, Hematological and Biochemical parameters

INTRODUCTION

The welfare of exotic pets is becoming increasingly important in the professional work of biologists and veterinarians (Sotohira et al., 2017; Rendle et al., 2020a). Exotic species are characterized by various lifestyles, behaviors, and reproduction, necessitating special conditions for their care, maintenance, and feeding (Sherwen et al., 2015; Kido et al., 2018).

Among these exotic species is a wild Western grey kangaroo (*Macropus fuliginosus*), which historically inhabited Australia and the island of Tasmania until the 19th century. Today, kangaroos are also kept in national parks and private zoos worldwide (Sotohira et al., 2017).

In contrast to periodontal disease in humans, macropod progressive periodontal disease (MPPD) frequently advances to necrotizing osteomyelitis affecting the mandible or maxilla. This progression involves the development of sequestra and the extensive growth of subperiosteal bone, ultimately causing deformities in the jawbone. While the pathogenesis of MPPD is deemed comparable to that in humans, the uncommon occurrence of osteomyelitis, suppurative inflammation, and necrosis in adjacent soft tissues observed in macropods sets it apart from similar conditions in humans (Yip et al., 2021). The common cause of pathologies is often attributed to keeping animals in captivity. This requires special knowledge and professional skills from veterinarians to make an accurate diagnosis and conduct treatment. Limited published data on the results of hematological and biochemical changes during severe MPPD in kangaroos are available. Some authors have reported the risks of developing this pathology (Kido et al., 2013; Rendle et al., 2020b). Therefore, this study aimed to present the clinical signs and changes in hematological and biochemical indicators of MPPD in Western Gray Kangaroos.

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CASE REPORT

Ethical approval

This investigation was approved according to the Law of Ukraine (No. 3447-IV of February 21, 2006), according to the requirements of an Ethical Permit (Consensus Guidelines on Animal Ethics and Welfare for Veterinary Journals – International Association of Veterinary Editors, Geneva, Switzerland, 2010).

Case presentation and clinical signs

A male wild Western grey kangaroo *(Macropus fuliginosus)* aged 2.5 years was referred to the Animal Central Clinic City of Kyiv, Ukraine. It weighed 52.3 kg, measured 0.95 m in length with a 0.93 m tail, and a height of 1.27 m. The kangaroo had a history of lethargy, anorexia, hypersalivation, and anxiety in history. Physical examination of the kangaroo revealed fever (39.7 °C), dehydration, shortness of breath and tachycardia, and involuntarily clenching the jaw due to stress and anxiety. The initial dental exam indicated excessive secretion of frothy saliva, soft tissue edema on the left side of the lower jaw, and pain on palpation. A radiograph of the head revealed a large radiodense mass in the left part of the lower jaw. Proliferative lesions of the lower jaw with intraosseous opacity (osteolysis) were seen, which created expanding lesions (Figure 1). Hematological studies indicated significant changes in the main parameters (Table 1).

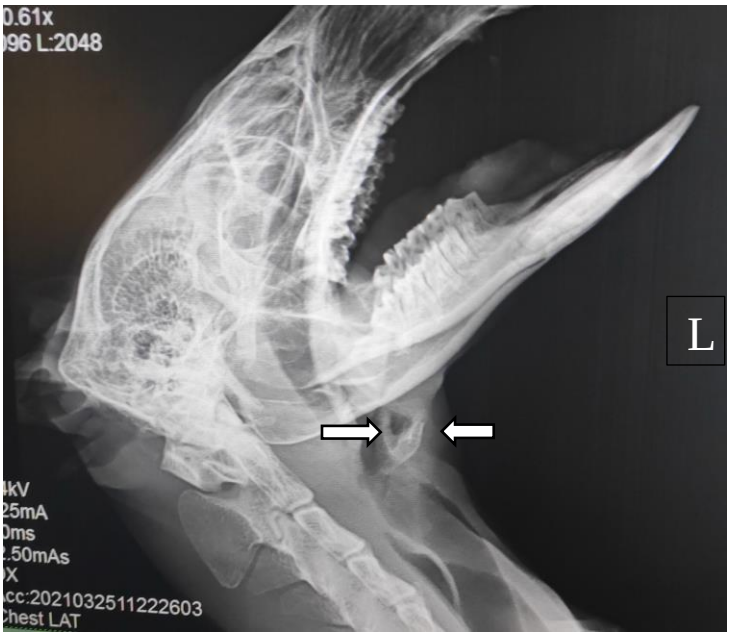

Figure 1. The lateral view of the skull of a wild Western grey kangaroo aged 2.5 years with signs of macropod progressive periodontal disease. The mandible proliferative lesions with intraosseous opacity (osteolysis) and periosteal new bone formation create expansile lesions (white arrows; lateral plane). Bar = 10 mm.

In particular, the acute inflammatory process of the periodontium was accompanied by a marked increase in the count of erythrocytes (6.05×10^{12}/L) and level of hematocrit (0.94 L/L). The laboratory outcomes revealed leukocytosis (13.17×10^{9}/L) with neutrophilia (88.4 %) and lymphopenia (6.1 %). Abnormalities of serum biochemistry included an increase in the concentration of total protein and globulin and a decrease in the albumin level (Table 2). Biochemical changes in the patient's body were characterized by an increase in alkaline phosphatase activity (at 4.45 mmol/L), alanine aminotransferase (at 2.52 mmol/L), and amylase (at 21.16 mmol/L). The activity of creatinine (194.48 μmol/L) and blood urea nitrogen level (at 16.07 mmol/L) were increased. The content of phosphorus and calcium exceeded the upper reference values. The laboratory results determined a decrease in the content of albumin, level of glucose, and total bilirubin (Table 2).

Microbial associations of bacteria were identified in the samples collected from the gums and tooth using a sterile curette or a swab from the subgingival margins. Bacterial species were identified in the microbiological laboratory. The microbiological laboratory utilized non-selective (Wilkens Chalgren Agar, WCA), selective, and enriched media. This included glucose-enriched thioglycollate medium (BBL™ Thioglycollate Medium, Enriched with Vitamin K1 and Hemin, and also Calcium Carbonate), anaerobic kanamycin-vancomycin blood agar (B.D. ™ Schaedler Kanamycin-Vancomycin Agar with 5% Sheep Blood (Schaedler-KV Agar) for selective isolation of gram-negative anaerobes, and Bacteroides Bile Esculin (BBE) agar. The colony morphology was studied using a stereoscopic microscope (Zeiss Discovery.V12 APO Stereo Motorized Microscope Stereoscope - A.V.), which was also used for the tentative

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differentiation of bacteria. Microbial strains (*Fusobacteriaceae* spp., *Porphyromonadaceae* spp., and *Bacteroidaceae* spp.) play a significant role in the pathogenesis of MPPD (NCBI, 2012).

The diagnosis of MPPD was established through a comprehensive assessment, incorporating the case history, clinical signs, hematology, and biochemical parameters. To provide intensive therapy, the animal was transferred to the surgical department of the biotechnological university (Kyiv, Ukraine). As reported by the surgical department, a successful operation was conducted to extract the diseased teeth. The operation was performed under general anesthesia successfully. Following the surgery, antibiotic therapy was administered using clindamycin (Cleocin, Med-Vet International, USA) in a single dose of 20 mg/kg intravenously (*v. coccygeal ventralis*) every 12 hours for 14 days.

Table 1. Hematological parameters of an adult male wild Western grey kangaroo (*Macropus fuliginosus*) with macropod progressive periodontal disease

Parameter	Unit	Normal mean (ranges)	MPPD
Erythrocytes	$\times10^{12}$/L	2.99 (1.52–4.83)	6.05
Leukocytes	$\times10^{9}$/L	7.1 (2.16–14.36)	13.17
Neutrophil	%	55.83 (10.17–68.2)	88.4
	$\times10^{9}$/L	2.47 (0.45–5.7)	11.64
Lymphocyte	%	27.0 (9.39–61.32)	6.10
	$\times10^{9}$/L	3.85 (1.34–8.75)	0.81
Eosinophils	%	5.16 (0.37–6.04)	4.50
	$\times10^{9}$/L	0.56 (0.04–1.41)	0.61
Monocytes	%	7.33 (0–8.1)	0.92
	$\times10^{9}$/L	0.13 (0-0.52)	0.12
Basophils	%	4.66 (0–5.1)	0.10
	$\times10^{9}$/L	0.01 (0-0.09)	0.01
Haemoglobin	µmol/L	1290.21 (967.11-1640.14)	1600.0
Haematocrit	L/L	0.27(0.14-0.41)	0.94
MCV	fl	91.12(62.15-107.91)	82.0
MCH	fmol	2.79 (1.39-4.59)	1.77
MCHC	mmol/L	30.89 (21.31-50.31)	20.10
Thrombocyte	$\times10^{9}$/L	155.94 (66.95-286.1)	258.0

MCV: Mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, MPPD: Macropod progressive periodontal disease

Table 2. The blood biochemical parameters of an adult male wild Western grey kangaroo (*Macropus fuliginosus*) with macropod progressive periodontal disease

Parameters	Normal mean (ranges)	MPPD
Albumin (mmol/L)	0.71 (0.70-0.72)	0.57
Alkaline phosphatase (mmol/L)	1.62 (1.61-1.63)	4.45
Alanine aminotransferase (mmol/L)	1.27 (1.2-1.35)	2.52
Amylase (mmol/L)	3.01 (2.66-3.56)	21.16
Total bilirubin (µmol/L	7.5 (7-8)	0.61
Blood urea nitrogen (mmol/L	8.60 (8.4-8.8)	16.07
Ca^{2+} (mmol/L	2.19 (2.17-2.22)	2.34
P (mmol/L)	1.96 (1.77-2.15)	2.57
Creatinine (µmol/L)	125.0 (121.0-129.0)	194.48
Glucose (mmol/L)	8.35 (7.0-9.7)	4.10
Na^{+} (mmol/L)	161.00 (157.0-165.0)	139.0
K^{+} (mmol/L)	4.0 (3.1-4.9)	4.80
Total protein (g/L)	58.0 (56.0-60.0)	80.10
Globulin (g/L)	10.50 (8.0-13.0)	42.10

MPPD: Macropod progressive periodontal disease

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DISCUSSION

Macropod progressive periodontal disease (MPPD), commonly referred to as lumpy jaw in Western gray kangaroos, tends to be a persistent and potentially lethal condition in captive kangaroos (Vogelnest and Portas, 2019; Purcarea and Sovaila, 2020; Rendle et al., 2020a). The MPPD is a severe complication involving the inflammation and infection of bone tissue. In the context of the jaw, this condition can lead to the formation of sequestra and the proliferation of subperiosteal bone, ultimately resulting in bone deformities, which may manifest as the characteristic 'lumpy jaw.' Gingivitis and periodontitis, if left untreated or in specific cases, can lead to complications such as periodontitis-osteomyelitis, where the infection and inflammation extend from the gums and periodontal tissues into the underlying bone, potentially contributing to osteomyelitis (Antiabong et al., 2013; Rendle et al., 2020b).

The distinct purulent and necrotizing lesions are believed to result from infection from anaerobic bacteria, such as *Fusobacterium necrophorum* (Antiabong et al., 2013; Kido et al., 2013; Rendle et al., 2018; Ward et al., 2018). The disease initiates as periodontitis with the invasion of the mucosa by saprophytic bacteria, such as *Corynebacterium pyogenes*, and *Dichelobacter nodosus*, which then extends to adjacent bones, resulting in osteomyelitis (Yip et al., 2021). Protective factors and the progenitor microbiome are important in local immune protection of the mucous membrane (Zhelavskyi, 2021). These lesions typically manifest in the jaws, feet, and less commonly, in stomach. Secondary infections can develop in the intestinal wall, lung, liver, and brain. While wallabies are not uniquely susceptible to *Fusobacterium necrophorum*, predisposing factors such as trauma, fecal contamination of the environment, and stress play a crucial etiological role.

During differentiation, gingivitis, periodontitis, and periodontal abscesses initiated by plaque bacteria should be considered. According to the published studies, gingivitis is a reversible inflammation of the gingival margins, while periodontitis involves the periodontal ligament, connective tissue attachment, and loss of alveolar bone (Antiabong et al., 2013; Hoyer et al., 2020). Diagnosis primarily relied on clinical appearance, radiography, and hematology of suspected cases (Hao et al., 2022). Determination of α-amylase is of great clinical and diagnostic importance in diagnosing and monitoring acute and exacerbations of chronic pancreatitis. However, elevated serum α-amylase levels may be present in pancreatitis, inflammatory salivary glands, and various oral tissues (Dave et al., 2021). Alanine aminotransferase enzyme is found in many animal body cells. However, its highest concentration is in liver cells and kidneys, to a lesser extent, in the heart, pancreas, and skeletal muscles. An increase in transferase activity is a sign of the development of a systemic pathology (Dugar et al., 2020; Zhelavskyi et al., 2020; Zhelavskyi et al., 2023).

All wallabies are thoroughly examined for signs of MPPD during any handling procedure. Special attention is focused on the gingival mucosa where pinhead-sized sinuses might indicate extensive submucosal and perialveolar infection. The diagnostic potential of clinical hematology indicates raised fibrinogen levels and abnormalities of neutrophil morphology as more consistent findings in MPPD cases than in neutrophilia (Antiabong et al., 2013; Rendle et al., 2020b). Septic conditions lead to the malfunctioning of all systems and organs, resulting in severe pathology and frequently serving as the primary cause of the animal's demise. Hematological and biochemical parameters indicate the progression of pathology in afflicted animals (Rendle et al., 2020b; Jevon et al., 2021; Hao et al., 2022).

Treatment of kangaroos with MPPD is also carried out with antibiotics. Literature reports highlight the successful use of clindamycin (Watson et al., 2017; Birot et al., 2022). Currently, intravenous oxytetracycline at 10 mg/kg every day, plus oral metronidazole at 60-70 mg/kg is used daily for MPPD. Antibiotic therapy can continue until at least 2-3 weeks after clinical signs have resolved (Watson et al., 2017). Additionally, there is a mention of adjunctive therapy involving parenteral vitamin A. In cases where repeated handling of untamed animals for antibiotic administration causes stress, the use of long-acting oxytetracycline or amoxicillin every 3-4 days is recommended. Whenever possible, oral metronidazole is administered prophylactically to wallabies that have suffered trauma, and MPPD is a complication (Birot al., 2022).

Even minute sequestra of alveolar bone within granulating lesions significantly impede healing and must all be removed (Birot et al., 2022). To prevent continual re-contamination of an open or 'dry' socket by food material, a packing of zinc oxide/oil of cloves/oil of wintergreen/oil of cinnamon can be useful ('Chlorbutanol', Produits dentaires, S.A. Vevey). The chlorobutanol pack has strong analgesic and antiseptic properties and can be left *in situ* for up to 2 weeks without becoming putrid. In cases of extensive bony involvement, human destruction should be considered.

Macropod progressive periodontal disease can be identified during routine oral cavity examinations. Removal of calculus through ultrasonic or hand-scaling is recommended. In severe cases, parenteral antibiotic therapy is often necessary. Dental abscesses are frequent in macropods, often necessitating surgical debridement of the abscess and sometimes dental extraction (Rendle et al., 2020b; Birot al., 2022).

CONCLUSION

The insights from this case report can contribute to achieving a successful diagnosis outcome for macropod progressive

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periodontal disease in Western grey kangaroos by considering the clinical signs, radiography, and particularly hematological and biochemical parameters. Animal owners and veterinarians must consider all risk factors that can cause this disease.

DECLARATIONS

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Availability of data and materials

The data of the current study are available by request from the authors.

Authors' contribution

Mykola Zhelavskyi originated the presented concept, validated medical history, contributed to data collection, and conducted the experiment. Serhii Kernychnyi authored the manuscript and handled the submission process. Tamara Betlinska participated in designing and coordinating the study as well as providing assistance in drafting the manuscript. All authors reviewed and approved the final edition of the manuscript.

Ethical consideration

The authors considered all necessary ethical issues (e.g., plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy).

Competing interests

The authors declare that they have no competing interests.

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Clinical and Molecular Detections of Lumpy Skin Disease: Possibilities of Co-infection with Foot-and-Mouth Disease

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CASE REPORT
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ABSTRACT

Lumpy skin disease (LSD) and foot and mouth disease (FMD) are notable viral diseases of cattle. This report aimed to highlight the possibilities of an uncommon case of LSD and FMD co-infection. The report also presents the clinical and molecular detection of LSD virus in six crossbred calves and LSD and FMD virus co-infection in a heifer at small-scale dairy farms located in northern Ethiopia. Nasal swabs and tissue samples were collected following aseptic techniques from the six calves suspected of having LSD and a tissue sample from one heifer suspected of having LSD-FMD co-infection and submitted to the laboratory for cell culture and real-time polymerase chain reaction (PCR) tests. Different-sized, firm, painful skin nodules with necrotic centers were seen on different parts of the calves' body. Swelling of the prescapular and prefemoral lymph nodes, conjunctivitis, and corneal cloudiness were also observed. Uniquely, one heifer was seen with erosive lesions in the oral cavity and tongue, salivation, lameness, and skin nodules. Intracytoplasmic inclusion bodies, a distinctive feature of LSD virus, and the formation of syncytia, a characteristic of FMD virus, were observed in the cell lines. The heifer was diagnosed with a rare co-infection of LSDV and FMDV based on clinical signs, cell culture, and real-time PCR test results. The other six calves were diagnosed with the LSD virus. Treatment with broad-spectrum antibiotics, local wound cleansing, and anti-inflammatory drugs was initiated. Unfortunately, the heifer with LSD-FMD co-infection died while under treatment, and just three calves with LSD were recovered. It can be concluded that vaccination of animals against both diseases and promotion of bio-security protocols in farms is more helpful than treatment, and early case reporting is also warranted to avoid losses related to the diseases.

Keywords: Calves, Co-infection, Foot and mouth disease, Heifer, Lumpy skin disease

INTRODUCTION

Foot and mouth disease (FMD) is a severe, clinically acute, vesicular disease of both domestic and wild cloven-hoofed animals (Pacheco et al., 2010). The disease has the potential for rapid and widespread transmission within and between countries, as well as severe economic consequences (Sarker et al., 2011). The disease is distinguished by vesicular lesions on the feet, mouth, snout, and teats, as well as fever, lameness, loss of appetite, and salivation (Arzt et al., 2011). Furthermore, vesicles (blisters) on the tongue, dental pad, or another area of the skin that burst after 3 days leave shallow erosions (Tolawak and Pal, 2022). Despite the fact that FMD does not have a high mortality rate in adult animals, it has major implications such as weight loss, a drop in milk yield, reproductive problems, and a loss of draught ability, which leads to lower productivity (Knight-Jones and Rushton, 2013). In young animals, the virus causes heart degeneration, which might result in a high mortality rate (Sarker et al., 2011). The diagnosis of FMD depends on characteristic clinical signs and laboratory testing to determine the serotypes of the causal virus (Admassu et al., 2015). The detection and isolation of the FMD virus are required to confirm the FMD outbreak and design prevention strategies (Kitching et al., 2005). Because FMD is a viral disease, treatment focuses on preventing secondary bacterial complications using broad-spectrum antibiotics and other supportive care (Ole-Miaron, 2003).

Lumpy skin disease (LSD) is another devastating viral skin disease of cattle caused by the Lumpy skin disease virus (LSDV), a member of the *Poxviridae* family of the genus *Capripoxvirus* (Gupta et al., 2020). The virus can cause infection mainly in cattle and buffaloes; there are also reports in other wild ruminant species, such as giraffes, and springboks (Ratyotha et al., 2022). Lumpy skin disease is a notifiable disease by the World Organization for Animal Health (WOAH) because of the significant economic impact on the cattle industry related to decreased milk volume and meat production, abortions, infertility, and damaged hides (Whittle et al., 2023). It is the second most economically significant viral disease in cattle after FMD in Ethiopia (Tamire, 2022).

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Infected animals may exhibit a range of clinical indications, from asymptomatic to severe morbidity and mortality (Ratyotha et al., 2022). In natural infections, the incubation period of the disease lasts between 2 and 5 weeks, whereas in the experimental setting, it lasts between 7 and 14 days (Gupta et al., 2020). Lumpy skin disease can take three forms, including acute, subacute, and chronic (Salib and Osman, 2011). The mild form of LSD manifests as fever, naso-ocular discharge, and nodular lesions on the skin, skeletal, and mucosal tissue (Coetzer and Tuppurainen, 2004). Later, nodular lesions on the other body parts, particularly the skin of the muzzle, nares, back, legs, scrotum, perineum, eyelids, lower ear, nasal and oral mucosa, and tail, may be detected (Salib and Osman, 2011). Furthermore, necrotic plaques in the mucous membranes of the oral and nasal cavities can cause purulent or mucopurulent nasal discharge and excessive salivation (Salib and Osman, 2011). Ulcerative lesions in the cornea of one or both eyes can also occur, resulting in vision impairment and even blindness (Tuppurainen et al., 2021). Oedema of the limbs induced by lymphangitis and vasculitis can occur in calves (Ratyotha et al., 2022). Calves may also develop mucosal lesions within 23 and 48 hours following infection (Al-Salihi, 2014). The subsequent lesions can result in necrotic tissue and scarring, which may lead to complications, such as corneal opacity (keratitis), mastitis, lameness, pneumonia, and myiasis (Salib and Osman, 2011; Ratyotha et al., 2022; Whittle et al., 2023). Lumpy skin disease virus is primarily spread through mechanical transmission by arthropod vectors, mainly mosquitoes, ticks, and biting flies (Khan et al., 2021; Tuppurainen et al., 2021). In addition, indirect transmission through contaminated water and feeding through saliva or nasal discharge from infected animals can also occur (Sprygin et al., 2019).

Morbidity and mortality of LSD vary from 3 to 85% and 1 to 3% (up to 40% in heavy outbreaks), respectively (Ochwo et al., 2019). However, according to a recently published review document by Dubey et al. (2023), the mortality rate of LSD is around 10% (rarely 40% in severe outbreaks), and the morbidity rate ranges from 5% to 45% (occasionally even up to 100%). According to Dubey et al. (2023), these variations in severity were likely to be due to variations in cattle breed, age, immunological condition or health status, viral isolates, insect vectors involved in the transmission, and stage of development.

Lumpy skin disease can be diagnosed based on the characteristic clinical signs, which include circumscribed skin nodules and enlarged superficial lymph nodes, which will later be confirmed by laboratory tests (Tamire, 2022). Conventional and real-time polymerase chain reaction (PCR) have been developed for efficient and rapid molecular diagnosis of LSD (Orlova et al., 2006). However, it should be distinguished from pseudo-lumpy skin disease, urticaria, dermatophilosis, dermatophytosis, hypoderma bovis infection, photosensitization, bovine papular stomatitis, FMD, bovine viral diarrhea and malignant catarrhal fever (Abutarbush, 2017).

Co-infections in viral diseases of cattle were uncommon and/or less reported. A mixed infection of LSD with pseudocowpox was reported by Omoniwa et al. (2023). Based on common clinical symptoms and in accordance with the clinical hallmarks of both disorders, FMD and LSD were clinically diagnosed (Abas et al., 2021). However, the confirmed diagnosis of FMD and LSD co-infection was not reported so far. Therefore, this case report presents the clinical and molecular detection of LSD in six crossbreed calves and a rare co-infection of LSD and FMD in a heifer at two small-scale dairy farms located in Northern Ethiopia.

CASE REPORT

Ethical approval and consent to participate

This clinical casework was conducted following the World Organization for Animal Health's (WOAH) animal welfare guidelines. The farmers or owners of the animals were informed of the objectives of the work, and they were willing to allow sample collection from the animals.

Lumpy skin disease case description in calves

Six crossbred calves from a dairy farm located in Kombolcha City, Northern Ethiopia, were examined for skin nodules on different body parts, lesions on the hind limb, and eyes. The lesions were first seen in the two calves (one male and one female). The ages of two calves were 11 months and six to nine months for other calves. The calves were living with other dairy cattle of different ages of the same breed in an intensive farming system. Based on the owner's information, one male calf died within a week of infection. There had been a history of similar cases before two years at the farm, as well as complaints from another nearby farm before one month. All animals on the farm aged above six months, including two of the diseased calves, had been vaccinated against the LSD virus. In addition, the calves started treatment with short-acting oxytetracycline, diclofenac sodium, and multivitamin injections one day prior. On clinical examination, the calves had a rectal body temperature ranging from 38.7 to 40.5°C, a respiratory rate of 18 to 64 breaths per minute, and a pulse rate of 72 to 120 beats per minute. Variably-sized skin nodules were seen on the neck, ear, teat, perineum, face, back, muzzles, and scrotum. The nodules were firm, painful, raised above the surrounding skin, and sometimes separated by a narrow ring of hemorrhage with a characteristic intact central area. The findings of physical examinations, including the specific identification and clinical signs, are summarized in Table 1.

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Table 1. Identification, clinical signs, and treatment outcome of calves affected by Lumpy skin disease

No	Sex	Age (Month)	Observed clinical signs	Body temperature (°C)	Respiratory rate (breath/min)	Pulse rate (beat/min)	Vaccination history	Outcome
1	F	11	Skin nodules from the neck, face, ear, muzzle, back, perineum, and teat, ulcerative nodular lesions from the muzzle and external nose, ocular and nasal discharge, swollen lymph node and necrotic lesions over oedematous limb with skin peel off and lameness.	39.9	64	120	Yes	Died
2	M	9	Skin nodule from the neck, rectum, and scrotum, ocular discharge, swollen lymph node.	40	36	98	No	Recovered
3	F	6	Skin nodules on the neck, face, and ear.	38.9	28	104	No	Died
4	F	11	Skin nodules on the neck, face, ear, perineum, and teat, ocular discharge, conjunctivitis and corneal cloudiness, and salivation.	40.5	48	92	Yes	Killed by the owner
5	F	8	Skin nodules on the neck and face.	39.3	18	84	No	Recovered
6	F	6	Skin nodules on the neck, swollen lymph node	38.7	22	72	No	Recovered

No: Number

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The nodular skin lesions were identified at various stages of growth and in varying numbers and sites among afflicted calves. Typical ring-like ulcerative lesions from the muzzle and external nose (only two calves), ocular-nasal discharge that stains the face, swelling of the prescapular and prefemoral lymph nodes, conjunctivitis, and corneal cloudiness (both eyes of one calf) were also observed. Severe depression and oedematous swelling of the left hind limb with pits on palpation, large areas of necrotic lesions over the oedematous limb with skin peeling off, and lameness were observed in one calf. The skin lesions and clinical features of LSD in the affected calves are indicated in Figure 1. Therefore, based on history, observed clinical findings, and the endemic nature of the disease in the area, LSD was tentatively diagnosed, and appropriate nasal swabs and nodular skin biopsy samples were collected for further laboratory confirmation.

Figure 1. Characteristic skin lesions and clinical features of Lumpy skin diseasein crossbred calves: Typical ring-like ulcerative lesions from the muzzle and external nose (**A**), Raised nodules separated by a narrow ring of haemorrhage after the hair over the nodule was clipped off (**B**), Different sized and staged skin nodules on the neck and face (**C**), Necrotic lesions over an oedematous limb with skin peeling off (**D**), Nodules on the testicle (**E**), Conjunctivitis and corneal opacity (**F**), and the affected calves at day one (**G**).

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Lumpy skin disease and foot and mouth co-infection in heifer

A crossbred heifer (2-year-old) with history of reduced feed intake, oral lesions, lameness, skin nodules, and salivation was presented to the Kombolcha City Veterinary Clinic, Northern, Ethiopia. The heifer was kept intensively with three cows and an ox. The heifer was purchased from the South Gondar Zone at one month ago with unknown vaccination history, although the rest of the animals were treated with ivermectin and long-acting oxytetracycline upon arrival on the farm. Based on the owner's complaint, a farm visit was made, and physical examinations were conducted at the heifer's barn. Upon physical examination, the heifer was depressed and febrile, with a rectal body temperature of 40.5°C. Respiratory and heart rates were 56 breaths per minute and 120 beats per minute, respectively. The prefemoral and prescapular lymph nodes were swollen. There were typical multiple and different-sized nodular lesions, particularly on the skin of the neck, shoulder, teat, around the vulva, and on the face, including the ear and mouth. The heifer was depressed, and the left forelimb was swollen and pitted on palpation. There were extensively necrotized lesions on the exterior of the nose, ruptured tissue on the gum, erosive lesions on the dorsal epithelium of the tongue, drooling of saliva, ruptured vesicles on the digits, and swelling on the lower jaw. Ocular and nasal discharge, reddened ocular mucous membranes, and lameness were also observed. The clinical hallmarks are presented in Figure 2. Based on the history and typical clinical findings observed, lumpy skin disease and foot and mouth disease co-infections were suspected.

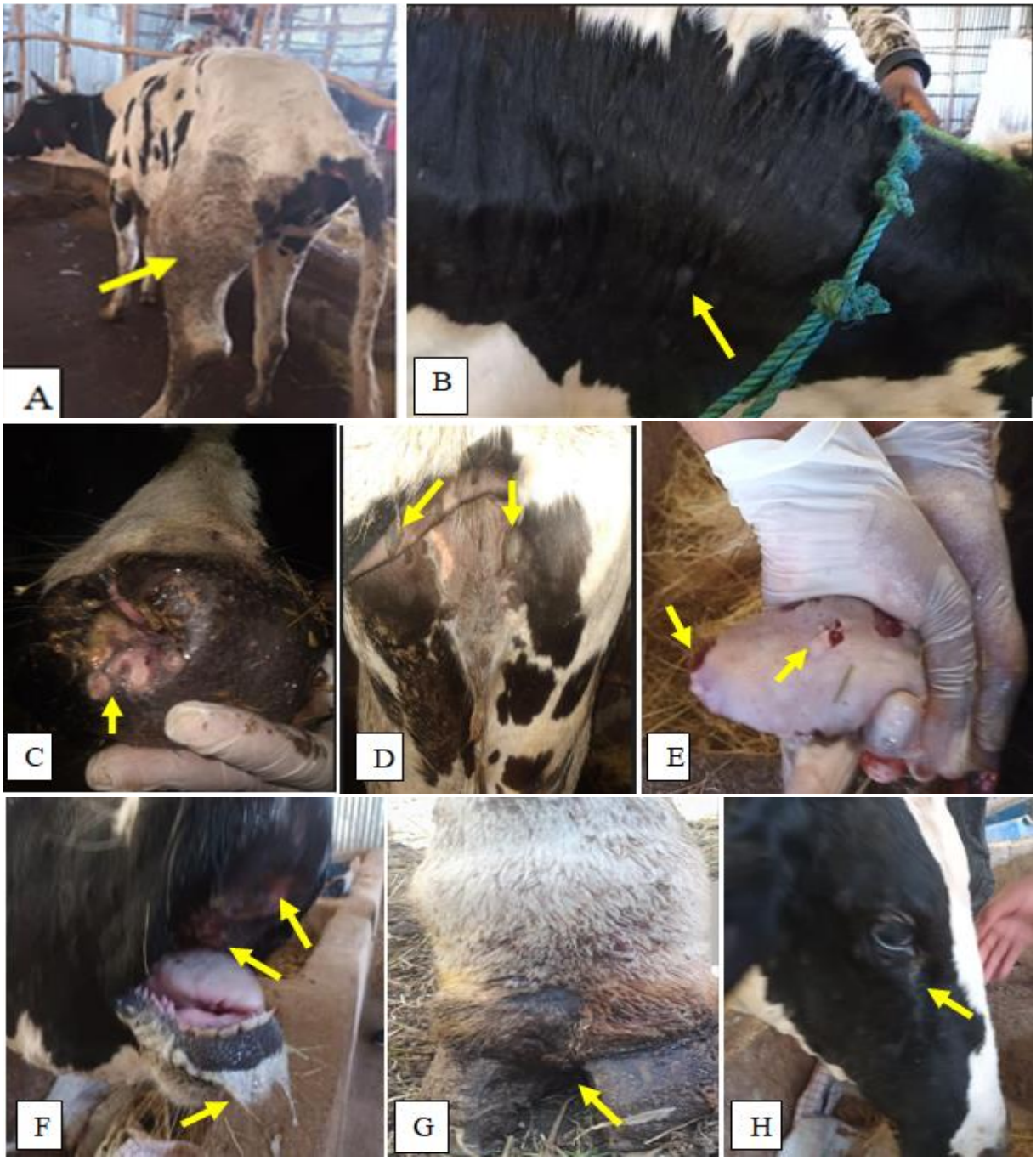

Figure 2. The different clinical indications of both Foot and mouth disease and LSD in crossbreed heifer. A heifer with a swollen left hind limb (**A**), Skin nodules around the neck (**B**), Nasal discharge and necrotized lesions on the exterior of the nose (**C**), Nodules around the perineum (**D**), Erosive lesions on the tongue (**E**), Lesions on the oral cavity with salivation (**F**), Lesion on the digit (**G**) and Lacrimation (**H**).

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Sample collection and laboratory findings

Nasal swabs and tissue samples were collected following aseptic techniques from the six calves suspected of having LSD and a tissue sample from one heifer suspected of having LSD-FMD co-infection and submitted to the laboratory for further testing. The animals were restrained properly before collecting the sample. Accordingly, for the laboratory diagnosis of LSD, a nasal swab and an excisional biopsy of cutaneous nodules were collected. The nasal swab samples were collected by vigorously rubbing the swab on the lining inside the nose. For cutaneous nodule collection, the selected nodule was prepared aseptically, and the hair over the nodule was clipped off. The area was anesthetized locally using 2% lidocaine, and the entire nodular biopsy was surgically removed and taken aseptically using scalpel blade number 24 and thumb forceps. The area was sutured and cleaned with 1% iodine. Similarly, for the laboratory diagnosis of FMD, about 1 g of epithelial tissue from the tongue was collected aseptically with the help of tissue forceps. After collecting the tissue samples, they were properly labeled with a history, including the provisional diagnosis, kept in sterile universal bottles that contain viral transport media, and then sent with a cold chain to the National Veterinary Institute (NVI), Ethiopia, for laboratory examination. Finally, the real-time PCR amplification result and melting curve analysis showed the presence of the LSD virus in both nasal swabs and tissue samples in both cases, as shown in Figure 3.

Further, the samples were then inoculated on the Embryonic Sheep Skin cell line (ESH-L), and the intracytoplasmic inclusion bodies, a distinctive feature of LSDV, were observed on the cell line, as indicated in Figure 4. The epithelial tissue sample from a suspected heifer was inoculated into the Baby Hamster Kidney (BHK-21) cell line, and syncytia formation, a characteristic cytopathic effect for FMDV, was observed (Figure 5). The test results confirmed LSD and FMD virus co-infection in the heifer.

Treatment and its outcomes

As LSD and FMD are viral diseases, the treatment in both cases was aimed at avoiding secondary bacterial infection by applying broad-spectrum antibiotics, dressing lesions with antiseptics, and practicing proper animal managment, reducing pain to the animal and losses due to the disease. Therefore, short-acting oxytetracycline (Herbei Hobe Harmony Pharmaceutical, China) at 10 mg/kg/day, intramuscular, for 5 days was prescribed. A multivitamin (Aether Centre [Beijing] Biology, China, 10-20 ml/animal, intramuscular) was given to improve the appetite. Diclofenac sodium (Reyong Pharmaceutical, China), at 2.5 mg/kg, intramuscular, was given once daily for three to five days. All the doses were prescribed based on manufacturer indications. The skin wound was cleaned and disinfected daily with an iodine solution. Topical eye ointment was applied to manage ocular lesions in the affected calf. The owners were advised to provide soft, palatable feed and isolate the animals from the others. Unfortunately, the heifer died on the third day of therapy while under treatment. Three calves among the LSD-affected were fully recovered after two months from the last treatment (Figure 5).

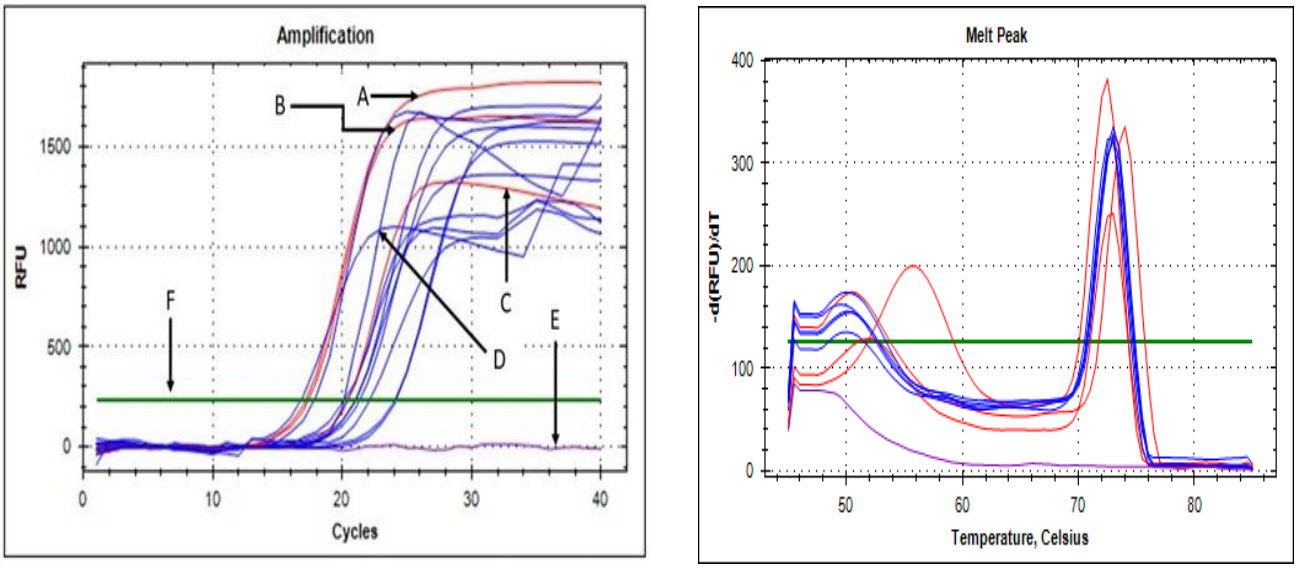

Figure 3. Detection of LSDV from nasal swab and skin nodule samples (crossbred calves [mean age ~ 9 months], five females, and one male). Real time polymerase chain reaction amplification result (left) and melting curve analysis (right). **A**: Known lumpy skin disease virus, **B**: Known goat pox virus, **C**: Known sheep pox virus, **D**: Field samples (blue colours), **E**: Negative control, **F**: Threshold value.

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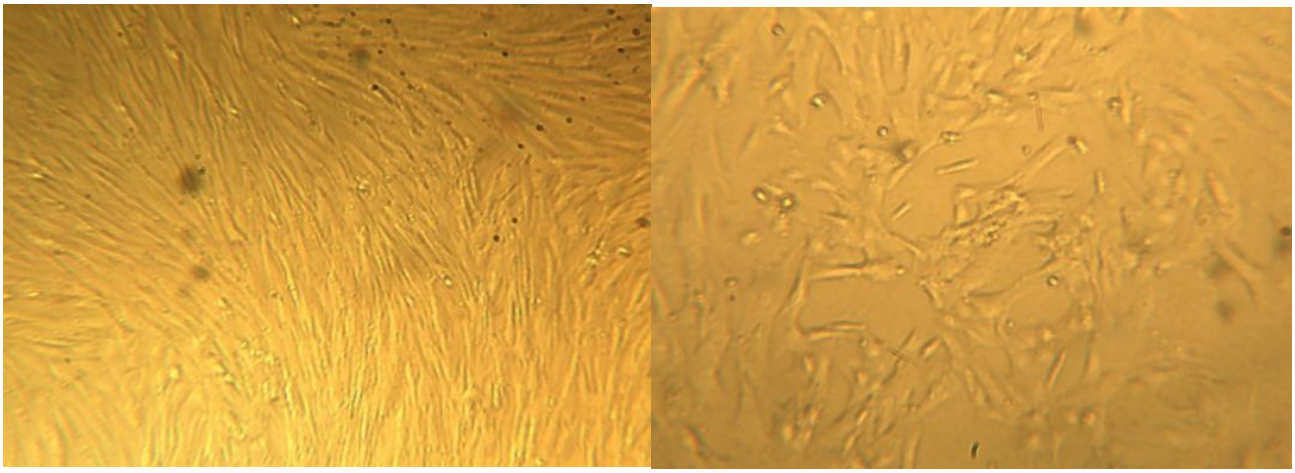

Figure 4. Isolation of Lumpy skin disease virus (LSDV) on Embryonic Sheep Skin cell line (ESH-L, left) and cytopathic effect provoked by LSDV (Crossbred dairy cattle, heifer, right).

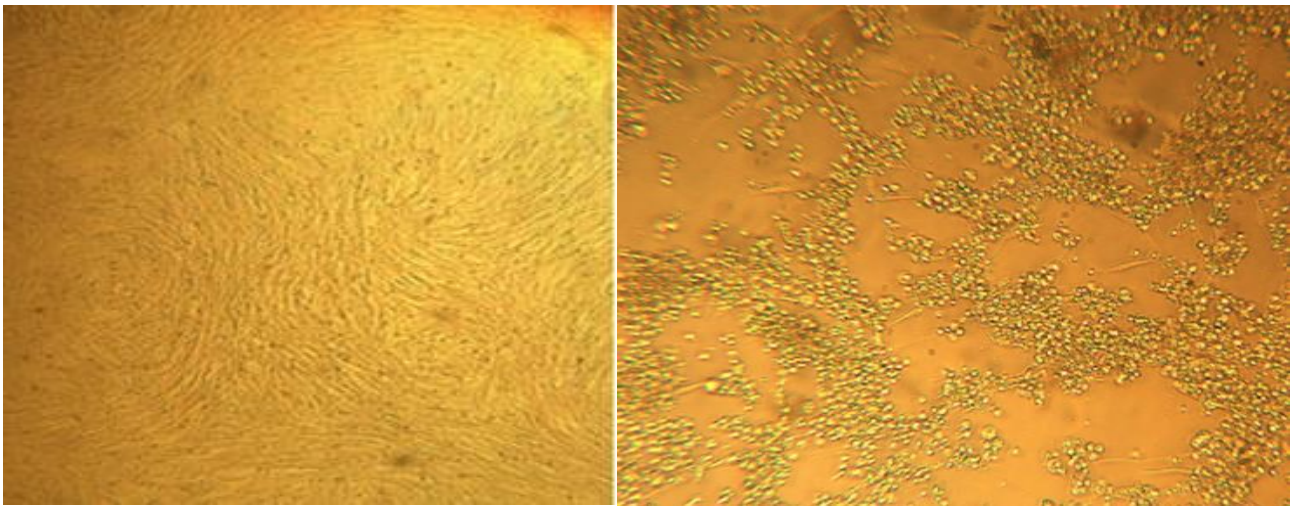

Figure 5. Noninfected Baby Hamster Kedney (BHK-21) cell line (left) and the cytopathic effect of FMDV on the cell line crossbred dairy cattle, 2-year-old heifer (right).

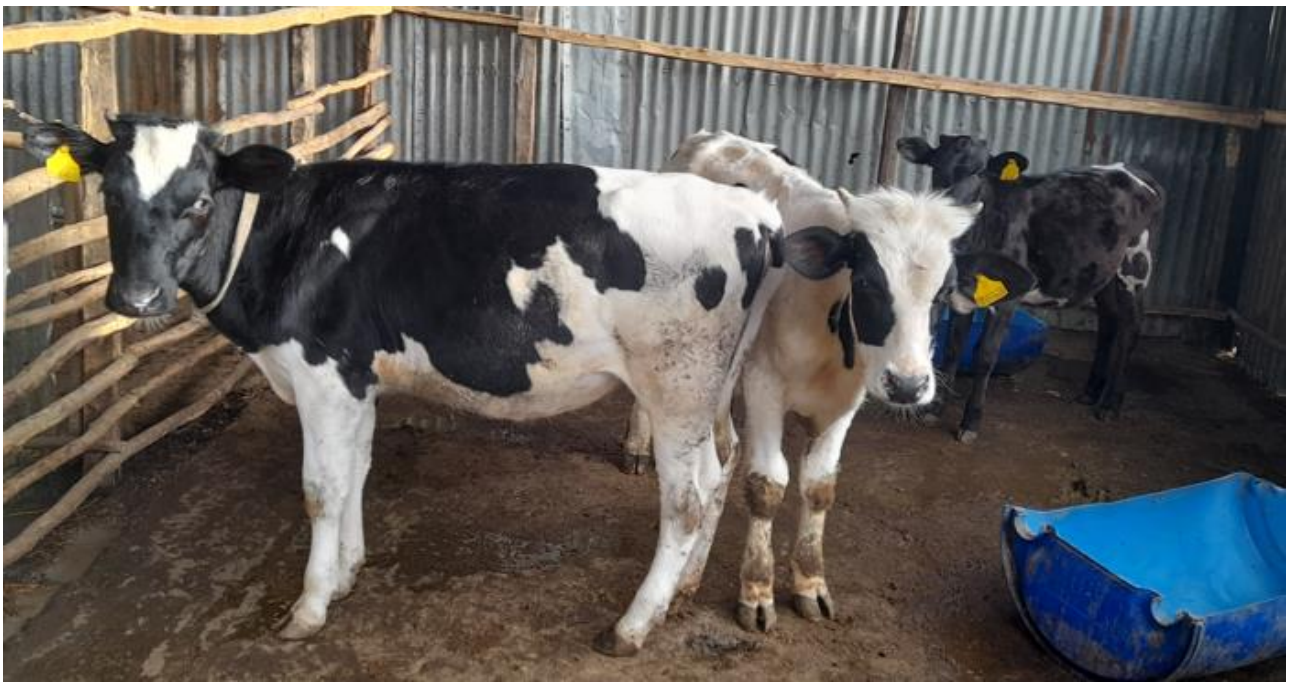

Figure 5. Calves recovered from Lumpy skin disease virus infection at two months of the follow-up period

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DISCUSSION

The LSDV and FMDV lack effective treatments, posing a substantial threat to the dairy and beef farming industries with potential for significant economic losses upon occurrence. Therefore, implementing routine vaccinations against both FMDV and LSDV emerges as the most efficacious strategy to curb the spread of these viruses (Sevik, 2017). Lumpy skin disease is a serious infectious viral disease in cattle. It occurs in an acute or chronic form and causes serious economic losses (Al-Salihi and Hassan, 2015). In this case study, the clinical infection of LSD was examined in crossbreed calves under one year old on a dairy farm. Likewise, LSD was found in four-month-old calves with nodular growth on different parts of the body, reduced appetite, emaciation, nasal discharge, and lameness in India (Halder and Seikh, 2022). Previously, a clinical case report of LSD was reported in Ethiopia in a bull with fever and the presence of large-sized circumscribed nodules on different body parts, particularly in the neck area, by Feyisa (2018). In this case study, varying-sized skin nodules, typical ring-like ulcerative lesions from the external nose, salivation and oculo-nasal discharge, and swelling of prescapular and prefemoral lymph nodes were observed on clinical examinations of the affected calves. Besides, conjunctivitis, corneal cloudiness, oedematous swelling of the limb, large areas of necrotic lesions over the oedematous limb with skin peeling off, and lameness were observed. Fever, depression, and reduced feed intake were also distinguished. As a result, the clinical findings in the current case report were in agreement with the LSD signs reported and described in previous studies (Feyisa, 2018; Mulatu and Feyisa, 2018; Tuppurainen et al., 2021).

In the present case, typical ring-like ulcerative lesions were seen on the muzzle and external nose. This is due to the sloughing of the necrotic lesions from the surrounding healthy epithelium (Al-Salihi, 2014). In this case report, conjunctivitis and corneal opacity were investigated in a nine-month-old calf. A similar sign was reported by Sudhakar et al. (2020) from outbreaks of LSD in cattle in India. The observed clinical signs, such as ulcerative lesions, conjunctivitis, corneal opacity, and lameness, might occur as severe clinical symptoms with complications of secondary bacterial infections (Al-Salihi and Hassan, 2015; Ratyotha et al., 2022; Whittle et al., 2023). The oedematous swelling of the limb in this case study was observed in one calf, which might be due to lymphangitis and vasculitis (Ratyotha et al., 2022). The presence of LSD-like nodules after vaccination ranges from 0.38% to 12% (Bamouh et al., 2021). In the present case study, two of the affected calves had a five-month vaccination history against LSDV. This is probably due to the incomplete effectiveness of the vaccine, the incorrect dosage of the vaccine at the time of vaccination, the low response level of the calves to the vaccine (Namazi and Khodakaram, 2021), or the fact that the calves might be missed during vaccination.

In the current case study, the calves were treated using short-acting oxytetracycline, multivitamins, and diclofenac sodium with the aim of avoiding secondary bacterial infection with antibiotics and reducing losses due to the disease and pain to the animal for welfare reasons. Similarly, the treatment protocol was in agreement with the management of the LSD case by Salib and Osman (2011). The most recent assessment by Dubey et al. (2023) revealed that while the mortality rate of LSD is typically 10%, it can occasionally rise to 40%. During the follow-up period, three calves were recovered and the others died. The case of death in the present instance might be due to lesions in the ocular and respiratory tract that probably prolong the period of anorexia and develop secondary pneumonia, which is a common sequel to LSD (Al-Salihi, 2014) that arises from the late detection and report of the cases by the owner.

In the present case report, a heifer was examined with clinical manifestations indicating both FMD and LSD in the study area. The presence of a cytopathic effect on BHK-21 and molecular examination of a tissue sample from an infected heifer revealed both FMD and LSD viruses, respectively. Thus, the results indicated that the clinical symptoms were co-infections caused by both LSDV and FMDV. The diagnosis of the case is also supported by the fact that FMD was present in the area at the time the current case occurred. Similar FMD-LSD co-infection was previously reported by Sevik (2017) in calves with clinical signs of fever (40°C), limping, nasal secretions, and lesions on the skin and vesicles on mucous membranes of the mouth.

CONCLUSION

Lumpy skin disease and foot and mouth disease are noticeable viral diseases that cause significant economic loss in the cattle industry. The current case report has shown the possibility of unusual LSD and FMD co-infections. Therefore, prevention by vaccination of animals against LSDV and FMDV and promotion of bio-security are more helpful than treatment, and early case reporting is also warranted to avoid losses due to death.

DECLARATIONS

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Authors' contribution

Abdi Feyisa Fufa and Asmamaw Bihonegn have generated the proposal. Both participated in designing the treatment and laboratory test protocol. Asmamaw Bihonegn did the follow-up and the write-up of the draft manuscript. Abdi Fufa reviewed and edited the documents. Both authors have read and approved the final version of the manuscript for publication in the World`s Veterinary Journal.

Competing interest

The authors declare that they have no competing interests.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked and compiled by the authors.

Availability of data and material

All clinical findings and laboratory results were included in the manuscript.

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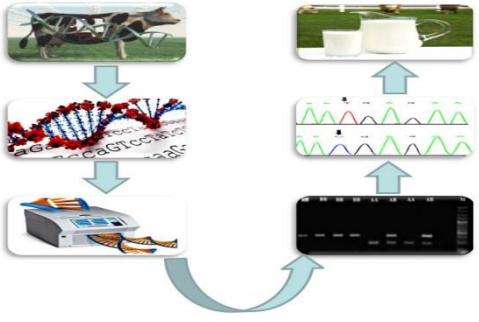

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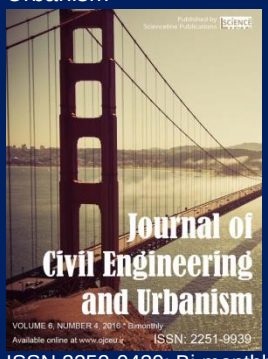

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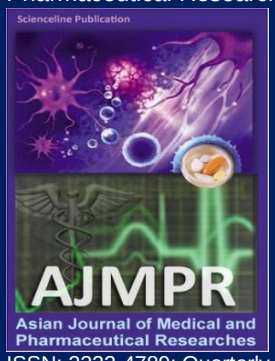

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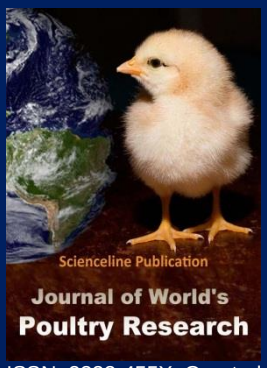

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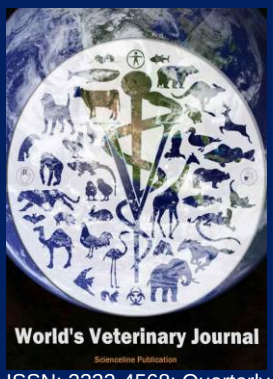

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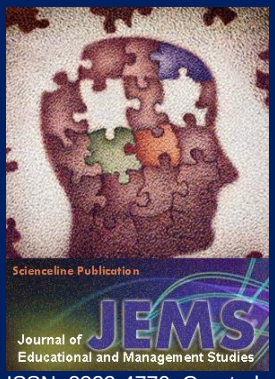

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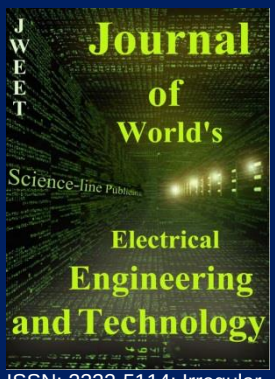

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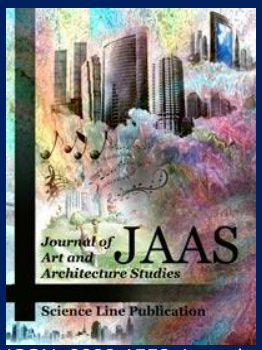

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