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Volume 14 (4); December, 2024

Research Paper

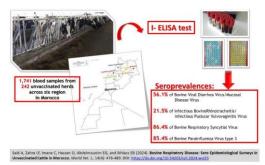
Bovine Respiratory Disease: Sero-Epidemiological Surveys in Unvaccinated Cattle in Morocco

Alali S, Laabouri FZ, Choukri I, Outenrhrine H, El Ghourdaf A, and El Berbri I.

World Vet. J. 14(4): 476-489, 2024; pii:S232245682400055-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj55

ABSTRACT: Viral respiratory diseases are a major cause of severe clinical symptoms and substantial economic losses in cattle breeding. This study aimed to evaluate the epidemiological status of four respiratory viruses in cattle including Bovine Viral Diarrhea Virus/Mucosal Disease Virus (BVDV/MDV), Infectious Bovine Rhinotracheitis/Infectious Pustular Vulvovaginitis Virus (IBR/IPV), Bovine Respiratory Syncytial Virus (BRSV), and Bovine Parainfluenza Virus type 3 (PI3). A total of 1,741 blood samples were collected from 242 unvaccinated herds. The animals comprised 1314 females and 427 males, aged between 6 months and 5 years. The herds included both pure breeds (Holstein or Montbéliarde breeds) and crossbreeds (local-Holstein or local-Montbéliarde), and were located in 89 rural communes of six regions of Morocco including Casablanca-Settat, Rabat-Salé-Kénitra, Marrakech-



Safi, Béni Mellal-Khénifra, Fès-Meknès, and Oriental. The samples were analyzed using the I-ELISA technique. The results indicated seroprevalence rates of 56.1, 21.5, 86.4, and 85.4% for BVD/MD, IBR/IPV, BRSV, and Parainfluenza-3, respectively. Co-infections were observed in 88% of the infected cattle, and 95% of the cattle were infected with at least one of the four viruses. Seroprevalence rates varied significantly with age, sex, breed, breeding systems, and practices. These findings confirmed the endemic status of bovine respiratory viral diseases and highlighted their direct and indirect impacts on livestock losses in Morocco.

Keywords: Bovine respiratory syncytial virus, Bovine viral diarrhea Virus/mucosal disease virus, Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis virus, Parainfluenza-3 Virus, Serology

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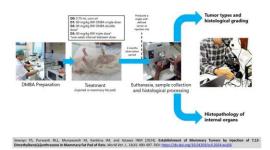
Research Paper

Establishment of Mammary Tumors by Injection of 7,12-Dimethylbenz[a]anthracene in Mammary Fat Pad of Rats

World Vet. J. 14(4): 490-497, 2024; pii:S232245682400056-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj56

ABSTRACT: Chemical-induced mammary cancer models are widely used mimic human carcinogenesis, with breast dimethylbenz[a]anthracene (DMBA) being a commonly used agent. The oral administration of DMBA frequently results in the formation of tumors at random locations and carries significant risks, including high mortality rates and damage to various organs. To address these issues, this study employed a subcutaneous DMBA administration protocol to induce mammary cancer in rats. A total of twenty-four female Sprague-Dawley rats aged 45-55 days weighing 112-130 g were divided into four groups, including the control group injected with 0.75 mL corn oil (D0), a single dose of DMBA at 80 mg/kg BW (D1), two doses with a one-week interval (D2), and three doses with one-week intervals (D3), all administered via



subcutaneous in mammary fat pad. Control groups (D0) did not show any tumor growth. Mammary tumor incidence increased with dosage (D1 33.33%, D2 66.67%, and D3 100%). Histopathological examination revealed the presence of various mammary tumor types without evidence of metastasis in all induced rats. All tumors originated from the injection site, and only a single nodule was observed in each rat. There were no significant differences in tumor grades between the treatment groups, and no mortality was recorded during the study. The D3 group showed the highest tumor incidence over the three-month observation period. These findings suggest that subcutaneous DMBA administration effectively induces mammary cancer in rat models with controlled tumor localization and minimal systemic effects, making it a promising method for experimental breast cancer studies.

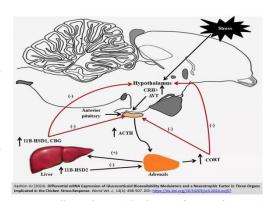
Keywords: 7,12-dimethylbenz[a]anthracene, Animal model, Breast cancer, Mammary fat pad, Rat

Differential mRNA Expression of Glucocorticoid Bioavailability Modulators and a Neurotrophic Factor in Three Organs Implicated in the Chicken Stress Response

World Vet. J. 14(4): 498-507, 2024; pii:S232245682400057-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj57

ABSTRACT: The hypothalamus-pituitary-adrenal (HPA) axis remains active despite the elevated corticosterone (CORT) levels during immobilization stress (IMS). This indicates that the HPA axis activity is dependent not only on CORT concentrations but also on the availability of free active CORT that is unbound of corticosteroid-binding globulin (CBG) and is activated by the 11β -hydroxysteroid dehydrogenase (11β -HSD) enzyme. The study examined the CORT levels in the blood and the mRNA expression of both *proopiomelanocortin* gene in the anterior pituitary gland (APit) and *brain-derived neurotrophic factor (BDNF)* in the septohypothalamus during IMS. Additionally, the expression of *glucocorticoid receptor (GR)*, 11β -HSD, and CBG were analyzed in the septohypothalamus, APit, and liver. The experiment included three male Cobb 500 chicken groups, a control group, and two treatment groups



exposed to 60 or 120 minutes of IMS. Blood, brain, APit, and liver were collected at 35 days of age (N= 12 samples/group). CORT concentrations in blood were quantified using radioimmunoassay, while reverse transcription-quantitative PCR was used to measure mRNA levels of CBG, 11β -HSD1, 11β -HSD2, and BDNF in the septohypothalamus, APit, and liver. The findings suggested that the IMS activated the HPA axis, as demonstrated by increased CORT levels and changes in *proopiomelanocortin* expression within the APit of stressed chickens compared to unstressed ones. The septohypothalamus of stressed chickens showed an increase in the CBG, BDNF, and 11β -HSD1 mRNA levels, whereas 11β -HSD2 and GRs expression remained stable compared to the control group. Although CBG and BDNF expression decreased from peak levels, their mRNA remained significantly elevated in the 120-minute group. In liver tissue, the treatment groups showed higher levels of 11β -HSD1 and CBG expression, but 11β -HSD2 expression decreased. Overall, CORT levels and the expression of GR and CORT modulators seemed to have a significant influence on the stress response. Notably, increased mRNA levels of CBG and 11β -HSD1 could improve the availability of free active CORT. Furthermore, a positive correlation between CORT levels and BDNF expression was demonstrated, highlighting the role of BDNF in neuronal protection during IMS. Additionally, the liver may contribute to stress regulation through the functions of CBG and 11β -HSD, which are vital for CORT activation and transport.

Keywords: 11β-hydroxysteroid dehydrogenase, Brain-derived neurotrophic factor, Corticosteroid binding globulin, Corticosterone, Immobilization stress

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Research Paper

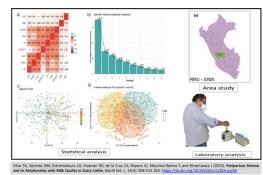
Peripartum Ketone and its Relationship with Milk Quality in Dairy Cattle

Villar FA, Sánchez NM, Estremadoyro LG, Huamán RD, de la Cruz CA, Payano IU, Mauricio-Ramos Y, and Ninahuanca J.

World Vet. J. 14(4): 508-515, 2024; pii:S232245682400058-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj58

ABSTRACT: Ketosis is a common metabolic disorder in dairy cows and has been associated with alterations in milk composition and a decrease in milk quality, impacting both the economic and nutritional value of dairy products. The purpose of this study was to evaluate ketone levels before and after calving and their effect on milk quality in cattle in the district of El Mantaro, Jauja in the Peruvian highlands. Multivariate analyses, including Principal Component Analysis (PCA) and clustering, were employed to explain the variability in the data better. The study was conducted at the Instituto de Investigaciones Tropicales y de Altura (IVITA) and El Mantaro experimental stations cattle in the district of El Mantaro, Jauja, using a total of 72 Brown Swiss cattle, all of which received similar management. Blood and milk samples were collected from all cows studied and analyzed in the laboratory for ketone levels



and milk quality such as density, non-fat solids, protein, freezing point, solids, and lactose. The descriptive analysis revealed significant variations in the evaluated variables, highlighting a reduction in ketone levels after calving and consistency in milk composition, such as non-fat solids and density. The PCA showed that the first two principal components explained 49.8% of the total variability, dominated by compositional variables, while subsequent components contributed smaller proportions, reaching 100% with 11 components. The reduction in ketone levels after calving suggested metabolic stabilization associated with energy recovery during this stage, while differences in compositional variables such as protein and non-fat solids reflected the influence of factors such as diet, genetics, and physiological status. Although ketones showed weak to moderate correlations with the evaluated variables, the negative relationships with body condition and non-fat solids indicated that better nutritional and metabolic status might be associated with lower ketone levels.

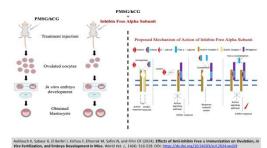
Keywords: Brown Swiss cattle, Correlation, Milk composition, Milk quality, Subclinical ketosis

Effects of Anti-inhibin Free a Immunization on Ovulation, in Viro Fertilization, and **Embryo Development in Mice**

World Vet. J. 14(4): 516-528, 2024; pii:S232245682400059-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj59

ABSTRACT: Inhibin is a dimeric glycoprotein comprised of two subunits, a and B. Immunization against dimeric inhibin is mainly used in assisted reproductive technology to induce superovulation. However, the specific function of immunoreactive-free inhibin a subunit remains unclear. In this study, two main investigations were conducted (first on ovulation and the other on fertilization) using a novel monoclonal antibody targeting free inhibin a subunit (Pro-aN-aC). The ovulation study was conducted in 6 replicates, involving a total of 48 female CD1 mice aged 4-6 weeks. In each replicate, 4 control mice received PMSG/hCG treatment, and 4 treated mice received PMSG/hCG with mAb- Free a subunit. The fertilization study was conducted in 3 replicates, involving a total of 22 female CD1 mice. In each replicate, there were 4, 3, and 4 mice



respectively for both control and treatment groups. In both investigations, female mice were injected intraperitoneally with 50 units/ml of Pregnant Mare Serum Gonadotropin (PMSG), alone or combined with 400ug of mAb- Free a subunit, followed by an injection of 50 units/ml of Human Chorionic Gonadotropin (hCG) 48 hours later. Seventeen hours postinjection, the females from all groups were sacrificed, and the ovulated oocytes were collected from the oviducts. For the fertilization study, in vitro fertilization was performed using fresh sperm from male CD1 mice. The results revealed that neutralization of the free inhibin a subunit significantly decreased the ovulation rate by 47.29% compared to the control group, while immunoneutralization significantly increased the fertilization rate by 55.68% and the blastocyst development by 43.85% compared to the control group. This study suggests that immunization against free inhibin a subunit decreases ovulation, in contrast to the effect of immunoneutralization of dimeric inhibin. The authors hypothesize that the free a subunit may function as an inhibin antagonist, competing with inhibin for binding to its co-receptor.

Keywords: Activin, Betaglycan, Fertilization, Immunoneutralization, Inhibin, Ovulation

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Research Paper

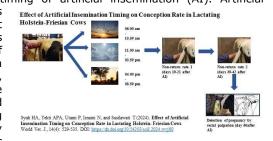
Effect of Artificial Insemination Timing on Conception Rate in Lactating Holstein-**Friesian Cows**

Syah HA, Yekti APA, Utami P, Isnaini N, and Susilawati T.

World Vet. J. 14(4): 529-535, 2024; pii:S232245682400060-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj60

ABSTRACT: Successful pregnancy in dairy cows requires accurate timing of artificial insemination (AI). Artificial insemination conducted in the morning, midday, and afternoon exposes cows to different environmental temperatures with high ambient temperatures can potentially reduce the viability of spermatozoa, thus disrupting the fertilization process and increasing the possibility risk of pregnancy failure. The purpose of the present study was to ascertain and determine how the effect of various AI timings (during the morning, midday, and afternoon) affected the pregnancy success of the pregnancies in Holstein-Friesian cows. The purposive sampling was used to select a total of 191 Holstein-Friesian cows based on the following specific criteria; cows showing symptoms of estrus, having healthy reproductive organs, having one parturition at least once, aged 2-6 years



old, having body condition score of 2.5-3.5 (on a 1-5 scale). The cows were divided into 3 three treatment groups including T1 cows inseminated from 06.00 am to 10.59 am (n=38 cows), T2 cows inseminated from 11.00 am to 03.59 pm (n=82), and T3 cows inseminated from 04.00 pm to 08.59 pm (n=71). The non-return rate was monitored at 19-22 days post-insemination (NRR-1) and 39-42 days post-insemination (NRR-2) was monitored, while the conception rate (CR) was assessed on day 60 post-insemination. Artificial insemination was performed 8 hours after estrus. NRR-1 values for T1, T2, and T3 were 82%, 80%, and 89%, respectively, The NRR-2 values were 71% in T1, 66% in T2, and 79% in T3. The CR for T1, T2, and T3 were 50%, 48%, and 54%, respectively. Although AI timing did not yield conception rates based on AI timing did not show a significant difference in conception rates, artificial insemination performed in the morning insemination is recommended due to its higher likelihood of successful pregnancy compared to other times. Keywords: Artificial insemination, Conception rate, Dairy cow, Insemination timing, Lactating dairy cows, Non-return rate

Research Paper

Gastrointestinal Parasitic Infections of Ruminants in Pastoral Communities of Ondo State, Nigeria

World Vet. J. 14(4): 536-543, 2024; pii:S232245682400061-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj61

ABSTRACT: Livestock are important for food production and agricultural systems; however, helminth infections are a major constraint on their productivity and value. The present study aimed to investigate the prevalence and associated risk factors for helminth infections in four pastoral communities in Ondo State, Nigeria. A total of 1,165 fecal samples from livestock in four pastoral rural/peri-urban communities (Akungba, Ikare, Oka, and Supare) in the Akoko area of Ondo State were screened for gastrointestinal parasitic infections via the sedimentation technique. The overall prevalence of parasitic infection (72.8%). The prevalence of parasitic infection was significantly associated with the breed of livestock. Important parasites of public



health, including Fasciola species, Strongyloides species, Trichuris species, hookworms, Schistosoma species, Trichostrongylus species, and Fasciola species, which are the most common, were isolated in the current study. Two-thirds (75.5%) of the observed prevalence was due to infection with a single helminth species, whereas the remaining one-third (24.5%) comprised infections with two or three species of helminths. Among multiple infections, Fasciola species plus Strongyloides species coinfection had the highest prevalence (46%). The present findings suggest the need for regular epidemiological surveillance and treatment of infected ruminants with gastrointestinal helminths. **Keywords:** Helminth, Infection, Livestock, Prevalence, Ruminant

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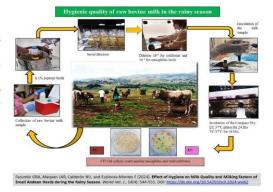
Research Paper

Effect of Hygiene on Milk Quality and Milking Factors of Small Andean Herds during the Rainy Season

World Vet. J. 14(4): 544-551, 2024; pii:S232245682400062-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj62

ABSTRACT: Dairy production in the Peruvian Andes is mainly based on small herds. However, there is little information on how hygiene affects milk quality during the rainy season. The study's objective was to evaluate the hygienic factors of milk and milking practices during the rainy season in small herds located at two high Andean altitudinal levels. The characteristics of the factors related to the milking process were recorded using an observation guide, and 108 raw milk samples were collected from 18 herds with Brown Swiss cows at two altitude levels. Samples were obtained from the milk collection containers and milk. The results showed that 56.5% of the samples analyzed were unhygienic, with no significant differences in bacterial counts between altitudinal levels. However, a strong correlation was identified between precipitation and the presence of coliforms (0.726) and mesophilic aerobes (0.861). Factors such as milking location, hand washing, and use of cleaning



agents were associated with microbial contamination, acquiring odds ratios (OR) of 4.04, 5.26, and 4.71, respectively, during the months of heavy rain. The study concludes that the hygienic quality of milk in small high Andean herds significantly deteriorates during the rainy season, with counts of total coliform bacteria and mesophilic aerobes exceeding recommended levels, particularly during peak rainfalls. This finding highlights a direct relationship between the intensity of rainfall and the quality of milk, underscoring the need for improved milking practices in the rainy season to ensure the safety of the products.

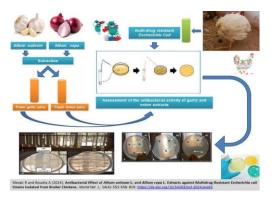
Keywords: Andean ecosystem, Milk quality, Rainy season, Small farmer

Antibacterial Effect of Allium sativum L. and Allium cepa L. Extracts against Multidrug-Resistant Escherichia coli Strains Isolated from Broiler Chickens

World Vet. J. 14(4): 552-558, 2024; pii:S232245682400063-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj63

ABSTRACT: Over the past decades, the incidence of avian colibacillosis caused by multidrug-resistant *Escherichia coli* (*E. coli*) has increased dramatically worldwide. The present *in vitro* study focused on evaluating the antibacterial properties of *Allium sativum* L. and *Allium cepa* L. extracts against multidrug-resistant *E. coli* strains isolated from broiler chickens suffering from colibacillosis. The confirmation of *E. coli* isolates and their antibiotic resistance was performed using conventional methods. Furthermore, the antimicrobial activity of both extracts was assessed through the disk diffusion method, along with the determination of the minimum inhibitory concentration (MIC) via liquid macrodilution and the minimum bactericidal concentration (MBC) using solid media. The obtained results showed that the multidrug-resistant *E. coli* strains were extremely sensitive to garlic extract with a MIC of 41.5 mg / mL and CMB of 166 mg / mL and very sensitive to the combination of garlic and onion extracts. However, onion extract was ineffective against the resistant *E. coli* strains.



The findings of the present study suggested the possibility of using garlic as an alternative to antibiotics in the treatment of colibacillosis caused by resistant *E. coli* strains.

Keywords: Allium cepa L., Allium sativum L., Broiler chicken, Colibacillosis, Escherichia coli, Multidrug-resistance

[Full text-PDF]

Research Paper

Phenotypic Variability of Native Guinea Pig (*Cavia porcellus*) Lines Associated with Productive and Reproductive Variables in the Traditional Production Systems of the Pastos Indigenous Reserve

Rosero J, Rosero-Alpala MG, Rosero D, Rosero A, and Tapie WA.

World Vet. J. 14(4): 559-571, 2024; pii:S232245682400064-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj64

ABSTRACT: Genetic improvement seeks to meet human needs, resulting in a loss of genetic variability, affecting indigenous communities' biodiversity and food sovereignty. Therefore, this study aimed to determine the phenotypic variability of native guinea pig (*Cavia porcellus*) lines associated with productive and reproductive variables in the traditional production systems of the Pastos Indigenous Reserve in the Department of Nariño, southwestern Colombia. A total of 2007 guinea pigs older than 3 months were divided into 2 batches. 1934 individuals (batch 1) were randomly selected for phenotypic characterization, including hair length, leg size, body size, and behavior. Seventy-three individuals (batch 2) were used to evaluate productive and reproductive variables, and the lines with the highest similarity were clustered using the UPGMA method. In addition, ethnoveterinary information obtained through interviews within the production systems



was described. As a result, nine traditionally known phenotypes were described including Shinhuzo, Pelochon, Zambo, Guarico, Chocolate, Peruvian, Coral, Piño, and Moro. The lines were clustered into 4 groups, highlighted by lines with high production and reproductive potential (group 1), lines with low progeny mortality (group 2), a line with low reproductive potential (group 3) a line that presented a unique coat and high productive potential as Group 1 (Group 4). As a result, local knowledge was shown to be crucial for the conservation of native guinea pig lines, as it includes traditional feeding techniques and disease treatment. The native lines Shinhuzo and Coral, with morphological differences between them, showed the potential to reach productive and reproductive parameters similar to the improved Peruvian line, according to the UPGMA dendrogram. However, a detailed analysis of the specific nutritional requirements of each guinea pig line is necessary to improve the traditional breeding of guinea pigs, enhancing the production of all native lines already adapted to the indigenous territory, maintaining the important genetic variability that, in the context of climate change, is relevant to promoting research on sustainable production strategies using resilient native species adapted to local conditions for the future exploration of differentiated markets.

Keywords: Animal conservation, Ethno-veterinary, Genetic variability, Traditional knowledge

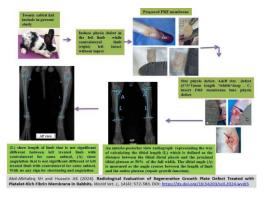
Research Paper

Radiological Evaluation of Regenerative Growth Plate Defect Treated with Platelet-Rich Fibrin Membrane in Rabbits

World Vet. J. 14(4): 572-583, 2024; pii:S232245682400065-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj65

ABSTRACT: Bony bar formation after growth plate injuries leads to shortening and angulation of the long bone, which is considered one of the most critical sequelae affecting animals' and humans' lives in adulthood. The objective of the present study was to evaluate radiographically the role of using an autologous platelet-rich fibrin membrane in regenerating growth plate defects to prevent the formation of bony bars. A total of 20 kit rabbits, aged between 6-12 weeks and weighing 500-1100 g, were included in the current study. They were experimentally exposed to approximately $5 \subseteq 5 \subseteq 1$ mm growth plate defects, which were filled with an autologous platelet-rich fibrin membrane previously prepared at the time of the surgery. A radiological follow-up was conducted weekly at the first, second, third, fourth, sixth, and eighth weeks post-surgery to examine the growth plate defect area. The tibial length and angulation were measured during this period of the study and compared to the contralateral limb of the same animal. The



radiological results showed no bony bar formation in most cases and the presence of the growth plate up to the end of the study (week 8 post-surgery) in the injured area. In addition, no significant differences were identified in the tibial length and angulation of the affected limb in comparison to the contralateral limb of the same animal throughout the study. In conclusion, treating serious growth plate injuries by PRF membrane may prevent angular deformity and length discrepancy in limbs.

Keywords: Angulation, Bony bar, Growth plate, Platelet-rich fibrin membrane, Shortening

[Full text-PDF]

Research Paper

The Toxicity Assessment of Chicken Liver-Based Drug Filler Supplemented with Itraconazole

Pringgodigdoyo PT, Manalu W, Andriyanto, Mustika AA, and Sutardi LN.

World Vet. J. 14(4): 584-591, 2024; pii:S232245682400066-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj66

ABSTRACT: Itraconazole is a systemic antifungal often used for fungal infection treatment in cats. This study aimed to evaluate the safety of chicken liver paste as a drug filler through an acute toxicity test when supplemented with itraconazole. A total of 25 female mice were used and divided into five groups, each consisting of five mice. The control group received chicken liver paste without any itraconazole supplementation. In contrast, the treatment groups were administered chicken liver paste supplemented with itraconazole at 5, 10, 15, and 20 g/kg body weight dosages. Observations were conducted for two weeks. The evaluated included clinical parameters abnormal signs, mortality, temperature, weight gain, and hematology profile. During the 14 days of observation, no mortality or abnormal clinical signs were observed. Other parameters such as body temperature and weight increase showed no



significant difference. Hematology profile including red blood cells (RBC), hemoglobin (HB), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), thrombocyte (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), white blood cells (WBC), lymphocytes, monocytes, and granulocytes remained within the standard ranges and showed no significant difference. The present study indicated that chicken liver paste supplemented with itraconazole falls into the practically nontoxic category with an estimated LD50 value exceeding 20 g/kg body weight.

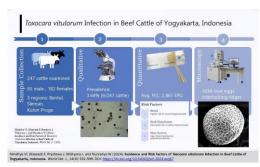
Keywords: Chicken, Hematology, Itraconazole, LD50, Liver, Mice

Incidence and Risk Factors of *Toxocara vitulorum* Infection in Beef Cattle of Yogyakarta, Indonesia

World Vet. J. 14(4): 592-599, 2024; pii:S232245682400067-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj67

ABSTRACT: *Toxocara vitulorum* (*T. vitulorum*), an Ascarid nematode, infects the small intestine of cattle and buffalo, particularly in newborn calves. The present study aimed to identify the occurrence of *T. vitulorum* collected from cattle in Yogyakarta, Indonesia, and to examine the surface structure of its eggs by scanning electron microscopy (SEM). The present study did not observe asymptomatic clinical signs of toxocariasis, including diarrhoea and weight loss. Fecal samples were collected from 247 cattle of various breeds, consisting of 65 males and 182 females across three regions including Bantul (78 cattle), Sleman (63 cattle), and Kulon Progo (106 cattle). Qualitative and quantitative



methods, including flotation and modified McMaster methods, were respectively employed to analyze nematode egg counts. SEM was utilized to characterize the surface morphology of *T. vitulorum* nematodes. A total of 9 cattle were found to excrete *T. vitulorum* eggs in their feces (3.64%). The average fecal egg count was 2.861 eggs per gram (EPG), with positive cases observed exclusively in female cattle. The risk factors influencing toxocariasis in this study were breeds and frequency of cleaning the stall. A higher odd ratio of *T. vitulorum* infection was found in mixed Ongole breeds than in Limousin or Simmental breeds. Moreover, cattle housed in rarely cleaned stalls showed a higher odd ratio than those in regularly cleaned ones. Factors such as age, fecal consistency, and population density factor showed no significant association with toxocariasis. The SEM analysis of *T. vitulorum* eggs revealed an oval shape with distinct surface ornamentations, including interlocking ridges and depressions. The cage cleanliness and cattle breed were the most common risk factors associated with infected cattle.

Keywords: Ascarid, Cattle, Prevalence, Risk factor, Scanning Electron Microscopy, Toxocariasis

[Full text-PDF]

Research Paper

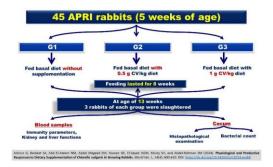
Physiological and Productive Responses to Dietary Supplementation of *Chlorella vulgaris* in Growing Rabbits

Ashour G, Barakat SA, Abd El-Azeem NM, Abdel Mageed EM, Younan GE, El-Sayed HGM, Morsy SH, and Abdel-Rahman SM.

World Vet. J. 14(4): 600-610, 2024; pii:S232245682400068-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj68

ABSTRACT: Chlorella vulgaris (CV) acts as an immuno-modulator and growth enhancer, however, studies were concerned about its impact on growing rabbits. The present study was undertaken to evaluate CV addition on physiological responses and productive performance (feed intake, feed conversion ratio, body weight, mortality rate, and other parameters) of APRI rabbits. A total of 45 growing rabbits at their weaning age with an initial body weight of $574.8 \pm 11.79g$ were investigated. The rabbits were divided into three equal groups; the first group (G1) received a basal diet without any additions. While, the other two groups, G2 and G3 received basal diets that contained 0.5g and 1.0g CV/kg diets. The results revealed the positive impact of CV on immunity (IgA, IgM, and IgG) status, especially IgG, which was significantly higher in G3 than in G1. No negative effects of CV on kidney and liver functions,



since the lowest levels of creatinine, blood urea, aspartate aminotransferase, and alanine aminotransferase were recorded in G3 compared with G1. Throughout the experimental period (8 weeks), G3 was the best group in feed intake with the lowest feed conversion ratio reflected on achieving the highest body weight compared to other experimental groups. No mortality cases were recorded in G3, while, G1 and G2 almost showed the same mortality rate (%). The histopathological examination of rabbits' intestines indicated that a less inflammation presence of rabbit intestinal cells has been noticed in G3 compared to G1 and G2. Therefore, it could be concluded that using CV at a level of 1.0 g/ kg in diet is the best level that can be used as a natural feed additive. This contributes to the health of growing rabbits by protecting their intestines against inflammation, lowering the mortality rate, and ultimately improving their overall productivity.

Keywords: APRI rabbit, Chlorella vulgaris, Intestinal histopathology, Physiological responses, Productive performance

Research Paper

Effects of Ethanolic Extracts of Tithonia diversifolia and Azadirachta indica on Haemonchus contortus in Goats

World Vet. J. 14(4): 611-616, 2024; pii:S232245682400069-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvi69

ABSTRACT: Infestation of gastrointestinal worms (helminths) contributes significantly to neonatal mortality and reduced growth performance in livestock animals. The present study was conducted to determine the phytochemical composition and the in vitro potency of ethanolic extracts of Tithonia diversifolia and Azadirachta indica on motility inhibition on Haemonchus contortus. Tithonia diversifolia and Azadirachta indica were obtained from farmers in Bulambuli district of Uganda and were treated with 70% ethanol as an extraction solvent. A standard phytochemical procedure was used for qualitative analysis. The in-vitro experiment was conducted using 2.5 mg/ml, 5 mg/ml, 10 mg/ml, and 20 mg/ml for each plant ethanolic extract. Moreover, phosphate buffer saline (PBS) was utilized as the control. Phytochemical analysis revealed the presence of tannins, alkaloid salts, saponins, flavonoids, steroid glycosides, anthracenosides, coumarins, and anthocyanosides in ethanolic extracts. A dosage of 20 mg/l of Tithonia diversifolia and Azadirachta indica indicated motility inhibition of adult *Haemonchus contortus* after 2.55 hours and 2.1 hours, respectively, compared to the PBS control group. In conclusion, both plant extracts showed anthelmintic activity leading to the mortality of the worms. The ethanolic extracts of Azadirachta indica were faster in causing mortality of Haemonchus contortus than those of Tithonia diversifolia at the same dose rate of 20 mg/ml.

Keywords: Azadirachta indica, Haemonchus contortus, Plant extract, Tithonia diversifolia, Wormicide

[Full text-PDF]

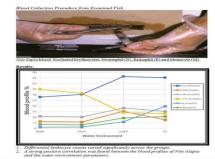
Research Paper

Influence of Different Water Environments on the Differential Leukocyte Counts in Nile Tilapia

World Vet. J. 14(4): 617-625, 2024; pii:S232245682400070-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj70

ABSTRACT: Aquaculture production has become increasingly important for ensuring food security, supported by the expanding variety of cultivated species. This study evaluated the effects of different water environment characteristics on the differential leukocyte counts of the Nile tilapia (Oreochromis niloticus), a key farmed fish species in Africa. Fish from four different water sources were compared with healthy individuals from the natural water of the Nile River. A total of 64 Nile tilapia, each weighing 80-100 grams, were evenly divided into four groups and maintained at temperatures ranging from 25.00± 2.5°C to 30.00 ± 2.5 °C. The groups included fish from experimental tanks (ET), ponds managed by the General Administration of Fisheries Ponds (GAFP), the Fisheries Research Center Ponds (FRCP), and the White Nile River (WNR, control). Significant differences in water quality parameters,



Adam Sulieman HM and Habeeb TH (2024). Influence of Different Water Environments on the Differential Leukocyte Counts in Nile Tilapia. World Vet. J., 14(4): 617-625, DOI: https://dx.doi.org/10.54203/scii.2024.wvi70

particularly NH₄, NH₃, NO₃, and NO₂ concentrations, were observed among the water sources. GAFP and ET waters showed higher concentrations of these compounds (NH₄, NH₃, NO₃, and NO₂) compared to FRCP and WNR. Differential leukocyte counts varied significantly across the groups. The ET group showed the highest eosinophil percentage (9.68 ± 0.44%), while the GAFP group exhibited the highest percentages of lymphocytes (46.40 \pm 0.13%), monocytes (15.43 \pm 0.14%), and neutrophils (18.31 \pm 0.16%) compared to WNR. In contrast, the FRCP group recorded the highest platelet percentage (32.34 \pm 0.49%), while the ET group had the lowest (13.65 \pm 0.15%). Additionally, the ET group recorded the highest overall white blood cell count (191.46 \pm 0.61 \times 10 3). A strong positive correlation was found between the blood profiles of Nile tilapia and the water environment parameters. This study highlighted significant differences in water quality among experimental groups, with FRCP and WNR showing lower parameters. In addition, examining white blood cells in fish is crucial for biological monitoring of surface water pollution.

Keywords: Differential leukocyte count, Nile tilapia, Water quality deterioration

[Full text-PDF]

Research Paper

Incidence of Hepatitis Hydropericardium Syndrome in Broiler Chickens Caused by a New Fowl Adenovirus Strain in Iraq

World Vet. J. 14(4): 626-636, 2024;

pii:S232245682400071-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj71

ABSTRACT: Hepatitis Hydropericardium syndrome (HHS) is an acute



Oraibi MI, Khaleel MH, and Al-Baldawi AAA (2024). Incidence of Hepatitis Hydropericardium by a New Fowl Adenovirus Strain in Iraq. World Vet. J., 14(4): 626-636. DOI: https://dx.doi.org

infectious disease affecting broiler chickens. It is caused by a fowl adenovirus (FAdV) of Group I, serotype 4. This disease is characterized by sudden deaths in broilers as young as three weeks, with mortality rates reaching up to 65%. The current study aimed to evaluate the outbreak of HHS in three broiler farms in southern Iraq. It also sought to identify the specific serotypes of fowl adenovirus (FAdV) responsible for this outbreak, primarily focusing on its genetic characteristics and diversity. Ten liver and heart tissue samples were collected from broiler chickens (Ross 308) that had displayed clinical signs of depression, ruffled feathers, and a tendency to huddle in corners before death. Viral DNA was extracted from liver tissues for further virus detection using PCR and RT-PCR. A post-mortem examination showed a turmeric-yellow discoloration in the dividing lines between the pectoral muscles and the abdominal cavity. The livers of infected chickens were markedly enlarged, and clear, yellow-colored fluid was observed in the pericardial sac. Histopathological analysis of stained liver and heart tissues revealed small multifocal areas of necrosis and mononuclear cell infiltration, including basophilic intranuclear inclusion bodies in hepatocytes and lymphocytic infiltrates. Conventional PCR analysis of liver tissues confirmed the presence of FAdV serotype 4, identifying all samples as the Melad strain, a novel strain responsible for the ongoing epidemic in Iraq. This study confirmed the presence of FAdV serotype 4 and identified all samples as the Melad strain. This research also addresses the need to investigate FAdV with molecular techniques for a better understanding of the epidemiology of the disease.

Keywords: Fowl adenovirus, Hepatitis Hydropericardium Syndrome, Melad strain serotype 4.

[Full text-PDF]

Research Paper

Sebaceous Adenitis in an Akita: Symptoms and Therapeutic Approaches

World Vet. J. 14(4): 637-644, 2024;

pii:S232245682400072-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj72

ABSTRACT: Sebaceous adenitis is observed in different animal species, with dogs being the most commonly diagnosed. This study aimed to report a case of sebaceous adenitis in a 5-year-old male Akita dog weighing 35.8 kg, initially presented with pruritus and alopecia on the inner ear surfaces, face, and head. Over time, signs of changes in sebaceous glands appeared in other body parts. The dog was sent to a veterinary clinic in Kamyanets-Podilsky, Ukraine. During the clinical examination, the body temperature was 38.4°C, heart rate 78 bpm, and a respiratory rate 27 breaths per minute, with no change in appetite.



Zhelavskyi M, Maryniuk M, and Drobot M (2024). Sebaceous Adenitis in an Akita: Symptoms and Therapeutic Approaches. World Vet. J., 14(4): 637-644. DOI: https://dx.doi.org/10.54203/scii.2024.wv/72

Clinical and dermatological methods and histopathological examination were used to detect the disease. The hair on the head was curling, and the lesions appeared on the dorsal tail, the distal front and hind legs, the groin, and the abdomen. Initially, redness and itching occurred in the affected areas, and the skin lost elasticity and became dry. The hair was stuck together with gray crusts. The diagnosis was confirmed based on pathohistological studies. An inflammatory infiltrate with migration of lymphocytes, histiocytes, neutrophils, and atrophy of sebaceous glands was diagnosed, focusing on the pathology of the dermis. Treatment included topical therapy with shampoo applied for 3-5 minutes, then rinsed and followed by Conditioner for 2 minutes, over 30 days. Omega-3 was administered orally at 1000 mg twice daily. Isotretinoin (Roaccutane®, 20 mg) was administered orally twice daily for 30 days. Moreover, Cyclosporine was dosed at 5 mg/kg once daily orally, on an empty stomach, for 30 days. During the treatment, signs of inflammation gradually disappeared, with itching and hyperemia disappearing from day 3. From day 7, desquamation and hair loss decreased, and on day 12, signs of new hair growth appeared. The areas were completely restored on day 28 of treatment. Sebaceous adenitis was found to lead to the destruction of sebaceous glands, causing scaling, hair loss, and skin inflammation, which could be a hereditary condition in Akitas. Immunomodulation and normalization of trophic processes in the skin are crucial in the treatment.

Keywords: Akita, Diagnosis, Dog, Sebaceous Adenitis, Treatment

[Full text-PDF]

Research Paper

An Overview of Cyanide Poisoning in Humans and Animals

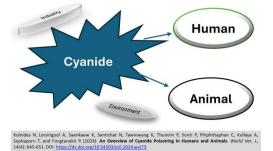
Kulnides N, Lorsirigool A, Saenkaew K, Santichat N, Tawinwang K, Thumrin P, Sonti P, Phiphittaphan C, Kullaya A, Sophaporn T, and Fongtanakit R.

World Vet. J. 14(4): 645-651, 2024;

pii:S232245682400073-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj73

ABSTRACT: Cyanide poisoning poses a significant global health risk, affecting both humans and animals due to its rapid and often fatal effects. Cyanide compounds, such as hydrogen cyanide, potassium cyanide, and cyanogenic glycosides found in certain plants, interfere with cellular respiration by blocking cytochrome oxidase, causing cytotoxic hypoxia and organ failure. Human exposure to cyanide is primarily linked to industrial activities such as mining and electroplating, as well as smoke inhalation during fires. Symptoms of poisoning include respiratory distress, dizziness, and, in severe cases, cardiac arrest and death if left untreated. In animals, particularly livestock, poisoning often results from



consuming cyanogenic plants such as sorghum and specific grasses. Different species have varying susceptibility to cyanide, with cattle showing signs such as respiratory distress, seizures, and death after ingesting these plants. Detection methods for cyanide, including spectrophotometry, gas chromatography, and ion-selective electrodes, which analyze cyanide levels in blood, urine, or tissues. Treatment generally involves the administration of antidotes, such as hydroxocobalamin, sodium thiosulfate, and sodium nitrite, which neutralize cyanide in the body. The current study aimed to highlight the importance of stringent regulatory measures on cyanide usage in industrial and agricultural contexts to prevent accidental poisoning. Environmental monitoring and processing of safe food are vital to reduce the incidence of cyanide poisoning in both humans and animals.

Keywords: Animal, Antidote, Cyanide, Human, Law, Poisoning

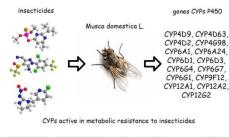
Research Paper

Contribution of Cytochrome P450s to Development of Insecticide Resistance in *Musca domestica L.*: A Review

World Vet. J. 14(4): 652-658, 2024; pii:S232245682400074-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj74

ABSTRACT: *Musca domestica L.* (*Diptera: Muscidae*) is recognized as one of the most prevalent fly species globally, playing a significant role in the transmission of infections and pathogens that are important in both veterinary and medical contexts. This includes the dissemination of eggs from intestinal helminths, as well as ectoparasites, endoparasites, and protozoan cysts. Several approaches to combating insect pests include biological, physical, chemical, and agrotechnical methods. Chemical methods remain the main strategy for controlling the population of insect pests; however, excessive use, increased dosages, and frequency of treatments have led to the development of resistance. To date, numerous documented cases of resistance to insecticides have been registered in natural populations. A significant mechanism for resistance



Krestonoshina K and Melnichuk A (2024). Contribution of Cytochrome P450s to Development of Insecticide Resistance in Musca domestica L.: A Review. World Vet. J., 14(4): 652-658. DOI: https://dx.doi.org/10.54203/scii.2024.wvj74

development is the detoxification of xenobiotics by enzymes of the cytochrome system. This study aimed to summarize the current knowledge on the role of *P450* monooxygenase in developing insecticide resistance in houseflies. This overview focuses on the diversity of Cytochrome *P450* monooxygenases in *Musca domestica* that contribute to resistance against the most popular classes of insecticides and their location in the genome. Throughout this work, the main *P450* candidate genes associated with insecticide resistance were identified and described. The authors also summarized and systematized recent research results in this area.

Keywords: Gene expression, Housefly, Insecticide, Insecticidal resistance, Monooxygenase, Cytochromes *P450* [Full text-PDF]

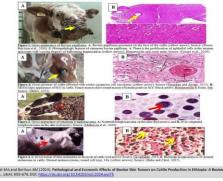
Research Paper

Pathological and Economic Effects of Bovine Skin Tumors on Cattle Production in Ethiopia: A Review

World Vet. J. 14(4): 659-679, 2024; pii:S232245682400075-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj75

ABSTRACT: A tumor is an abnormal mass of tissue that exceeds normal boundaries, resulting from uncoordinated and uncontrolled cell proliferation. Tumors can affect various parts of cattle animals, including the skin, bones, glands, and visceral organs. The present study aimed to explore the pathology of bovine skin tumors and their health and economic impacts on cattle. Skin tumors are the most frequently diagnosed neoplastic disorders in bovine species. The most common skin tumors in bovine include bovine papilloma, squamous cell carcinoma, and bovine lymphosarcoma. These tumors pose significant health challenges and have a negative economic impact on cattle production and its byproducts. Clinical features of skin tumors often include hyperkeratosis,



acanthosis, elongated rete pegs, large nodular structures, exophytic and cauliflower-like lesions, and friable lesions. Melanomas, another type of proliferative skin tumor, are characterized by spindle to round cell shapes containing abundant black pigment. More than 90% of skin tumors are linked to prolonged exposure to ultraviolet radiation. Diagnosing a skin tumor in cattle typically involves skin biopsy and fine needle aspiration cytology. Histologically, skin tumor cells exhibit an increased nuclear-to-cytoplasmic ratio, cellular and nuclear pleomorphism, and a discohesive arrangement of cells. In addition to their health implications, skin tumors in cattle result in significant economic losses due to reduced productivity, decreased reproduction rates, carcass condemnation, and the downgrading of skins and hides. Common treatment options for skin tumors include chemotherapy, radiation, and surgical removal. Given that skin tumors are an economically significant disease in Ethiopia, they require increased attention from researchers and the centers for control and prevention. Early diagnosis and effective management of these tumors are crucial issues that must be addressed.

Keywords: Bovine, Cattle, Diagnosis, Skin tumor, Tumor

[Full text-PDF]

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ABOUT JOURNAL



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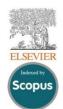
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Bovine Respiratory Disease: Sero-Epidemiological Surveys in Unvaccinated Cattle in Morocco

Said Alali¹, Fatima Zahra Laabouri¹, Imane Choukri^{2*}, Hassan Outenrhrine³, Abdelmounim El Ghourdaf⁴, and Ikhlass El Berbri²

ABSTRACT

Viral respiratory diseases are a major cause of severe clinical symptoms and substantial economic losses in cattle breeding. This study aimed to evaluate the epidemiological status of four respiratory viruses in cattle including Bovine Viral Diarrhea Virus/Mucosal Disease Virus (BVDV/MDV), Infectious Bovine Rhinotracheitis/Infectious Pustular Vulvovaginitis Virus (IBR/IPV), Bovine Respiratory Syncytial Virus (BRSV), and Bovine Parainfluenza Virus type 3 (P13). A total of 1,741 blood samples were collected from 242 unvaccinated herds. The animals comprised 1314 females and 427 males, aged between 6 months and 5 years. The herds included both pure breeds (Holstein or Montbéliarde breeds) and crossbreeds (local-Holstein or local-Montbéliarde), and were located in 89 rural communes of six regions of Morocco including Casablanca-Settat, Rabat-Salé-Kénitra, Marrakech-Safi, Béni Mellal-Khénifra, Fès-Meknès, and Oriental. The samples were analyzed using the I-ELISA technique. The results indicated seroprevalence rates of 56.1, 21.5, 86.4, and 85.4% for BVD/MD, IBR/IPV, BRSV, and Parainfluenza-3, respectively. Co-infections were observed in 88% of the infected cattle, and 95% of the cattle were infected with at least one of the four viruses. Seroprevalence rates varied significantly with age, sex, breed, breeding systems, and practices. These findings confirmed the endemic status of bovine respiratory viral diseases and highlighted their direct and indirect impacts on livestock losses in Morocco.

Keywords: Bovine respiratory syncytial virus, Bovine viral diarrhea Virus/mucosal disease virus, Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis virus, Parainfluenza-3 Virus, Serology

INTRODUCTION

Bovine respiratory disease (BRD) is one of the most prevalent and economically significant diseases affecting both beef and dairy cattle worldwide (Fulton, 2009; Mehinagic et al., 2019). Among all possible causes of bovine respiratory disease, viruses are by far the primary etiological agents as they change the respiratory mucosa, produce cytokines, and impair the functioning of immune system cells (Bosch et al., 2013). Key viral contributors to this condition include bovine viral diarrhea virus/mucosal disease (BVDV/MD), infectious bovine rhinotracheitis/infectious pustular vulvovaginitis virus (IBRV/IPV), bovine respiratory syncytial virus (BRSV), and bovine parainfluenza virus type 3 (PI3) (Fulton, 2009).

Bovine viral diarrhea virus (BVDV) is endemic in cattle populations worldwide. BVDV is a *pestivirus* within the family of *Flaviviridae*, related to the classical swine fever virus and the border disease virus (Vilcek et al., 2004). The economic impact of BVDV has grown with the emergence of more virulent strains since the 1980s and 1990s (Al-Kubat et al., 2021). Significant economic impacts associated with BVDV infection include reduced fertility, abortions, growth retardation, and the birth of calves that remain persistently infected with the virus. These calves are uniquely prone to contracting the lethal condition known as "mucosal disease" (Al-Mubarak et al., 2023).

Infectious bovine rhinotracheitis (IBR/IPV) is a contagious infectious disease caused by bovine herpesvirus 1 (BoHV-1), which belongs to the *Herpesviridae* family and the *varicellovirus* genus (Nandi et al., 2009). Its first discovery is traced back to the early 1950s in fattening units in the Western United States. In Europe, the first IBR/IPV outbreaks were reported in 1970 (Straub, 1975). IBR/IPV can cause respiratory, ocular, reproductive, central nervous system, enteric, neonatal, and cutaneous symptoms in cattle (Iscaro et al., 2021).

Bovine respiratory syncytial virus (BRSV) is one of the main viral infectious agents responsible for the onset of bovine respiratory disease (Sudaryatma et al., 2018). It was first identified as a cause of respiratory disease in the 1970s (Larsen, 2000). BRSV often results in primary infection of the respiratory tract and predisposes cattle to secondary

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infections by bacterial pathogens (Tjønehøj et al., 2003; Agnes et al., 2013). This virus is a member of the *orthopneumovirus* genus within the family of *Pneumoviridae* (Rima et al., 2017). The disease resulting from infection with BRSV can range from subclinical causes to severe manifestations of clinical signs including nasal discharge, anorexia, coughing, pyrexia, and respiratory distress (Valarcher and Taylor, 2007).

The association of bovine parainfluenza virus 3 (PI3) with respiratory disease in cattle has been documented since its first discovery in 1959 (Gaudino et al., 2023). It is classified under the *respirovirus* genus within the *Paramyxoviridae* family (Albayrak et al., 2019). PI3 is now recognized as a widespread infectious agent in cattle populations around the world (Erid et al., 2024). The range of symptoms associated with PI3 infection can vary greatly, from mild or even unnoticed to severe respiratory illness, characterized by a runny nose, elevated body temperature, and persistent coughing (Newcomer et al., 2017). Moreover, cattle infected with PI3 often experience concurrent viral or bacterial infections, suggesting that PI3 may compromise the immune system and facilitate these subsequent infections (Newcomer et al., 2017). In Morocco, few studies have been conducted on bovine respiratory diseases. An early study on 524 cattle from different areas of the country reported seroprevalences of 48.5% for BVDV, 62.8% for Bovine Herpesvirus Type 1, 70.4% for BRSV, and 68.1% for PI3 (Mahin et al., 1985). Alali et al. (1992) conducted a clinical study on BRD in the Gharb region in Morocco and reported morbidity, mortality, and lethality rates of 30.6%, 1.6%, and 5.1%, respectively. Lucchese et al. (2016) identified the primary causes of abortion in dairy cattle in Morocco and reported seroprevalence rates of up to 37.71% for BVDV and 50% for BHV-1. The findings suggest that bovine respiratory viruses are endemic and may have circulated at high levels in the country for a long time.

In 1975, the Moroccan government launched the 'Dairy Plan' to intensify dairy farming and develop milk production, particularly in large urban areas and irrigated coastal regions (Chatibi, 2011). In 2008, the 'Green Morocco Plan' was introduced to bridge the gaps between small, medium, and large farms by modernizing and aggregating them into competitive agro-industrial chains (FAO, 2011). These government initiatives transformed the structure of livestock farming in Morocco, not only enhancing production and productivity but also increasing the prevalence and severity of diseases associated with the industrialization and intensification of livestock farming, particularly respiratory infections (Moroccan Ministry of Agriculture, 2015). Respiratory diseases often occur discreetly and without indicative clinical signs (Patrick and Gorden, 2010). However, under stressful conditions, particularly when transporting and assembling animals at livestock markets, one or more of these diseases may show clinical manifestation, occasionally with very severe symptoms leading to animals' deaths (Patrick and Gorden, 2010). Significant indirect losses such as decreased milk and meat production and increased medical services are often associated with these diseases (ONSSA, 2018). Vaccines against respiratory viruses are available in the Moroccan pharmaceutical market, but only IBR/IPV is a notifiable disease under Moroccan legislation, requiring adherence to a code of procedure established by national authorities (ONSSA, 2018). However, vaccination alone is insufficient to control BVD/MD, IBR/IPV, BRSV, and PI3 if their risk factor management and biosecurity measures are not properly implemented (Makoschey and Berge, 2021). In this regard, the present study aimed to assess the epidemiological status of BVD/MD, IBR/IPV, BRSV, and PI3 in cattle throughout Morocco. It also sought to evaluate the impact of various risk factors associated with these diseases, such as age, sex, breed, mode of reproduction, rearing method, rearing conditions, interaction and movement between farms, usable agricultural area, herd size, and the origin of the cattle on their transmission.

MATERIALS AND METHODS

Ethical approval

All animals were handled according to international laws concerning animal welfare and handling. Blood samples were collected by a veterinarian in a non-stressful environment. Breeders and owners were informed of the objectives and nature of the analysis.

Study period and regions

This cross-sectional study encompassed 242 farms across 89 rural communities in six regions of Morocco (out of a total of 12 regions) known for their high concentration of livestock production, namely Casablanca-Settat, Rabat-Salé-Kénitra, Marrakech-Safi, Béni Mellal-Khénifra, Fès-Meknès, and Oriental. The study was conducted during the cold season from December 2018 to March 2019. A total of 1,741 blood samples were collected (Figure 1).

Selection of farms and animals

Farms were selected to cover as many rural communities as possible and to represent various types of farming and cattle categories. No clinical correlation was sought in this study, which was designed to determine seroprevalence

without regard to the clinical aspects of the viral infections under study. Farm sizes ranged from small herds of 10 animals to large herds of 170. In each farm, a random sample comprising approximately 30% of the total number of cattle in each farm was chosen. As illustrated in Figure 2, the sampling plan within each holding was designed to reflect the structure of cattle farming at the national level.

Furthermore, since there is no way (under this study conditions) to differentiate between vaccinated animals and animals that had/have the diseases, they were excluded from the present study: first, calves younger than 6 months of age (to avoid potential serological interference with the colostral antibodies); second, cattle that are from a region in Morocco (Souss-Massa region) where most animals are usually vaccinated against the four diseases; third, cattle from vaccinated herds within the study regions; and fourth, imported calves from endemic countries, where they typically receive vaccination against these viruses followed by a booster once they reach Morocco.

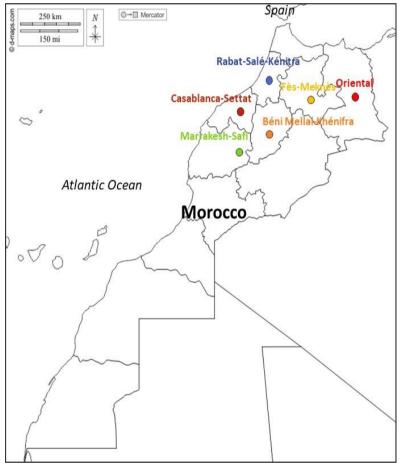


Figure 1. The different regions of the study area in Morocco during 2018-2019

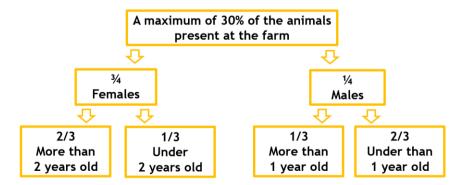


Figure 2. Survey methodology across the sampled farms in the different regions of the study area in Morocco during 2018-2019

Samples

Blood samples were collected from the jugular or coccygeal vein using 10 ml sterile dry tubes and transported to the laboratory at +4°C. After centrifugation (15 minutes at 3000 rpm) and serum collection, the samples were stored at -20°C. For each tested animal, an epidemiological survey was performed to determine (1) the age of the animal (> 6 months and < 1 year, 1 to 2 years, > 2 years), (2) sex, (3) housing conditions (good, average, poor), (4) contact with animals from other farms (yes, No), (5) herd size (1 to 10 cattle, 11 to 40 cattle, more than 40 cattle), (6) reproduction methods (artificial insemination, natural mating, mixed), (7) breeds (crossbred (local-Holstein or local-Montbéliarde), pure breeds (Holstein or Montbéliarde), (8) usable agricultural area (UAA; < 5 ha, 5 to 10 ha, > 10 ha), (9) origin of the animals (imported, born and raised in Morocco), (10) farming methods (extensive, semi-intensive, intensive, fattening, mixed [extensive- intensive- fattening]), and 11) the region under study.

Serological tests

The collected sera were analyzed utilizing indirect enzyme-linked immunosorbent assay (I-ELISA). The IBR-Ab, BVDV-Ab, BRSV-Ab, and PIV3-Ab kits from SvanovaBiotech AB (Uppsala, Sweden) were used according to the manufacturer's instructions to detect specific IgG antibodies against IBR/IPV, BVDV, BRSV, and PIV3, respectively.

The results obtained with the I-ELISA kits used required calculating the optical corrected densities (ODcorr) by subtracting the OD values of the wells containing the control antigen (ODctrl) from the corresponding viral antigencoated wells (ODatg), i.e. (ODcorr=ODatg-ODctrl). Percentage positivity (PP) was then calculated as follows:

 $PP = (OD_{corr} \text{ of test sample}) \times 100/ (OD_{corr} \text{ of positive control})$

The interpretation of the PP into a seropositive/seronegative infectious status was done using the tables provided by the manufacturer.

Statistical analysis

The chi-square independence (chi-square distribution) test was used to assess the possible association between the seroprevalence rates and the different risk factors considered. The study considered a p-value of less than 0.05 as the threshold for statistical significance. This test was conducted using the multiple correspondence factor analysis (MCFA) method. All statistical tests were performed using SPSS software (Modeler 16.0).

RESULTS

Seroprevalence overall and by study area

The overall infection seroprevalence rates found were 56.1% for BVD, 21.5% for IBR/IPV, 86.4% for BRSV, and 85.4% for PI3. The variation of seroprevalence by region is presented in Table 1.

Transmission risk factors of the four studied diseases

Seroprevalence rates for the four studied diseases varied significantly by age, sex, breed of cattle, and breeding systems and practices. The seroprevalence rates according to the different epidemiological factors are presented in Table 2

a. Bovine viral diarrhea/mucosal disease

The results of the analysis showed a significant association (p < 0.05) between BVD/MD seroprevalence and the breeding methods. The fattening farms had the highest rate (73.50%), while extensive farms had the lowest rate (33.33%). In terms of age and sex, older animals (67.38%) and females (59.97%) had the highest rates compared to younger animals (39.72%) and males (44.03%, p < 0.05). The breed of animals also significantly impacted BVD/MD seroprevalence, with pure breeds (62.05%) more frequently infected than crossbreds (54.33%, p < 0.05).

However, no statistically significant association was observed between the seroprevalence of BVD/MD and herd size, reproductive methods (artificial insemination, natural mating, mixed), housing conditions, and whether there was contact or not with animals from other farms (p > 0.05). The multiple correspondence factor analysis (MCFA) identified two factors of the first and second dimensions with an explained percentage variation of 44.6%, which is considered quite satisfactory. In other words, this method explains 44.6% of the total variation of the cloud of points representing the risk factors likely to influence the observed BVD/MD seroprevalence. The projection of the studied parameter modalities on axis 1 (explaining 25.67% of this variation) and axis 2 (explaining 18.89% of the same variation, Figure 3) showed a correspondence between the practice of artificial insemination, animals over 2 years old, females and the high seroprevalence of BVD/MD in the Rabat-Salé-Kénitra and Casablanca-Settat regions. In these two regions, the farms most affected by BVD/MD were those that practiced artificial insemination on adult females (over two years old). In contrast, cattle purchased locally in the Fès-Meknès region were moderately infected with BVD/MD, while male cattle under one year of age from the eastern region were the least affected.

Table 1. Seroprevalence of bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, bovine respiratory syncytial virus, and bovine parainfluenza virus according to the different studied regions in 2018-2019.

Region	Number of samples	BVD seroprevalence (%)	IBR/IPV seroprevalence (%)	BRSVs seroprevalence (%)	PI3 seroprevalence (%)
Casablanca-Settat	446	59.42	24.77	88.79	89.91
Béni Mellal-Khénifra	283	48.76	15.41	88.34	87.28
Rabat-Salé-Kénitra	365	59.73	20	83.01	82.19
Fès-Meknès	78	60.26	25	78.2	82.05
Oriental	221	50.23	22.83	81.9	80.09
Marrakech-Safi	348	56.61	22.09	89.94	85.34
Total	1741				
Overall seroprevalences		56.06	21.48	86.39	85.35

BVD: Bovine Viral Diarrhea virus, IBR/IPV: Infectious bovine rhinotracheitis virus/Infectious Pustular Vulvovaginitis Virus, BRSV: Bovine respiratory syncytial virus and PI3: Bovine para-influenza virus

Table 2. Seroprevalence of BVD, IBR/IPV, BRSV, and PI3 according to the categories of epidemiological studied factors in the different regions of the study area in Morocco during 2018-2019.

Breeding parameters	Categories	Number of	Seroprevalences (%)			
breeding parameters	Categories	sera tested	BVD*	IBR/IPV*	BRSV*	PI3*
	< 5 ha	1080	56.85	18.94	86.02	85.09
TT	5 to 10 ha	351	50.43	26.72	86.61	88.03
UAA	> 10 ha	310	59.68	24.34	87.42	83.23
			(p > 0.05)	(p > 0.05)	(p > 0.05)	(p > 0.05)
	Imported	347	55.33	22.51	86.17	83.57
0:: 64 : 1	Born and raised in Morocco	1237	57.72	21.16	86.18	84.56
Origin of the animals	Mixed origin	157	44.59	21.66	88.53	95.54
			(p < 0.05)	(p > 0.05)	(p > 0.05)	(p < 0.05)
	Extensive	12	33.33	36.36	91.67	83.33
	Semi-Intensive Dairy	872	58.14	16.76	86.47	83.72
5	Dairy Intensive	384	55.47	24.01	89.84	89.58
Breeding methods	Fattening	83	73.50	35.8	89.16	93.98
	Mixed	388	49.22	26.31	82.22	82.99
			(p < 0.05)	(p < 0.05)	(p < 0.05)	(p < 0.05)
	1 to 10 cattle	292	57.88	17.3	81.16	78.08
	11 to 40 cattle	1073	55.36	17.64	87.5	86.58
Herd size	More than 40 cattle	376	56.65	35.77	88.56	87.5
			(p < 0.05)	(p < 0.05)	(p < 0.05)	(p < 0.05)
	AI	1191	56.09	21.14	86.98	87.49
	Natural mating	279	57.35	16.55	85.3	77.78
Mode of reproduction	Mixed	201	47.76	32.49	84.58	82.59
			(p > 0.05)	(p < 0.05)	(p > 0.05)	(p < 0.05)
	Crossbreed	1351	54.33	20.23	86.38	85.05
Breed	Pure breed	390	62.05	25.78	86.41	86.41
			(p < 0.05)	(p > 0.05)	(p > 0.05)	(p > 0.05)
	> 6 months and < 1 year	287	39.72	7.34	64.46	63.07
	1 to 2 years	568	46.65	14.59	80.28	79.05
Age	>2 years	886	67.38	30.57	97.4	96.61
			(p < 0.05)	(p < 0.05)	(p < 0.05)	(p < 0.05)
	Female	1314	59.97	24.73	91.7	90.49
Sex	Male	427	44.03	11.56	70.02	69.55
			(p < 0.05)	(p < 0.05)	(p < 0.05)	(p < 0.05)
	Good	143	57.34	32.12	86.71	83.92
D	Average	1196	55.6	21.49	86.7	85.03
Breeding conditions	Bad	393	58.26	18.2	85.75	86.77
Breeding conditions			(p > 0.05)	(p < 0.05)	(p > 0.05)	(p > 0.05)
G	Yes	55	56.36	3.7	92.73	92.73
Contact with animals from	No	1169	54.58	20.14	86.48	85.46
other farms			(p > 0.05)	(p < 0.05)	(p > 0.05)	(p > 0.05)

^{*}BVD: Bovine Viral Diarrhea virus, IBR/IPV: Infectious bovine rhinotracheitis virus/infectious pustular vulvovaginitis Virus, BRSV: Bovine respiratory syncytial virus and PI3: Bovine para-influenza type 3. UAA: Usable agricultural area; ha: Hectare.

a. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis

The IBR/IPV seroprevalences observed in this study were significantly influenced by farm size (p < 0.05). Larger farms exhibited higher seroprevalence rates compared to smaller farms (35.77% versus 17.30%). The type and conditions of husbandry also significantly affected seroprevalence (p < 0.05): paradoxically, the virus was more prevalent in optimal husbandry conditions than in average or poor conditions (32.12%, 21.49%, and 18.20%, respectively). Additionally, the sex and age of the animals had a significant impact (p < 0.05) with females (24.73%) and older animals (30.57%) showing higher rates than males (11.56%) and younger animals (7.34%). However, the origin and breed of the animals did not significantly affect the seroprevalence of IBR/IPV in this study (p > 0.05).

In MCFA, all variables are loaded onto two factors representing the first and second dimensions, explaining 44.5% of the variance, which is considered satisfactory. These two dimensions effectively summarize 44.5% of the information provided by the parameters for IBR/IPV, reflecting an important relationship between IBR/IPV seroprevalence and the studied parameters. From the projection of the breeding parameter modalities onto the two axes (Figure 4), it can be concluded that farms in the Marrakech-Safi region, which were less affected by the IBR/IPV virus, practice natural mating and have more male cattle under one year of age. Farms significantly affected by IBR/IPV in the Rabat-Salé-Kénitra region had average breeding conditions, practiced artificial insemination, and kept females over two years old (p < 0.05).

b. Bovine respiratory syncytial virus infection

The analysis of the seroprevalence of BRSV according to different epidemiological parameters showed a significant impact of sex and age on the seroprevalence rates (p < 0.05). Females and older animals had higher rates compared to males and younger ones (91.70% and 97.40% versus 70.02% and 64.46%, respectively). Likewise, herd size significantly impacted BRSV infection (p < 0.05), with larger farms being more frequently affected than smaller farms (88.56% versus 81.16%, respectively). However, no significant dependence was observed between BRSV seroprevalences and UAA, the origin of the animals, mode of reproduction, breed of the animals, housing conditions, or contact between cattle from different farms (p > 0.05). The analysis conducted using MCFA revealed two primary factors: the first accounts for 25.69% of the variability, while the second accounts for 19.42%. Together, they explain 45.1% of the total variance, which is deemed to be quite satisfactory. The data visualization indicates that in the Casablanca-Settat region, the Bovine Respiratory Syncytial Virus (BRSV) significantly impacted farms with female cattle older than two years (Figure 5). In contrast, farms in the Rabat-Salé-Kénitra region, which were moderately affected, maintained average hygiene standards and used artificial insemination. Meanwhile, in the Fès-Meknès region, farms that were minimally affected by the virus tended to use natural breeding methods and had a higher presence of crossbred cattle. Notably, the lowest rates of seroprevalence were observed in male cattle aged between six months and one year.

c. Infection with bovine parainfluenza virus 3

The statistical analysis demonstrated a strongly significant association between the seroprevalence of PI3 and several factors, including herd size, reproductive methods, age, and sex of the animals (p < 0.05). Larger herds (87.57%) were more affected by PIV-3 compared to smaller herds (78.08%). Farms practicing artificial insemination showed a higher seroprevalence (87.49%) compared to those practicing natural mating. Similarly, cattle over two years of age (96.61%) and females (90.49%) had higher seroprevalence rates compared to those under one year of age (79.05%) and males (69.55%). However, no statistically significant association was observed between PI3 seroprevalence and UAA, animal breed, husbandry conditions, and contact between animals from different farms (p > 0.05). The evaluation of all variables revealed two primary factors on the first dimension, accounting for 25.7% of the variance, and two factors on the second dimension, explaining 19.4% of the variance. Together, these factors contributed to a total explained variance of 45.1%, which is considered quite satisfactory. The analysis of the modalities across two dimensions (Figure 6) indicates that farms performing artificial insemination (AI) and those with a greater population of females over two years old had higher seroprevalence rates of PI3. This trend was particularly noticeable in Casablanca-Settat and Rabat-Salé-Kénitra regions. In the Fès-Meknes region, the most affected farms were those with locally purchased crossbred animals in substandard hygienic conditions. The lowest levels of seropositivity were found among the youngest male animals, particularly those aged between six months and one year.

d. Co-infections

In this study, only 12% of the tested animals were seropositive for one single virus. Co-infections were frequently observed (88%) across all the regions under study. The most frequent associations were BRSV-PIV-BVD (34.06%), followed by BRSV-PIV (23.78%), and BRSV-PIV-BVD-IBR/IPV (15.33%, Figure 7). Furthermore, it should be noted that only 5% of the studied animals were not seropositive for any of these four infections. This finding highlights the large distribution of these diseases across the studied regions.

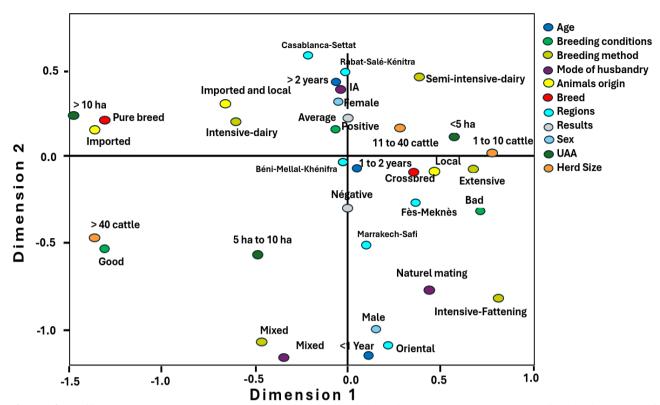


Figure 3. Different parameter modalities concerning bovine viral diarrhea/mucosal disease infection in cattle of Morocco during 2018-2019. The projection of the studied parameter modalities on axis 1 (explaining 25.67% of the variation) and axis 2 (explaining 18.89% of the variation) revealed a correspondence between the practice of artificial insemination, animals over 2 years old, females, and Rabat-Salé-Kénitra region.

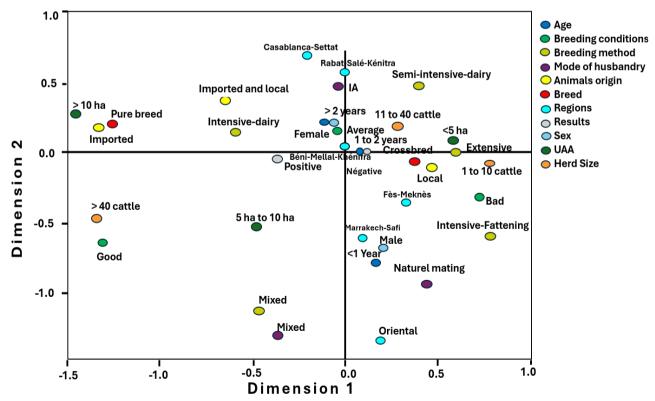


Figure 4. The modalities of the different parameters for bovine rhinotracheitis infection in cattle of Morocco during 2018-2019. The breeding parameter modalities onto the two axes: The Marrakech-Safi region that was weakly affected by the IBR/IPV virus practices natural mating and has more male cattle under one year of age. Farms significantly affected by IBR/IPV in the Rabat-Salé-Kénitra region had average breeding conditions, practiced artificial insemination, and kept females over 2 years old.

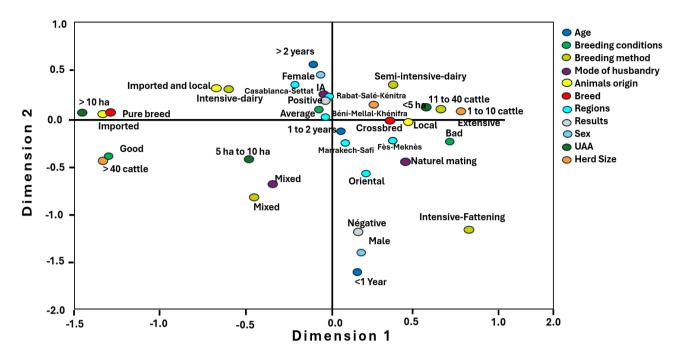


Figure 5. The modalities of the different breeding parameters with respect to bovine respiratory syncytial virus infection in cattle of Morocco during 2018-2019. The data visualization indicates that BRSV significantly impacted farms with female cattle older than two years in the Casablanca-Settat region, while farms in Rabat-Salé-Kénitra region (moderately affected), maintain average hygiene standards and use artificial insemination and farms in the Fès-Meknès region (minimally affected) use natural breeding methods and have more crossbred cattle.

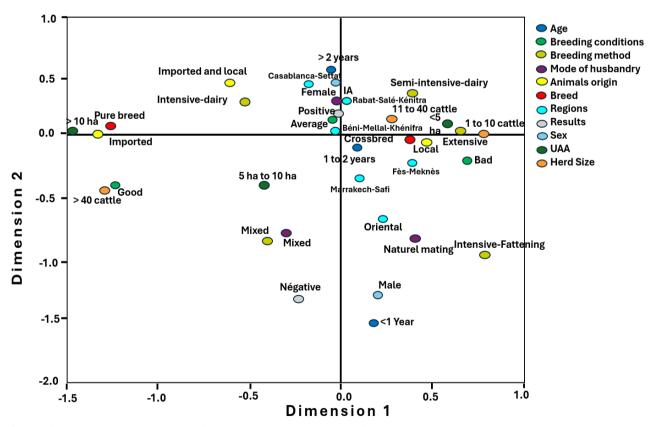


Figure 6. The modalities of the different breeding parameters for parainfluenza virus type 3 infection in cattle of Morocco during 2018-2019. Farms that engaged in artificial insemination and maintained a larger population of females over 2 years old demonstrated higher seroprevalence rates of PIV-3. This trend was especially pronounced in the Rabat-Salé-Kénitra and Fès-Meknès regions. The farms most impacted were those housing locally purchased crossbreed animals in below-average hygienic conditions. Conversely, the lowest levels of seropositivity were observed in the youngest male animals, particularly those aged between 6 months and one year.

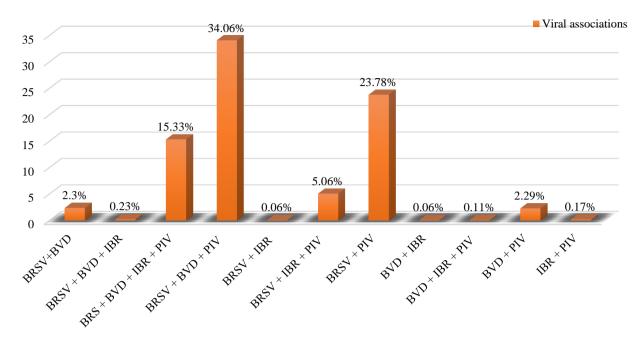


Figure 7. The distribution of various co-infection combinations by the four studied viruses in cattle of Morocco during 2018-2019. The occurrence of co-infection was frequently observed (88%). The most frequent associations were BRSV-PIV-BVD (34.06%), followed by BRSV-PIV (23.78%, and BRSV-PIV-BVD-IBR/IPV).

DISCUSSION

The results of this study indicated that the four viruses involved in bovine respiratory syndrome, namely BVD, IBR/IPV, BRSV, and PIV-3, are circulating in the studied regions with relatively high seroprevalence rates of 56.1%, 21.5%, 86.4%, and 85.4%, respectively. Additionally, 95% of the animals were infected with at least one of these viruses. This underscores the impact of both direct and indirect losses associated with these diseases on livestock in Morocco. However, there have been few comprehensive studies at the national level to assess the epidemiological status, transmission routes, and extent of these diseases, which are critical for managing and controlling their spread with worldwide effects (Gaudino et al., 2022).

Seroprevalence rates of the four diseases

BVDV is a cosmopolitan infection that has been reported on all continents, with seroprevalences ranging from 20% to 90% (Mahmoud and Allam, 2013). Similar seroprevalence rates to those observed in the present study were reported in Brazil (57.7%, Frandoloso et al., 2008), and Switzerland (56%, Schweizer et al., 2011). Higher seroprevalence rates were reported in many other countries such as Thailand (73%, Kampaa et al., 2004), Ethiopia (80 %, Tesfaye et al., 2021), Mexico (87.8%, Gael et al., 2016), and Canada (90%, Ahmad et al., 2011). However, lower seroprevalence rates were reported in Egypt (31.5%, Maher et al., 2023), Saudi Arabia (26%, Mahmoud and Allam, 2013), Belgium (32.9%, Sarrazin et al., 2013), and Jordan (31.6%, Talafha et al., 2009). Moreover, the rate observed in this study was higher than those reported in similar previous studies conducted in Morocco, particularly those conducted by Aiyar (2006) and Mahin et al. (1985) with respective rates of 39.53% and 48.5%. This suggests an increasing spread of the BVD/MD virus at the national level. The observed seroprevalence of bovine herpesvirus type 1 in this study (21.48%) is lower than those reported in several countries, such as Mexico (89.85%, Cordova et al., 2009), Uruguay (37%, Guarino et al., 2008), Thailand (67%, Kampaa et al., 2004), China (35.8%, Yan et al., 2008), and Egypt (75.80%, Amal et al., 2008). However, this rate is similar to those observed in Mexico (85.6%, Calderon et al., 2007) and Turkey (88.82%, Okur et al., 2007), yet it is significantly higher than the rate reported in Saudi Arabia (69.1%, Mahmoud and Allam, 2013). For BRSV, the seroprevalence rate observed in this study (86.39%) was higher than that found previously in Morocco by Aiyar (2006, 78.68%) and Mahin et al. (1985, 70.40%). However, it is similar to those reported in Mexico (85.5%) and Venezuela (85%; Calderon et al., 2007) and higher than those observed in the United States (56.6%; Grubbs et al., 2001) and Saudi Arabia (75.5%; Mahmoud and Allam, 2013).

The seroprevalence rate of PIV-3 infection observed in this study (85.35%) is higher than the rates recorded by previous studies in Morocco, namely those conducted by Aiyar (2006) (67.93%) and Mahin et al. (1985) (68.1%), which suggests an increase in the circulation of this infection during the last years among cattle in Morocco. Nonetheless, this rate is similar to those observed in Mexico (85.6%; Calderon et al., 2007) and Turkey (88.82%; Okur et al., 2007) although it is significantly higher than the rate reported in Saudi Arabia (69.1%; Mahmoud and Allam, 2013).

Evaluation of the impact of different epidemiological factors

The analysis of the seroprevalence variation of the four studied diseases according to different epidemiological parameters revealed that the most significant and common influence on these rates was due to the age and sex of the tested animals. Indeed, for the studied infections, seroprevalence rates increased significantly with age, and females were more frequently seropositive than males. This finding indicated that the risk of animals being infected by the viruses increases over time and suggests that the animals are regularly infected. Furthermore, the observed greater incidence of infections in females relative to males may be attributed to the fact that females are typically older than males at the time of slaughter. This is because female cattle are often kept for extended durations for reproductive purposes, allowing multiple births before they are slaughtered. In this study, more than 75% (1,314/1,741) of the studied animals were females; male cattle are fattened and sold when they are still young. Seemingly, then, the age of animals influences the variation of the seroprevalence rates of the four diseases more than their sex. This finding is supported by many other studies that have reported that the risk of infection by the four viruses increased with the age of animals (Calderon et al., 2007; Nandi et al., 2009; Talafha et al., 2009). Previous studies, including those of Autorino et al. (2002) and Houoiten et al. (2021), indicated that a significant increase in seroprevalence rates for a specific disease or infection correlating with the age of animals in a given area suggests consistent exposure to the pathogen over the years. Essentially, this means that in areas where a disease is endemic, it is common for animals to be regularly infected. This observation, coupled with the high seroprevalence rates identified in the present study, serves as evidence that the four diseases are indeed widespread and established within the country.

Animal breeds have also shown a significant impact on seroprevalence rates. Indeed, the seroprevalence rates, as mentioned before, were higher among the imported pure breeds (Holstein, Montbeliarde) than the local crossbreds (local-Holstein and local-Montbeliarde). According to previous studies, the breed of the animals (Snowder et al., 2006; Hussain et al., 2019) significantly influences the susceptibility or resistance of cattle to respiratory infections. Indeed, breeds such as Holstein, Charolais, Jersey, Friesian, and Friesian-Sahiwal seem to be more susceptible to the Bovine Respiratory Syndrome than the native local ones (Aiyar, 2006; Schelcher, 2008; Daves et al., 2016).

The present study also revealed that other factors, such as breeding methods and conditions, mode of reproduction, and the size of the herd, can also influence the seroprevalence rates of the four studied diseases. As mentioned earlier, several governmental programs have been launched in Morocco to intensify dairy farming and develop milk production. The industrialization and intensification of farming practices come with several factors favoring the transmission and spread of respiratory diseases, namely overcrowding, stress (from transport, feeding, etc.), confinement of animals in unfavorable hygienic and aeration conditions, etc. Moreover, many other studies have observed that the seroprevalence of respiratory infections is influenced by the employed breeding strategies and management practices. The impact of breeding systems and practices on respiratory disease infection rates was also observed in several other studies (Calderon et al., 2007; Schelcher, 2008; Cordova Izquierdo et al., 2009). Likewise, the present study revealed a significant association between artificial insemination practice, females aged more than two years, and the high infection rates of the studied respiratory diseases. The production and use of semen in cattle are consistently monitored for sexually transmitted diseases, which minimizes the likelihood of these infections spreading via semen. However, the observed results in the current study may be due to the use of artificial insemination primarily in adult female cattle that are kept for breeding purposes, and, as previously mentioned, they have a higher susceptibility to infection over time. Furthermore, the findings indicated a high prevalence of co-infections among the tested animals, with 88% showing seropositivity for multiple viruses. Co-infections can modulate disease outcomes, potentially exacerbating the severity of diseases. For instance, the presence of one pathogen can impair the host's immune response, making it more susceptible to other infections, or conversely, prompt an immune response that inhibits the replication of another pathogen. In terms of transmission dynamics, co-infections can influence the rate and method of disease spread. The interaction between different pathogens may lead to synergistic effects that enhance transmission or competitive interactions that reduce it (Kumar et al., 2018; Devi et al., 2021). Consequently, understanding the specific interactions between co-infecting pathogens is crucial for developing effective control measures.

CONCLUSION

This study has established that the four viruses involved in bovine respiratory syndrome are widespread in Morocco, with current seroprevalence rates significantly surpassing previous records. Consequently, it is imperative to manage and mitigate the transmission of these diseases, given the substantial direct and indirect losses they inflict on Moroccan livestock. While vaccination is strongly advised, it becomes futile without the concurrent implementation of biosecurity protocols, which include optimized architectural design for animal housing, improvement of hygiene conditions, reduction of stress and confinement, regular cleaning and disinfection of instruments and equipment used, AI control, elimination or isolation of infected animals, and animal movement control. However, the application of these control strategies is not without its challenges and limitations. Factors such as limited resources, infrastructural deficits, and cultural practices should be acknowledged. Indeed, these factors can significantly influence the practicality and success of the control measures. On the other hand, understanding the genetic characterization of these viruses may lead to more targeted and effective prevention strategies, ultimately contributing to better management of bovine respiratory diseases. Indeed, further studies focusing on the genomic characterization of the circulating viruses for medical prevention purposes are necessary at the national level.

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Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

Authors' contributions

Said Alali made a major contribution to the study design, realization, and supervision of laboratory work, data analysis and interpretation, and redaction of the manuscript. Ikhlass El Berbri made a major contribution to study design, data analysis, and interpretation, redaction of the manuscript, and preparation of the final version of the manuscript. Fatima Zahra Laabouri contributed to the study design, data analysis and interpretation, and redaction of the manuscript. Imane Choukri revised the final version of the manuscript, submission of the manuscript, and coordinated the authors. Outenrhrine Hassan coordinated between commune chiefs and farm managers, conducted fieldwork, collected blood samples, and participated in data acquisition. El Ghourdaf Abdelmouni coordinated between commune chiefs and farm managers, conducted fieldwork, collected blood samples, and participated in data acquisition. All authors have read and given final approval for the last edition of the article to be published.

Ethical considerations

All authors were screened for ethical issues, including plagiarism, consent for publication, misconduct, fabrication of data, and duplicate publication or submission.

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Establishment of Mammary Tumors by Injection of 7,12-Dimethylbenz[a]anthracene in Mammary Fat Pad of Rats

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ABSTRACT

Chemical-induced mammary cancer models are widely used to mimic human breast carcinogenesis, with 7.12dimethylbenz[a]anthracene (DMBA) being a commonly used agent. The oral administration of DMBA frequently results in the formation of tumors at random locations and carries significant risks, including high mortality rates and damage to various organs. To address these issues, this study employed a subcutaneous DMBA administration protocol to induce mammary cancer in rats. A total of twenty-four female Sprague-Dawley rats aged 45-55 days weighing 112-130 g were divided into four groups, including the control group injected with 0.75 mL corn oil (D0), a single dose of DMBA at 80 mg/kg BW (D1), two doses with a one-week interval (D2), and three doses with oneweek intervals (D3), all administered via subcutaneous in mammary fat pad. Control groups (D0) did not show any tumor growth. Mammary tumor incidence increased with dosage (D1 33.33%, D2 66.67%, and D3 100%). Histopathological examination revealed the presence of various mammary tumor types without evidence of metastasis in all induced rats. All tumors originated from the injection site, and only a single nodule was observed in each rat. There were no significant differences in tumor grades between the treatment groups, and no mortality was recorded during the study. The D3 group showed the highest tumor incidence over the three-month observation period. These findings suggest that subcutaneous DMBA administration effectively induces mammary cancer in rat models with controlled tumor localization and minimal systemic effects, making it a promising method for experimental breast cancer studies.

Keywords: 7,12-dimethylbenz[a]anthracene, Animal model, Breast cancer, Mammary fat pad, Rat

INTRODUCTION

Chemical-induced mammary cancer models are widely used to simulate human breast cancer carcinogenesis, effectively representing the associated cellular and molecular changes (Abba et al., 2016) compared to tumor-transplanted models (Costa et al., 2020). A variety of chemical compounds, such as 7,12-dimethylbenz[a]anthracene (DMBA), N-methyl-N-nitrosourea (NMU), 2-amino-1-methyl-6-phenylimidazo[4,5-B] pyridine (PhIP3), and methylcholanthrene (MC), have been demonstrated to induce mammary cancer in animal models (Bazm et al., 2018). Among these, DMBA, a member of the polycyclic aromatic hydrocarbon (PAH) family, is frequently employed due to its ability to mimic the multistep carcinogenic process observed in human breast cancer (Liu et al., 2015).

Significant efforts have been made to develop breast cancer models, with studies examining various doses and administration routes of DMBA. Oral administration is the most common but has notable drawbacks, including high mortality (Fidianingsih et al., 2022), pathological lesions in multiple organs (Budi and Widyarini, 2010; Batcioglu et al., 2012; Costa et al., 2020; Allam et al., 2023), and risks of acute death from lung exposure due to administration errors. Additionally, oral DMBA induces randomly located tumors with varying nodule numbers (Barros et al., 2004; Costa et al., 2020), complicating studies requiring a single, well-defined nodule for targeted therapy. Systemic administration can also lead to tumors in mammary glands and other tissues (Dias et al., 1999; Tedardi et al., 2013).

To address these challenges, subcutaneous administration of DMBA into the mammary fat pad has been proposed, enabling localized tumor formation while minimizing the systemic effects and mortality. Several studies have employed this approach with varying doses (El-Makawy et al., 2022; Silihe et al., 2023; Sewoyo et al., 2024). However, detailed histopathological characteristics and their systemic effect remain underreported. The present study aimed to evaluate the

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mortality rate, and detailed histopathological characteristics of mammary cancer induced by subcutaneous administration of DMBA, comparing single dose versus repeated doses and evaluating its systemic effect on internal organs. In addition, the study aimed to provide a clearer understanding of the impact of this method on tumor localization and grading while minimizing systemic effects that could influence experimental outcomes.

MATERIALS AND METHODS

Ethical approval

The current study received ethical permission from the Animal Testing Ethics Committee of the Veterinary Medicine Faculty, Udayana University, Indonesia (No. B/145/UN14.2.9/PT.01.04/2024). All procedures were conducted in accordance with the strict guidelines and regulations of the committee.

Animals

Twenty-four nulliparous female Sprague Dawley rats (*Rattus norvegicus*), aged 45-55 days and weighing between 112-130 g, were used as experimental subjects. The animals were obtained from the Department of Pharmacology, Faculty of Medicine, Udayana University, Indonesia. The rats in the four treatment groups were housed in separate cages, each measuring 53 x 38 x 16 cm, in the Pathobiology Laboratory, Faculty of Veterinary Medicine, Udayana University, under a 12-h light/12-h dark cycle. The rats were provided with standard pellet feed and water *ad libitum*.

7,12-dimethylbenz[a]anthracene preparation

The rats were weighed to determine their body weight, and the corresponding DMBA dose was calculated (El-Makawy et al., 2022). The DMBA (Tokyo Chemical Industry, Tokyo, Japan) was weighed according to the calculated dose and dissolved in 0.75 mL of corn oil using a magnetic stirrer until the solution was homogeneous. An autoclave sterilized the solution (Sewoyo et al., 2024).

Treatment protocols

Before treatment, the rats were acclimatized for two weeks. The rats were divided into four groups including D0 received a single injection of vehicle (corn oil) 0.75 mL, D1 received a single injection of DMBA at 80 mg/kg BW, D2 who received two injections with one-week intervals, and D3 received three injections, also with one-week interval. Each treatment group consisted of six rats that were randomly allocated to each group. Before each injection, the rats were anesthetized with ketamine and xylazine at doses of 75 mg/kg BW and 10 mg/kg BW respectively, by i.m. injection (IACUC, 2011). The injection site was shaved with clippers, followed by aseptic preparation. DMBA was administered subcutaneously into the mammary fat pad. The study period lasted three months, during which tumor growth was monitored. Rats were euthanized when tumor masses exceeded 30 mm in diameter or when they exhibited poor general health.

Euthanasia and sample processing

Rats were euthanized by intraperitoneal injection of a lethal dose (10 times the normal dose) of ketamine and xylazine according to American Veterinary Medical Association (AVMA) guidelines (Leary et al., 2020). After rats were euthanized, tumor tissue was excised. Rats were also necropsied to collect internal organs, such as lungs, heart, liver, kidney, and stomach. All tissues were fixed in 10% neutral buffered formaldehyde for 24 hours, followed by routine histological preparation, and stained with Harris hematoxylin-eosin.

Histopathological examination

The histopathological characteristics of the tumors were evaluated based on Goldschmidt et al. (2011) and do Nascimento and Otoni (2020). Tumor grading was conducted according to the Nottingham grading system (Elston and Ellis, 1991). Multiple organs were evaluated for pathologic lesions and neoplastic transformation.

Data analysis

Histopathological types and tumor grades are presented in tabular form, while microscopic findings are described narratively. Differences in tumor incidence and histopathological grade between groups were assessed through the Kruskal-Wallis test and Mann-Whitney U-test. Statistical analyses were performed using SPSS version 26, 2018 for Windows, and a p-value < 0.05 was considered statistically significant.

RESULTS

Histopathology characterization and tumor grading

In this study, mammary tumors were induced via subcutaneous injections of DMBA into the mammary fat pad, and the effects of different dosing regimens were observed over three months. Tumor development and characteristics were subject to rigorous monitoring. The control group (D0), which received the vehicle solution (corn oil), did not develop any tumors (0%). In the D1 group, which received a single dose of DMBA, two out of six rats (33.33%) developed tumors. The D2 group, which received two doses, showed an increased tumor incidence, with four out of six rats (66.67%) developing tumors. The highest tumor incidence was observed in the D3 group, in which all six rats (100%) developed tumors after receiving three doses of DMBA.

The histopathological analysis revealed the tumor compositions in the D1 group. The tumors were found to consist of cribriform ductal carcinoma *in situ* (DCIS, 50%, Figure 1a) and papillary DCIS (50%, Figure 1b). The D2 group exhibited solid DCIS (25%, Figure 1c), cribriform DCIS (50%), and comedo DCIS (25%, Figure 1d). In the D3 group, a more diverse range of tumors was observed, including lipid-rich carcinoma (16.67%, Figure 1e), invasive cribriform carcinoma (16.67%, Figure 1f), comedo DCIS (33.33%), and cribriform DCIS (33.33%, Table 1).

The results revealed a statistically significant difference in tumor incidence between the groups (p < 0.05). The results confirmed a significant difference in tumor incidence between D0 and D2, D0 and D3, and D1 and D3 (p < 0.05). It can be concluded that DMBA injection significantly increased tumor incidence compared to the control group. Three repeated doses of 80 mg/kg of DMBA significantly increased tumor incidence compared to a single dose injection. Despite these differences in incidence, there was no significant variation in tumor grade across the dosing regimens as indicated by the Kruskal-Wallis test.

It is noteworthy that all tumors consistently originated at the injection site, with each rat developing only one tumor nodule. No mortality occurred during the study, and examination of internal organs, including the lungs, heart, kidneys, and stomach, revealed no evidence of tumor formation. The finding indicated that DMBA-induced tumors were localized specifically to the mammary glands. Moreover, no metastasis was detected throughout the three-month observation period.

In addition to the tumor formation, several organs in rats across all treatment groups exhibited mild lesions. These included pulmonary inflammation (Figure 2a), hepatic inflammation and vacuolar degeneration (Figure 2b), and renal inflammation and tubular degeneration (Figure 2c, 2d). No lesion was observed in the heart and stomach of any of the rats in all the treatment groups (Figure 2e, 2f). All organs exhibited no indications of toxicity. A comprehensive description of the lesions observed in each treatment group is presented in Table 2.

Table 1. Histopathological classification and tumor grading in rats administrated with 7,12- dimethylbenz[a]anthracene during a three-month observation period

				Tumor		
Group	Rat no.	Histopathological type	Tubular Formation	Nuclear Atypia	Mitosis Count	grade
	1	Cribriform DCIS	2	1	1	1
	2	Papillary DCIS	2	2	2	2
D1	3	-	-	-	-	-
	4	-	-	-	-	-
	5	-	-	-	-	-
	6	-	-	-	-	
	1	Solid DCIS	3	1	1	1
	2	Cribriform DCIS	3	2	2	2
D2	3	Cribriform DCIS	3	2	2	2
DZ	4	Comedo DCIS	3	2	2	2
	5	-	-	-	-	-
	6	-	-	-	-	-
	1	Invasive Cribriform Carcinoma	3	3	3	3
	2	Comedo DCIS	3	2	2	2
D3	3	Comedo DCIS	3	2	2	2
DJ	4	Lipid-rich Carcinoma	3	2	2	2
	5	Cribriform DCIS	3	2	1	2
	6	Cribriform DCIS	3	2	1	2

Note: D1 received a single injection of DMBA at 80 mg/kg BW, D2 received two injections with a one-week interval, and D3 received three injections, also with a one-week interval. DCIS: Ductal carcinoma *in situ*. *Score criteria are based on the Nottingham Grading System. Tubular formation, nuclear atypia, and mitosis count scores were tabulated. Score 3-5: Grade 1; Score 6-7: Grade 2; Score 8-9: Grade 3.

Table 2. Lesions in the internal organ of rats after induction of 7,12-dimethylbenz[a]anthracene

Group	Organ	Lesions						
		Deg.	Infl.	Nec.	Met.	Neo.		
D0	Lungs	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Liver	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Kidney	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
D1	Lungs	(0/6)	(2/6)	(0/6)	(0/6)	(0/6)		
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Liver	(1/6)	(3/6)	(0/6)	(0/6)	(0/6)		
	Kidney	(1/6)	(2/6)	(0/6)	(0/6)	(0/6)		
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
D2	Lungs	(0/6)	(1/6)	(0/6)	(0/6)	(0/6)		
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Liver	(2/6)	(2/6)	(0/6)	(0/6)	(0/6)		
	Kidney	(2/6)	(1/6)	(0/6)	(0/6)	(0/6)		
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
D3	Lungs	(0/6)	(3/6)	(0/6)	(0/6)	(0/6)		
	Heart	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		
	Liver	(1/6)	(2/6)	(1/6)	(0/6)	(0/6)		
	Kidney	(2/6)	(2/6)	(0/6)	(0/6)	(0/6)		
	Stomach	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)		

Note: D0 received a single injection of vehicle (0.75 mL corn oil), D1 received a single injection of DMBA at 80 mg/kg BW, D2 received two injections with one-week intervals, and D3 received three injections, also at one-week intervals. Deg: Degeneration; Infl: Inflammation; Nec: Necrosis; Met: Metastasis; Neo: Neoplasia.

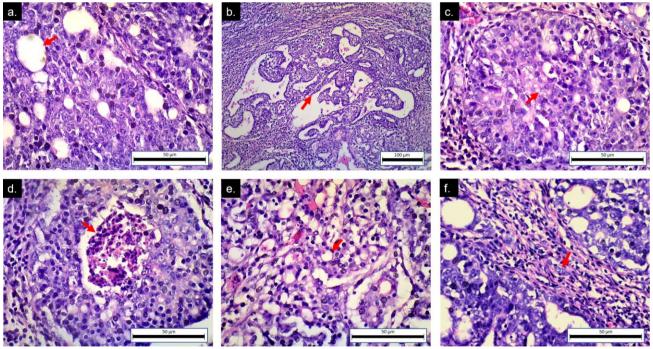


Figure 1. The mammary gland tumors induced by DMBA (7,12-dimethylbenz[a]anthracene) in rats. **a**: Cribriform ductal carcinoma *in situ* (DCIS), showing the proliferation of neoplastic cells with punched-out spaces within ductus (arrow) **b**: Papillary DCIS, where neoplastic cells form papillary structures with fibrovascular core within the ductus (arrow) **c**: Solid DCIS, characterized by a solid proliferation of neoplastic cells within the ductus (arrow) **d**: Comedo DCIS, marked with proliferation of neoplastic cells with central necrosis (arrow) **e**: Lipid Rich Carcinoma, marked with abundant lipid vacuoles within tumor cells (arrow) **f**: Invasive Cribriform Carcinoma, demonstrating cribriform carcinoma with neoplastic cells invading the stroma (arrow).

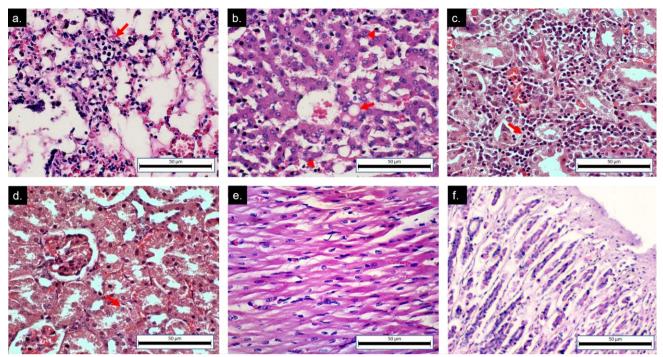


Figure 2. The internal organs of rats following subcutaneous administration of DMBA. **a**: Lungs, **b**: Liver, **c**, **d**: Kidney, **e**: Heart, and **f**: Stomach. The lungs (a) exhibit mild infiltration of mononuclear cells in the alveolar septa (arrow). The liver (b) shows vacuolar degeneration of hepatocytes (arrow) and mild inflammation (arrowhead). The kidney (c) exhibits tubular epithelial degeneration and mononuclear cell infiltration (arrow). The heart and stomach appear to be unremarkable.

DISCUSSION

The DMBA, a well-known member of the PAHs family, has been extensively studied through various administration routes to induce mammary cancer. The subcutaneous route, which directly targets the mammary fat pad, has demonstrated efficacy in producing localized tumors with minimal off-target effects. As reported by Barros et al. (2004), the oral administration of DMBA in rats resulted in an average of 4.9 nodules per animal, with a range of 1-15 nodules located at various sites. Similarly, Costa et al. (2020) reported that the induction of mammary cancer via oral administration of DMBA resulted in an average of 4.7 nodules per animal, with an average of 3.67 invasive tumors per animal. In contrast, in the current study, DMBA was injected subcutaneously into the mammary fat pad, which resulted in consistent tumor formation at the targeted site with minimal impact on other organs. This method produced a single tumor nodule per animal, making it a suitable model for studying localized cancer treatments like intratumoral therapy.

In the present study, increased DMBA dosage is associated with higher tumor incidence. However, there were no significant differences in tumor grades were observed among the various dosing groups, suggesting that while the subcutaneous method effectively induces localized tumors, it may not promote the progression to more aggressive cancer forms within the three-month observation period. The finding is consistent with Kubatka et al. (2002), who also demonstrated a dose-dependent relationship between DMBA dosage and tumor incidence, albeit with different administration routes and dosing regimens. Kubatka et al. (2002) found that administering repeated doses via oral induced a higher incidence of tumors compared to a single dose. The rarity of metastasis in DMBA-induced cancers suggests that a longer observation period might be necessary to observe metastatic progression (Abba et al., 2016).

In the present study, the histopathological analysis revealed a diverse range of tumor types, including papillary DCIS, solid DCIS, cribriform DCIS, comedo DCIS, lipid-rich carcinoma, and invasive cribriform carcinoma. Notably, cribriform DCIS was the most frequently observed type. The occurrence of lipid-rich carcinoma is consistent with the findings of Fidianingsih et al. (2022), who identified this rare tumor type following oral DMBA administration. Chemical induction of cancer generally results in a broader spectrum of tumor types compared to tumor transplantation in immunocompetent mice. Nevertheless, it effectively mimics the multistep carcinogenesis process observed in human breast cancer (Costa et al., 2020).

Molecularly, breast cancer is classified into distinct subtypes, including luminal A, luminal B, HER2-positive, and triple-negative based on the expression of specific biomarkers. The classification is based on the expression of estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 (do Nascimento and Otoni, 2020; Fidianingsih et al., 2022). Given that luminal subtypes are the most common in humans, further research is required to ascertain whether the

subcutaneous DMBA model accurately reflects human breast cancer subtypes by examining these proteins and receptor expression through immunohistochemistry.

The present study revealed that organs, including the lungs, liver, and kidneys exhibit indications of degeneration and inflammation. However, no evidence of necrosis, metastasis, or neoplasia was observed. Reactive metabolites formed during DMBA metabolism, such as 7,12-DMBA-3,4-diol-1,2-epoxides have been demonstrated to cause tissue damage (Batcioglu et al., 2012). The observed inflammation in certain organs is likely attributable to DMBA, which has been linked to an inflammatory response through increased pro-inflammatory cytokines (Youssef et al., 2022).

According to Tedardi et al. (2013), the oral administration of DMBA in Balb/c mice not only induces breast cancer but also leads to tumor development in other tissues, including the skin (1.43%), stomach (7.14%), lymphoid tissues (11.43%), and lungs (17.14%). Similarly, Dias et al. (1999) found that oral administration of DMBA in female Sprague Dawley rats can induce tumors beyond breast cancer, including benign tumors of the salivary glands (3.57%), ovary (5.35%), liver (1.78%), and epiploon (0.89%). They also documented the emergence of malignant tumors in the salivary gland (1.78%), lymphatic system (1.78%), eye (0.89%), lungs (0.89%), and pancreas (0.89%).

Batcioglu et al. (2012) found that intraperitoneal administration of DMBA can result in the formation of necrotic areas within the liver and the infiltration of inflammatory cells. Additionally, Allam et al. (2023) reported that oral DMBA administration can lead to hepatocyte vacuolization, apoptosis, and renal tubular epithelial degeneration. The administration of DMBA orally for the induction of mammary cancer induction has been associated with pathological changes in the stomach, including mucosal and submucosal hemorrhage, epithelial cell hyperplasia, necrosis, and the development of precancerous lesions (Budi and Widyarini, 2010). Furthermore, there have been reports of its ability to induce adrenal toxicity (Costa et al., 2020).

The lesions observed in the study were not severe, indicating that the subcutaneous administration of DMBA minimized the systemic impact while effectively inducing localized breast cancer.

CONCLUSION

The current study demonstrated that subcutaneous administration of DMBA at 80 mg/kg BW in the mammary fat pad of Sprague Dawley rats effectively induced mammary cancer with varying tumor types based on the dosing regimen. Tumors were observed to consistently arise from the injection site with no significant differences in tumor grade across treatments. Additionally, examination of internal organs, such as the lungs, heart, kidneys, and stomach revealed no evidence of tumor development or signs of toxicity. It is noteworthy that no mortality was observed during the study period. Further research is required to ascertain the molecular type of mammary cancer through immunohistochemical staining for Ki67, ER, PR, and HER2.

DECLARATIONS

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Authors' contributions

Palagan Senopati Sewoyo was responsible for the conceptualization, writing of the original draft, investigation, methodology, formal analysis, and visualization. Ni Luh Lasmi Purwanti contributed to the investigation, project administration, and funding acquisition. Muhammad Munawaroh handled the investigation, data curation, and funding acquisition. I Made Kardena contribute to supervision, validation, formal analysis, and writing of the review and editing. I Nyoman Mantik Astawa provided resources, supervision, writing of the review and editing, and validation. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that no competing interests exist.

Ethical considerations

All authors have diligently reviewed the manuscript for potential ethical issues, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication.

Availability of data and materials

All data generated during this research are pertinent and have been included in the published article. For further information or inquiries, please contact the corresponding author.

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The Differential of Glucocorticoid Bioavailability Modulators mRNA Expression and Neurotrophic Factors in Three Organs in Broiler Chicken under Immobilization Stress

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ABSTRACT

The hypothalamus-pituitary-adrenal (HPA) axis remains active despite the elevated corticosterone (CORT) levels during immobilization stress (IMS). This indicates that the HPA axis activity is dependent not only on CORT concentrations but also on the availability of free active CORT that is unbound of corticosteroid-binding globulin (CBG) and is activated by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme. The study examined the CORT levels in the blood and the mRNA expression of both proopiomelanocortin gene in the anterior pituitary gland (APit) and brain-derived neurotrophic factor (BDNF) in the septohypothalamus during IMS. Additionally, the expression of glucocorticoid receptor (GR), 11 β -HSD, and CBG were analyzed in the septohypothalamus, APit, and liver. The experiment included three male Cobb 500 chicken groups, a control group, and two treatment groups exposed to 60 or 120 minutes of IMS. Blood, brain, APit, and liver were collected at 35 days of age (N= 12 samples/group). CORT concentrations in blood were quantified using radioimmunoassay, while reverse transcription-quantitative PCR was used to measure mRNA levels of CBG, 11\(\theta\)-HSD1, 11\(\theta\)-HSD2, and BDNF in the septohypothalamus, APit, and liver. The findings suggested that the IMS activated the HPA axis, as demonstrated by increased CORT levels and changes in proopiomelanocortin expression within the APit of stressed chickens compared to unstressed ones. The septohypothalamus of stressed chickens showed an increase in the CBG, BDNF, and 11B-HSD1 mRNA levels, whereas 11B-HSD2 and GRs expression remained stable compared to the control group. Although CBG and BDNF expression decreased from peak levels, their mRNA remained significantly elevated in the 120-minute group. In liver tissue, the treatment groups showed higher levels of 11\(\beta\)-HSD1 and CBG expression, but 11β-HSD2 expression decreased. Overall, CORT levels and the expression of GR and CORT modulators seemed to have a significant influence on the stress response. Notably, increased mRNA levels of CBG and 11β-HSD1 could improve the availability of free active CORT. Furthermore, a positive correlation between CORT levels and BDNF expression was demonstrated, highlighting the role of BDNF in neuronal protection during IMS. Additionally, the liver may contribute to stress regulation through the functions of CBG and 11β -HSD, which are vital for CORT activation and transport.

Keywords: 11β-hydroxysteroid dehydrogenase, Brain-derived neurotrophic factor, Corticosteroid binding globulin, Corticosterone, Immobilization stress

INTRODUCTION

In vertebrates, the hypothalamus-pituitary-adrenal axis is a neuroendocrine system that controls homeostasis (Ulrich-Lai and Herman, 2009; Smulders, 2021). In avians, different stressors trigger neurons in the septal brain region and hypothalamus, causing activation of stress-related neurons that secret corticotrophin-releasing hormone (CRH) and arginine vasotocin (AVT) into the portal circulation. These hormones subsequently stimulate the anterior pituitary (APit) to cleave proopiomelanocortin (POMC) into a variety of peptides, including the adrenocorticotrophic hormone (ACTH, Kuenzel and Jurkevich, 2010; Bonfiglio et al., 2011). In avian, ACTH promotes CORT secretion from the adrenal cortex in response to both psychological and physiological stimulation (Romero, 2004; Herman et al., 2016). Furthermore, CORT interacts with glucocorticoid receptors (GRs) at various levels, including the APit, hypothalamus, and extrahypothalamic regions, to reduce the HPA axis stimulation and restore homeostasis (de Kloet et al., 2005; Vandenborne et al., 2005; Keller-wood, 2015).

According to studies, only unbound GCs can reduce the activity of the HPA axis because of their potential to cross the blood-brain barrier and interact with GRs in the target tissues (Willnow and Nykjaer, 2010; Groeneweg et al., 2011). Nevertheless, it is noteworthy that only 5% of GCs circulate freely in the blood, while 5-10% are binding non-specifically to albumin, and 80-90% are binding to corticosteroid-binding globulin (CBG; formally transcortin; Perogamvros et al., 2012; Hammond, 2016). CBG is primarily produced in the liver and many other tissues, including brain, heart, kidney, adipose tissue, lung, and pancreas (Tinnikov, 1999). Additionally, Chapman et al. (2013) demonstrated that local GCs can be modulated by two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). The

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first isozyme, 11β-HSD1, converts inert 11-dehydrocorticosterone and cortisone into active CORT and cortisol, respectively (Tomlinson et al., 2004). In avian studies, non-significant alterations in the mRNA levels of 11β-HSD1 were found in the hypothalamus following one hour of restraint stress (Krause et al., 2021). In contrast, rodent studies indicated an elevation of 11β-HSD1 mRNA levels in the extrahypothalamic regions, which subsequently inhibited HPA axis functions during acute stress (Spiers et al., 2016). The second isozyme, 11β-HSD2, plays a crucial role in transforming active GCs (cortisol and CORT) back to the inactive form, which is the ketoform. Additionally, it is worth noting that cortisol is the term applied to GCs in both fish and mammals, including humans. In contrast, corticosterone (CORT) is the nomenclature used for these hormones in birds, reptiles, amphibians, and rodents (Romero, 2004; Herman et al., 2016).

As previously stated, the primary drivers of the HPA axis in chickens are the CRH and AVT hormones that are secreted by hypothalamic paraventricular nucleus (PVN) neurons. Researchers have found that these neurons were activated by a range of stressors, including feed deprivation (Nagarajan et al., 2017a), heat stress (Cramer et al., 2015), and immobilization stress (IMS, Aman et al., 2016). Specifically, IMS, defined as a restriction in movement and aggressiveness, is an effective strategy for inducing both physical and psychological stress, potentially affecting the HPA axis and increasing CORT levels. Notably, recent stress studies have shown that CRH and AVT-producing neurons in the septohypothalamic region remain active despite the rise in the CORT levels (Kadhim et al., 2019; 2021). Furthermore, previous research showed that the brain-derived neurotrophic factor (BDNF) is essential for the activation of CRH neurons during stress sessions. Specifically, BDNF gene expression preceded CRH gene expression, suggesting its significance in the stress response (Nagarajan et al., 2017b; Miranda et al., 2019; Notaras and van den Buuse, 2020). According to the literature, stress has a variety of negative impacts on vital organs (Yaribeygi et al., 2017; Bohler et al., 2021). However, the mechanism by which neurons are protected in stressful situations has remained ambiguous. Miao et al. (2020) have emphasized BDNF's protective role in reducing stress-related neuronal damage. Furthermore, the relationship between CORT levels and BDNF expression is currently under investigation. Consequently, it would be intriguing to explore the effects of glucocorticoid modulators on the HPA axis functions. Thus, the current study hypothesized that the regulation of stress responses in chickens is influenced not only by the actions of CORT through GRs but also by the levels of free active CORT. As a result, the research aimed to measure CORT concentration in the blood and POMC mRNA in the APit during acute IMS as indicators of HPA axis activation. Additionally, the study sought to evaluate the expression of BDNF mRNA in the septohypothalamus to determine the correlation between BDNF mRNA expression and CORT levels. Furthermore, the mRNA expression of GRs, 11\(\theta\)-HSD, and CBG in the septohypothalamus, APit, and liver were analyzed to investigate the role of GC modulators in regulating the HPA axis activity.

MATERIALS AND METHODS

Ethical Approval

The University of Arkansas Institutional Animal Care and Use Committee (IUCAC/USA) protocol # 19054 has approved all procedures used in this experiment, including housing conditions, handling, immobilization, and sampling.

Animals, stress procedure, and sample collection

Male broiler chickens (one-day-old, Cobb 500) were obtained from a commercial hatchery located in northwest Arkansas /Arkansas/USA on the day of the hatch. To eliminate any potential for gender-related variables, only males were used in the current study. The chickens were reared in controlled environments with unrestricted access to water and feed. During the initial three days, the chickens were subjected to continuous lighting to help them find feed and water easily. Following this period, chickens were transitioned to a photoperiod that consisted of 16 hours of light and 8 hours of darkness, with lights turned on at 6:00 AM. The chickens were fed an ad libitum standard diet containing 3050 kcal/kg metabolic energy and 23.3% crude protein (NRC, 1994). Additionally, the ambient temperature was reduced by 2.5 degrees per week from 32 °C to 24 °C, while the humidity was kept between 30% and 40%. Initially, the chickens were housed in a battery system for three weeks before being relocated to cages measuring 85 cm in height, 60 cm in weight, and 70 cm in length, where they were grouped in sets of three. The chickens were then categorized randomly into three distinct groups including, a control group referring to unstressed chickens and two treatment groups. Chickens in treatment groups were exposed to immobilization stress (IMS) for either 60 minutes called 60 minutes or 120 minutes and called 120 minutes. Each group included 14 chickens; thereafter, two samples were excluded from each group because of technical issues during sample collection and/or processing, resulting in 12 chickens per group. The IMS protocol was initiated in week 5, during which the stressed chickens were secured in a harness towel that restricted their wing movement and standing ability while still allowing them access to feed and water throughout the period of the immobilization (Aman et al., 2016). Upon completion of the stress session, the weight of chickens was taken (2316 ± 215gm/chicken) for the control and treated groups. Then, without anaesthesia, blood (2-3 ml) was immediately drawn from the wing vein into heparinized tubes and refrigerated (4°C). On the same sampling day, plasma was isolated through the centrifugation of blood at 3000 rpm for 20 min at 4 °C, followed by storage at -20 °C for subsequent analysis of CORT concentration by radioimmunoassay. Following blood collection, the chickens were subjected to cervical dislocation, and their liver, APit, and brain were immediately sampled and frozen in liquid nitrogen (-196 °C). For brain samples, the septohypothalamus was isolated from frozen brains as described in previous studies (Kadhim et al., 2019; 2020). Importantly, throughout the eight hours of each sampling day (8:00 AM to 4:00 PM) at the Poultry Farm of the University of Arkansas, an equal number of chickens were sampled from each group to minimize variations in plasma CORT levels.

Quantification of corticosterone concentrations

Radioimmunoassay (RIA) was used to determine plasma CORT levels and performed in the duplicate assay for each sample, as documented previously (Madison et al., 2008; Kadhim et al., 2021). At room temperature, 200 µl of plasma was combined with 2 ml of ethyl ether in borosilicate tubes and vortexed for 30 min. Subsequently, the tubes were transferred to a bath of methanol/dry ice (-20 °C), and the soluble part was aspirated into the new tube and dried in a 37 °C evaporator. Thereafter, an assay buffer (400 µl) was added to the dried extracts and incubated overnight at 4 °C for equilibrations. Subsequently, the samples were mixed with two hundred microliters of ¹²⁵I CORT tracer (MP Biochemical Inc. Orangeburg, NY, USA) and one hundred of polyclonal rabbit anti-CORT (Fitzgerald Inc., Concord, MA, USA) and incubated at 4 °C. After 24 hours of incubation, two hundred microliters of sheep anti-rabbit (MP Biochemical Inc. Orangeburg, NY, USA) were added to the mixture, and bound tracers were precipitated using polyethylene glycol (6%). After removing the supernatant from each sample, the residual liquid was air-dried. Finally, the CORT concentration in samples was measured using a gamma counter (Perkin Elmer Wizard gamma counter, NY, USA).

RNA extraction, reverse transcription, and quantitative polymerase chain reaction

Total RNA was extracted from the septohypothalamus, APit, and liver using the TRIzol-chloroform (Life Technologies, CA, USA) protocol, as previously described (Kadhim et al., 2019, Kang et al., 2020). Then, deoxyribonuclease I (Ambion, Austin, TX, USA) was used to maintain RNA free from genomic DNA. Subsequently, the RNeasy mini kit (Qiagen, Valencia, CA, USA) was used to purify the extracted RNA. The RNA concentration in the samples was quantified using nanodrop spectrophotometry (Biotek, Winooski, VT, USA). Then, 1μg of extracted RNA was utilized for cDNA synthesis in 40 μl reaction volume using SuperscriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The primer sets for the examined genes and a housekeeping gene (GAPDH) are listed in Table 1.

Table 1. Primer set sequences for the real-time-quantitative PCR technique in the present study for evaluation of gene expression changes in chickens

Genes	Gene bank #	Primer sequence (5'_3')	Amplicon size (bp)	Annealing Temp. (°C)	Reference	
GR	NM_001037826	F: GCCATCGTGAAAAGAGAAGG R: TTT CAACCACATCGTGCAT	95	54	(Kang et al., 2020)	
CBG	KU_180444	F: CTTTTGCCTATGGCCAGCTT R: GGTCTTTAGGTTCATTTGGATCGT	66 58-59		(Vashchenko et al., 2016)	
BDNF	NM_001031616	F: GACATGGCAGCTTGGCTTAC R: GTTTTCCTCA CTGGGCTGGA	167	58-60	(Kadhim et al., 2019)	
11β-HSD1	XM_417988	F: CTGGGAACTGTCTGCACAAC R: GATTGCGAGGAACCATTTACAG	96	56	(Kang et al., 2020)	
11β-HSD2	XM_040680958	F: TGGACACGTTCCGCAGTG R: CGTAGTCCTCGCCGTAAGC	60	168	(Wang et al., 2014)	
POMC	NM_001031098	F: GCCAGACCCCGCTGATG R: CTTGTAGGCGCTTTTGACGAT	56	58-60	(Kadhim et al., 2019)	
GAPDH	NM_204305	F: CTTTGGCATTGTGGAGG GTC R: ACGCTGGGATGATGTTCTGG	128	58-60	(Kadhim et al., 2019)	

Temp: Temprature

The ABI 7500 system (Applied Biosystems 7500 Real-Time PCR System, CA, USA) was used in this experiment to measure the mRNA levels for *POMC*, *GRs*, *CBG*, *BDNF*, *11\beta-HSD1*, *and 11\beta-HSD2* in the dissected tissues. The real-time quantitative PCR assay was conducted in duplicate within a 30 μ l volume, following specific thermal cycling conditions. This included an initial denaturation step at 95 °C for 10 minutes, followed by 40 amplification cycles comprising 30 seconds at 95 °C, 1 minute at 60 °C, and 30 seconds at 72 °C. In the context of RT-qPCR, the average cycle threshold (Ct) and delta Ct values were calculated for all genes in each sample. Then, fold changes in mRNA expression were determined through normalization against the housekeeping gene using the $2^{-\Delta\Delta Ct}$ equation (Schmittgen and Livak, 2008). In detail, the initial delta Ct was computed by determining the difference in the Ct values between the target and housekeeping genes for each sample, expressed as [delta Ct = Ct (target gene) – Ct (housekeeping gene)]. Subsequently, delta Ct values were determined by calculating the differences between delta Ct in the treated groups and control group for each sample, represented as [delta Ct = delta Ct (treated sample #1) – delta Ct (control sample #1)]. Then, fold changes were calculated by using the $2^{-\Delta\Delta Ct}$ equation. It is important to note that the delta Ct value for the control group is equal to 0, thereby 2^0 is equivalent to 1. Therefore, relative gene expression in the control group is consistently maintained at a value of 1.

Statistical analysis

The results of the experiment were analyzed using John's Macintosh Project (JMP) Pro 18.0 (SAS Institute Inc., NC, USA). The Shapiro-Wilk test was used initially to validate the normal distribution. Subsequently, variations across three independent groups were assessed for the blood samples and examined tissues. The analyses started with a one-way ANOVA first, followed by Tukey's HSD test to determine the relative variations in mRNA levels for each gene across groups as well as for changes in the CORT levels. The findings were reported as the mean ± standard deviation (SD), with a p-value less than 0.05 indicating statistical significance. Additionally, the coefficients of variation for RIA were calculated, revealing inter and intra CV were 13% and 9%, respectively.

RESULTS

The corticosterone levels and pro-opiomelanocortin gene expression

In the samples examined, a significant rise in CORT levels was observed in the blood plasma of IMS groups when compared with the control group (p < 0.05). This increase was correlated with changes in the mRNA levels of *POMC* in the APit (Figure 1). Specifically, CORT levels peaked in the 60-minute group, showing a significant elevation compared to the control group (p < 0.05). Following this peak, CORT concentrations decreased in the 120-minute group yet remained significantly higher than those in the control group (Figure 1A). In the APit, *POMC* gene expression exhibited a significant downregulation in the 60-minute group (p < 0.05; Figure 1B). conversely, *POMC* mRNA levels were significantly elevated in the 120-minute group compared to the control groups (p < 0.05). The variations in CORT levels and *POMC* mRNA expression between stressed and unstressed groups were statistically significant (p < 0.05).

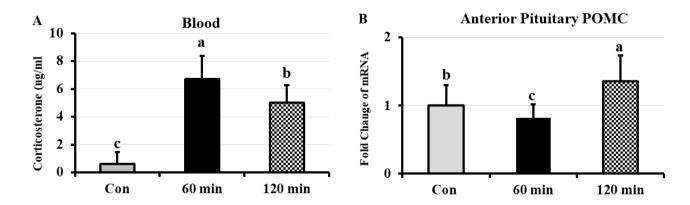


Figure 1. Plasma CORT levels and POMC mRNA expression during immobilization stress. **A**: CORT concentrations in the stressed groups compared with the control group (non-stressed chickens). **B**: Changes in the relative mRNA expression of POMC in the anterior pituitary were normalized with the housekeeping gene (GAPDH) and quantified using $2^{-\Delta\Delta Ct}$ equation. Data were presented as mean \pm the standard deviation (SD) and expressed as fold changes. Significant differences (p < 0.05) between groups were verified by different letters above the bars with a > b > c. (CORT = Corticosterone, POMC: Proopiomelanocortin, Con: Control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

Relative mRNA expression for septohypothalamic genes

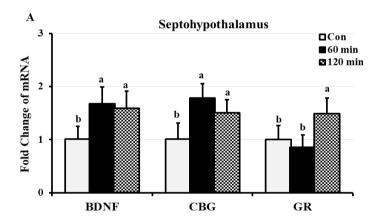
The examination of mRNA expression levels for target genes within the septohypothalamus revealed notable differences across the experimental groups. Specifically, the 60-minute group displayed a significant increase in the mRNA levels of *BDNF* and *CBG* in comparison to the control group, followed by a decrease from this peak in the 120-minute group. Nevertheless, the mRNA levels of *BDNF* and *CBG* in the immobilized groups remained significantly elevated (Figure 2A; p < 0.05 for both genes). In contrast, the mRNA levels of *GR* exhibited a non-significant decrease in the 60-minute group (p > 0.05), which was followed by a significant increase in the 120-minute group when compared to the control group (Figure 2A; p < 0.05). Additionally, the mRNA levels of 11 β -HSD1 and 11 β -HSD2 showed opposing responses to IMS. In the 60-minute group, there was a non-significant downregulation of 11 β -HSD1, accompanied by a significant upregulation of 11 β -HSD2. In the 120-minute group, 11 β -HSD1 mRNA levels were upregulated, while a significant downregulation of 11 β -HSD2 was detected (Figure 2B; p < 0.05 for both genes).

Relative mRNA expression for anterior pituitary genes

The expression of GR and CBG genes in the APit demonstrated a diverse response during IM stress (Figure 3A). In the 60-minute group, there was a significant decrease in GR mRNA levels (p < 0.05), which subsequently recovered to baseline levels in the 120-minute group (p > 0.05). In contrast to GR expression, CBG mRNA levels exhibited a significant increase in immobilized groups compared to the control group (p < 0.05). Remarkably, the mRNA levels of $II\beta$ -HSD1 and $II\beta$ -HSD2 maintained a consistent pattern throughout the different treatment conditions (Figure 3B). Specifically, the stressed groups showed significantly elevated mRNA levels of $II\beta$ -HSD1 and reduced mRNA levels of $II\beta$ -HSD2 compared to the control group (p < 0.05 for both genes).

Relative mRNA expression for liver genes

Gene expression data for multiple genes in the liver showed distinct variations in response to different times of IM stress. In detail, glucocorticoid receptors (GRs) displayed a significant downregulation in the 60-minute group compared to the control group (Figure 4A, p < 0.05), followed by a quick, considerable increase in the 120-minute group (p < 0.05). In contrast, *CBG* mRNA levels demonstrated a gradual increase, reporting a peak response in the 120-minute group (Figure 4A, p < 0.05). Compared with the control group, both 11β -HSD1 and 11β -HSD2 mRNA levels showed significant increases in the 60-minute group, followed by sustaining the upregulation of 11β -HSD1, while 11β -HSD2 mRNA levels decreased from their peak in the 120-minute group (Figure 4B, p < 0.05 for 11β -HSD1 and 11β -HSD2).



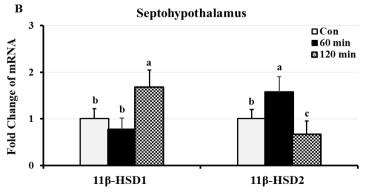


Figure 2. Alterations in the mRNA expression of septohypothalamus genes in immobilized chickens in comparison to control chickens. **A**: Brain-derived neurotrophic factor (BDNF), corticosteroid-binding globulin (CBG), and glucocorticoid receptor (GR). **B**: 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2). The relative mRNA expression of these genes was normalized with the housekeeping gene (GAPDH) and quantified using the $2^{-\Delta\Delta Ct}$ equation. Data were presented as mean ± the standard deviation (SD) and expressed as fold changes. Significant differences (p < 0.05) between groups were verified by different letters above the bars with a > b > c (Con: control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

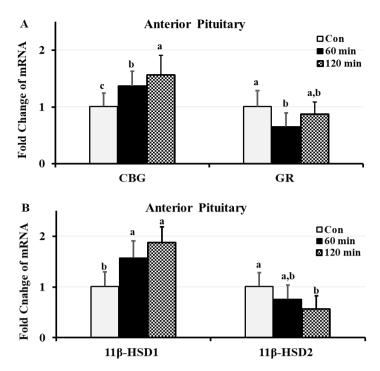


Figure 3. Alterations in the mRNA expression of anterior pituitary genes in immobilized chickens in comparison to control chickens. **A**: Corticosteroid-binding globulin (CBG) and glucocorticoid receptor (GR); **B**: 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2). The relative mRNA expression of the genes was normalized with the housekeeping gene (GAPDH) and quantified using the $2^{-\Delta\Delta Ct}$ equation. Data were presented as Mean ± the standard deviation (SD) and expressed as fold changes. Significant differences (p < 0.05) between groups were verified by different letters above the bars with a > b > c. While a,b is not different statistically from a or b (Con: Control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

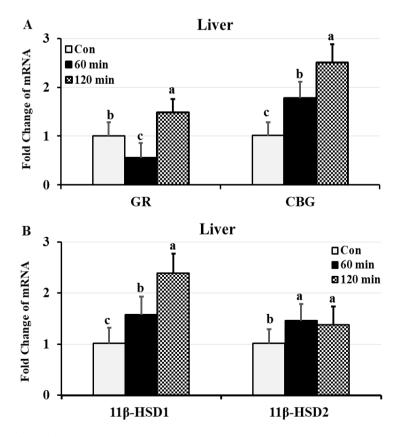


Figure 4. Alterations in the mRNA expression of liver genes in immobilized chickens in comparison to control chickens. A: Corticosteroid-binding globulin (CBG) and glucocorticoid receptor (GR); **B**: 11β-hydroxysteroid- dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2). The relative mRNA expression of the genes was normalized with the housekeeping gene (GAPDH) and quantified using the $2^{-\Delta \Delta Ct}$ equation. Data were presented as mean ± the standard deviation (SD) and expressed as fold changes. Significant differences (p < 0.05) between groups were verified by different letters above the bars with a > b > c. (Con: Control group; 60 min: 60 minutes of immobilization stress; 120 min: 120 minutes of immobilization stress).

DISCUSSION

The study utilized classical acute immobilization stress to examine the relationship between CORT levels and BDNF expression in the chicken hypothalamus. It also explored the changes in the mRNA expression of enzymes that influence the local bioavailability of CORT in stress-related organs during IMS. The findings revealed a significant increase in CORT levels across both stress groups. Supporting the finding of this study, Scanes et al. (2024) reported that restraint stress led to elevated CORT concentrations in the blood plasma of chickens, thereby activating both the HPA axis and the opioid system, which are integral to carbohydrate metabolism. Additionally, it was demonstrated that exposing chickens to IMS for one hour resulted in a fivefold increase in CORT levels in comparison with unstressed chickens (Aman et al., 2016). The high CORT levels in the 60-minute group, coupled with the downregulation of GR mRNA in the APit and septohypothalamus, may serve to inhibit CORT binding to GRs and facilitate continued CORT release. In this context, it has been noted that the release of GCs ceases once CORT binds to GRs (Lane et al., 2021). In contrast, the upregulation of GR expression in liver tissues may mediate the effects of CORT on liver function, thereby promoting the release of energy necessary for the maintenance of homeostasis. Feng et al. (2020) indicated that GCs influence liver functions, such as gluconeogenesis, through GRs. Concurrently, the mRNA levels of POMC within the APit exhibited a slight decrease in the 60-minute group, followed by an increase in the 120-minute group. Consistent with the results of the current study, Løtvedt et al. (2017) reported that a one-hour exposure to restraint stress did not significantly decline POMC expression in the APit of chickens. Notably, the observed CORT levels and POMC expression in the 60-minute group were mismatched phenomena that remained unexplained in avian species. Fortunately, research conducted by Harno et al. (2018) on rodents revealed that the precursor peptide of POMC is sequestered in vesicles within the corticotropic cells of the APit. Upon activation of corticotrophs, pro-hormone convertase enzymes cleave POMC into several peptides, including ACTH, which typically leads to an increase in plasma CORT levels within 60 minutes or less (Kadhim et al., 2021). Following the depletion of POMC in vesicles, changes in POMC gene expression occur, which appear to contribute to the maintenance of elevated CORT levels at 120 minutes of IM stress and beyond.

In the septohypothalamus, the mRNA levels of BDNF and CBG experienced a significant increase in the IMS groups. Despite the upregulation of both BDNF and CBG, their roles appear to be opposite. BDNF is essential for protecting neurons from the negative impacts of IMS. Furthermore, BDNF plays a pivotal role in the stimulation of CRH neurons, potentially facilitating their rapid response (Jeanneteau et al., 2012; Miranda et al., 2019; Miao et al., 2020). The elevation in BDNF mRNA levels coincided with an increase in CORT levels triggered by IM stress, displaying a positive correlation. Previous studies have shown that exposure to CORT can lead to neuronal damage in brain structures such as the hippocampus. Specifically, the administration of BDNF reduced neuronal death associated with CORT exposure (Nitta et al., 1999). Regarding CBG expression, its expression was upregulated significantly in the 60-minute group correlated with a downregulation of GR, which serves to mitigate the negative feedback effects of elevated CORT on CRH-producing neurons. Additionally, the increase in CBG mRNA levels within brain regions may reduce the capacity of CORT to penetrate the blood-brain barrier and sustain its release (Groeneweg et al., 2011), while the upregulation of BDNF mRNA levels during IMS might attenuate CORT-induced neuronal death (Miao et al., 2020). Remarkably, in the 120-minute group, there was an observed increase in the expression of 11β -HSD1, accompanied by a reduction in the mRNA levels of 11β-HSD2. This pattern of expression indicates that 11β-HSD1 plays a crucial role in converting CORT to its active form and aids the transport of active CORT into target cells within the APit tissue (Tomlinson et al., 2004). Consequently, CBG and 11β-HSD1 appear to work together in order to enhance the regulation of the HPA axis through the action of CORT on GRs. Previous studies have shown that the peak expression of CRH mRNA in PVN occurs at one hour following the initiation of IMS (Kadhim et al., 2021), with a subsequent decline in expression in the 120-minute group. This suggests that both CBG and GR are involved in the regulation of CRHproducing neurons located in the septohypothalamus.

In the anterior pituitary (APit), a reduction in the expression of GRs and 11β-HSD1 genes may facilitate the activation of corticotropic cells during IMS, as evidenced by the elevated levels of CORT in the blood of stressed chickens. Supporting the finding of this study, Vodička et al. (2014) stated that the downregulation of GRs and 11β-HSD1 expression could reduce the negative feedback of high CORT concentrations by limiting the accessibility of GRs within APit and maintaining CORT in its inert form. Furthermore, Michael et al. (2019) reported that the decrease in GRs and 11β-HSD1 expression functions as a protective response to elevated CORT concentrations. Additionally, the upregulation of local 11β-HSD2 and CBG genes is crucial for sustaining APit activation, as the main function of 11β-HSD2 is to inactivate GCs and convert them into their inactive ketoform (Cooper and Stewart, 2009; Sattler et al., 2018). Consequently, the APit remained activated, as only free CORT can cross the cell membrane and modulate the function of the HPA axis.

In the current study, the liver showed a significant increase in the mRNA levels of CBG and 11β -HSD1 in the IMS groups. This elevation in 11β -HSD1 mRNA levels may reduce or lessen cells' capacity to neutralize oxidants, potentially resulting in cellular death (Kratschmar et al., 2012; Chen et al., 2020). Furthermore, the upregulation of CBG and 11β -HSD1 in the liver matched with elevated CORT levels at both stress time points, indicating that CBG plays a crucial role in the CORT distribution to target organs through blood circulation. Supporting the findings of the current study, Satter et al. (2018) indicated that the elevation of 11β -HSD1 mRNA improved the translocation of active GCs into cells. On the other hand, there was a significant reduction in the expression of the 11β -HSD2 gene during IMS, suggesting a reduced ability to prevent CORT activation. Nonetheless, the decline in GRs may mitigate the effects of the active form of GCs (Cooper and Stewart, 2009; Sattler et al., 2018). Research suggests that elevated levels of 11β -HSD1 and GRs within the hepatic tissue may contribute to the development of metabolic disorders, a condition that can be exacerbated by elevated GC levels (Livingstone et al., 2000; Candia et al., 2012). Collectively, the findings of increased circulating CORT, the upregulation in 11β -HSD1 and CBG mRNA levels, alongside the downregulation of $I1\beta$ -HSD2 expression, suggest that the hepatic tissue is adversely affected by excessive GC levels, leading to a reduction of GR expression during periods of stress.

CONCLUSION

The results of the current study indicated that immobilization stress influenced glucocorticoid bioavailability by modulating local mediators such as CBG and 11β -HSD. Moreover, IMS was associated with elevated levels of BDNF mRNA and increased concentrations of CORT in chickens. Notably, there was a positive correlation between BDNF and CORT levels; the increase in BDNF appeared to be essential for neuronal protection and for mitigating neuronal damage induced by CORT during IM stress. Furthermore, the interaction between GCs with GRs within the HPA axis, along with the availability of free active GCs, played a significant role in the regulation of the HPA axis activity. Enzymes such as CBG and 11β -HSDs in the examined structures were vital for the generation of bioactive GCs. Furthermore, the modulators of CORT bioavailability, namely CBG, and 11β -HSD, were identified in the studied tissues, which include the brain, APit, and liver. Moreover, it is recommended to investigate the direct action of ACTH on the liver tissue and determine which ACTH receptors are expressed in the liver. Additionally, investigating the alterations in liver enzyme activity during stress episodes is of considerable importance.

DECLARATIONS

Acknowledgments

I would like to dedicate this paper to my supervisor, Dr. Wayne Kuenzel, in honor of his retirement. I also extend gratitude to the Poultry Science Department/the University of Arkansas for their invaluable support. The sampling, RT-qPCR, and statistical analyses were performed at the University of Arkansas/USA.

Authors' contributions

Hakeem Kadhim conceptualized the study, collected the samples, analyzed the data, conducted the study, and prepared the initial draft of the manuscript. Furthermore, Hakeem Kadhim has read, improved, and approved the submitted version of the manuscript.

Competing interests

There is no conflict of interest to declare.

Availability of data and materials

The original data presented in the study are available in the article.

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Peripartum Ketone and its Relationship with Milk Quality in Dairy Cattle

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ABSTRACT

Ketosis is a common metabolic disorder in dairy cows and has been associated with alterations in milk composition and a decrease in milk quality, impacting both the economic and nutritional value of dairy products. The purpose of this study was to evaluate ketone levels before and after calving and their effect on milk quality in cattle in the district of El Mantaro, Jauja in the Peruvian highlands. Multivariate analyses, including Principal Component Analysis (PCA) and clustering, were employed to explain the variability in the data better. The study was conducted at the Instituto de Investigaciones Tropicales y de Altura (IVITA) and El Mantaro experimental stations cattle in the district of El Mantaro, Jauja, using a total of 72 Brown Swiss cattle, all of which received similar management. Blood and milk samples were collected from all cows studied and analyzed in the laboratory for ketone levels and milk quality such as density, non-fat solids, protein, freezing point, solids, and lactose. The descriptive analysis revealed significant variations in the evaluated variables, highlighting a reduction in ketone levels after calving and consistency in milk composition, such as non-fat solids and density. The PCA showed that the first two principal components explained 49.8% of the total variability, dominated by compositional variables, while subsequent components contributed smaller proportions, reaching 100% with 11 components. The reduction in ketone levels after calving suggested metabolic stabilization associated with energy recovery during this stage, while differences in compositional variables such as protein and non-fat solids reflected the influence of factors such as diet, genetics, and physiological status. Although ketones showed weak to moderate correlations with the evaluated variables, the negative relationships with body condition and non-fat solids indicated that better nutritional and metabolic status might be associated with lower ketone levels.

Keywords: Brown Swiss cattle, Correlation, Milk composition, Milk quality, Subclinical ketosis

INTRODUCTION

Dairy cattle farming in the "Valle del Mantaro" Valley is characterized by being managed in small production units, which typically exhibit lower milk yield per cow compared to the major dairy regions of the country (Diego et al., 2024; Estremadoyro et al., 2024). The fresh milk produced is primarily directed towards a nascent artisanal or semi-industrial dairy industry, mainly focused on the production of cheese, yogurt, and other dairy derivatives, as well as for self-consumption (Carhuas et al., 2024; Payano et al., 2024). However, the pressure to increase milk production imposes greater metabolic demands on the animals, predisposing them to a higher incidence of metabolic diseases, commonly referred to as production diseases (Garcia-Olarte et al., 2024). These conditions arise from an imbalance between the intake, circulation, and excretion of one or more metabolites within the organism, pushing their concentrations beyond physiological limits.

Ketosis is a common metabolic disorder in dairy cows during the peripartum period, characterized by elevated levels of ketone bodies, particularly β -hydroxybutyrate (BHB), in blood, urine, or milk (Lei and Simões, 2021; Cascone et al., 2022). This condition arises from the high energy demands of late gestation and early lactation, combined with limited feed intake, leading to excessive mobilization of body fat reserves (Tufarelli et al., 2024). Beyond its direct health implications, ketosis has been associated with alterations in milk composition and a decline in its quality, impacting both the economic and nutritional value of dairy products (Cainzos et al., 2022). Milk quality, encompassing parameters such as fat, protein, lactose, and somatic cell count, is a critical indicator of herd productivity and consumer satisfaction (Pegolo et al., 2022). Emerging evidence suggests that ketone levels during the peripartum period can influence these parameters, underscoring the importance of early identification and management of ketosis to maintain optimal dairy production performance (Guliński, 2021). However, obtaining specific information for each production

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area is challenging due to environmental variations affecting quality. This highlights the necessity of understanding these values, particularly in the Junín region of Peru, where such data is lacking.

The analytical focus of this study was to evaluate the relationship between ketone levels during the peripartum period and milk quality parameters in dairy cattle, using descriptive and multivariate statistical analyses, models, and correlation analysis. This design enables the identification of patterns and associations between blood ketone concentrations and key quality variables, such as fat, protein, lactose content, and somatic cell count (Chapman et al., 2001). Although ketosis in cattle has been extensively studied, most research has focused on its impact on health and overall production, leaving a gap in the literature regarding its specific influence on milk quality. This study contributed to the understanding of metabolic health in dairy herds, offering potential strategies for the early detection and mitigation of ketosis and its impacts on milk quality.

MATERIALS AND METHODS

The procedures and ethical standards for animal use in this study were conducted in strict accordance with the "International and National Guidelines for the Care and Use of Research Animals," as outlined in LETTER No. 002-GRJ-DRA-AAC-PERÚ-2024. This ensured full compliance with established animal welfare protocols throughout the research process.

Study area and distribution

The study was conducted at the Instituto de Investigaciones Tropicales y de Altura (IVITA) and El Mantaro experimental stations, located in the district of El Mantaro, province of Jauja, Junín region, at an altitude of 3,200 meters above sea level (Senamhi, 2023). The study included 57 cows from the IVITA station and 15 cows from the El Mantaro Experimental Station (EEA), with five replicates for each cow. All of them were Brown Swiss. All cattle were fed a diet composed exclusively of alfalfa and received the same management. The cattle were selected 30 days before calving to measure ketone levels before calving, and the same animals were evaluated 30 days later to determine ketone levels after calving.

Data collection

Ketone

Blood samples (ml), one per cow, were collected using vacutainer tubes from the coccygeal artery of the animals and transported to the laboratory for processing. To ensure proper preservation, serum was separated and stored in cryovials at a freezing temperature of -40°C. Samples requiring additional processing were centrifuged prior to analysis. For ketone analysis, a NovaVET Xpress ketone/glucose meter (ket-mmol/L) was used to measure β -hydroxybutyrate (BHBA) levels in the blood (Zhuang et al., 2023). This parameter is widely regarded as the gold standard for detecting subclinical ketosis in dairy cattle. The ketone test strips contain a chemical reagent that reacts with the sample once inserted into the meter, and the reaction in the test strip's cell generates an electric current, which the device measures to calculate BHBA concentration.

Body condition and age

Body condition score (BCS) was assessed using the method described by Paul et al. (2020), which employs a scale from 1 to 5. Evaluations were performed by the same observer at 30 days before calving, at calving, and 15, 30, 45, and 60 days postpartum. The age of the cows was recorded by the experimental station records.

Milk quality

Milk quality was assessed using one sample per animal, collected at 6:00 a.m. before milking. The samples were analyzed with a Boeko Lactoscan (Germany) SP ultrasonic milk analyzer, which measured fat content (FAT), density, solids-not-fat (SNF), protein, pH, freezing point, total solids, and lactose levels (Kasmi et al., 2021).

Statistical analysis

A descriptive analysis was performed to examine the behavior of the data, followed by a Principal Component Analysis (PCA) to enable clustering and capture the maximum variability in the dataset. All analyses were conducted using R-Studio (Team et al., 2018) using version 4.3.0.

RESULTS

Table 1 summarizes the descriptive statistics for Age, Body Condition, Ketone Levels Before Delivery (KBD), Ketone Levels After Childbirth (KAC), Fat Content, Milk Density, Non-Fat Solids (NFS), Protein (PROT), Freezing Point (FP), Total Solids, and Lactose (LACT). The mean age of the subjects was 4.61 years, with a standard deviation of 2.81 years. The youngest individual was 1.9 years old, while the oldest was 15.4 years. The median age was 4.24 years. Body condition scores averaged 2.55, with variability of ± 2.69 , spanning from 2.0 to 3.0, with a median value of 2.60. Regarding ketone levels, the prepartum average was 0.91 ± 4.88 mM/L (range: 0.30-2.10; median: 0.80), while postpartum levels decreased to 0.60 ± 3.85 mM/L, with a range of 0.10-2.10 and a median of 0.45. The average fat content was 4.26 ± 1.97 (%) with a minimum of 0.08, a maximum of 9.45, and a median of 4.39. Milk density had an average of 26.83 ± 4.24 kg/m³ (range: 14.52–39.54; median: 26.77), while solids-not-fat (SNF) reached an average of 7.58 ± 1.56 (%), with values between 3.26 and 11.72 and a median of 7.58. Protein concentration had a mean of 3.59 \pm 9.72 (%) (range: 1.38–6.98; median: 3.46). Table 2 presents the findings from the Principal Component Analysis (PCA) conducted on milk quality indicators, including fat, protein, lactose content, and somatic cell count, alongside ketone body concentrations during the peripartum period, as well as variables such as age, calving condition, and seasonal grouping of the cows. The initial principal component (PC1) captured 35.12% of the overall variability, followed by the second component (PC2) with 14.70%, together explaining 49.80% of the variability. The third and fourth components (PC3 and PC4) contributed an additional 11.95% and 9.31%, respectively, reaching a cumulative 71.09%. From PC5 onward, the contribution of each component decreased, with variance proportions below 8%, until PC11 accounted for 100% of the variability in the dataset.

Table 1. The metabolic and milk quality parameters in dairy cattle during the peripartum period

Variable	Mean	sd	Min	Max	Median
Age	4.61	2.81	1.9	15.4	4.24
Body condition	2.55	2.69	2.0	3.00	2.60
Ketone before delivery (KBD) mM/L	0.91	4.88	0.30	2.10	0.80
Ketone after childbirth (KAC) mM/L	0.60	3.85	0.10	2.10	0.45
Fat (%)	4.26	1.97	0.08	9.45	4.39
Density kg/m ³	26.83	4.24	14.52	39.54	26.77
Non-fat solids (NFS) (%)	7.58	1.56	3.26	11.72	7.58
Protein (PROT) (%)	3.59	9.72	1.38	6.98	3.46
Freezing point (FP)	1.69	8.75	0.36	0.77	3.46
Solids (%)	0.67	1.18	0.41	0.98	0.66
Lactose (LACT) (%)	4.97	1.03	2.98	7.95	4.82

sd: Standard deviation; min: Minimum data value, max: Maximum data value.

Table 2. The principal component in the PCA analysis of Brown Swiss cows in the Mantaro district, to take the best PCs

Importance component	Proportion of Variance	Cumulative Proportion	SD	
PC1	0.3512	0.3512	1.9654	
PC2	0.1470	0.4980	1.2717	
PC3	0.1195	0.6177	1.1467	
PC4	0.0931	0.7109	1.0125	
PC5	0.0760	0.7869	0.9144	
PC6	0.0588	0.0845	0.8048	
PC7	0.0493	0.0895	0.7366	
PC8	0.0324	0.9276	0.5966	
PC9	0.0278	0.9554	0.5533	
PC10	0.0252	0.9806	0.5267	
PC11	0.0193	1.0000	0.4610	

PC1–PC11 represents the principal components generated from the principal component analysis (PCA), where each component captures a decreasing proportion of the total variability in the data, with PC1 explaining the largest share of the variance and subsequent components contributing progressively less. sd: Standard deviation.

The correlation matrix revealed significant relationships among the variables studied (Figure 1a). Notable correlations included Lactose (LACT) and Protein (PROT), with a positive correlation of 0.67, indicating that an increase in lactose was directly associated with an increase in protein levels. Non-fat Solids (NFS) and density (DENSITY) exhibited a positive correlation of 0.62, highlighting that higher non-fat solids content was associated with increased sample density. Fat (FAT) and Protein (PROT) showed a positive correlation of 0.56, suggesting that both variables were interconnected in their behavior. Ketones Before Calving (KBC) and Ketones After Calving (KAC) displayed a positive correlation of 0.39, indicating a direct relationship between ketone levels during both periods. LACT and density demonstrated a positive correlation of 0.58, showing that increased lactose levels had a direct impact on sample density. The strongest correlations (p < 0.05) were found among variables related to non-fat solids, density, lactose, and protein, underscoring their importance in the dataset. Conversely, variables such as ketones (KBD and KAC) exhibited weaker correlations with other variables, suggesting a lesser influence on the system studied. Before calving, ketone levels demonstrated moderate and weak correlations with the analyzed variables. A moderate negative correlation was observed with body condition (-0.15) and non-fat solids (-0.21), suggesting that better physical condition and higher non-fat solids content could be associated with lower ketone levels during this period. On the other hand, weak positive correlations were observed with variables such as fat (0.06), density (0.09), and total solids (0.06), indicating that these characteristics had no significant relationship with ketone levels. Correlations with lactose (0.04) and protein (0.08) were also too weak, suggesting a marginal influence of these variables before calving. After calving, ketone levels followed a similar trend, with a moderate negative correlation with body condition (-0.14), reaffirming that better body condition could be associated with lower ketone levels, moderate correlation is mentioned because it does not follow a strong correlation but there is some correlation. Correlations with non-fat solids (-0.10) and lactose (-0.02) were also negative but at a weak level, indicating a limited relationship. Conversely, correlations with protein (0.09), density (0.04), and total solids (0.06) were positive but at a weak level, suggesting an insignificant impact of these variables on ketone levels after calving. In general, ketone levels, both before and after calving, appeared to be inversely related to indicators of optimal metabolic health, although the relationships were mostly at a weak level. These correlations suggest that their variation may depend on other factors not evaluated in the current study.

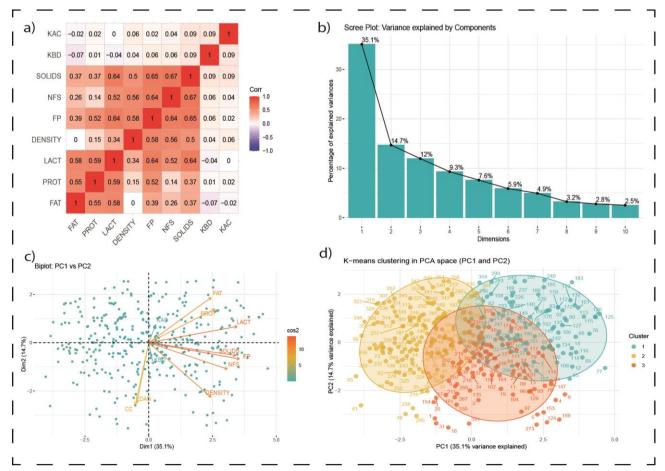


Figure 1. Comprehensive visualization of Principal Component Analysis (PCA) and clustering results for metabolic and milk quality parameters. **a:** Correlation Matrix, **b:** Scree Plot, **c:** Biplot (PC1 versus PC2), and **d:** K-means Clustering in PCA Space.

From Figure 1b, the Scree Plot, the variance analysis showed that the initial (PC1 and PC2) two principal components accounted for 35.1% and 14.7% of the total variance, respectively, accounting for a combined total of 49.8%. This suggested that a significant portion of the variability in the data could be accounted for through these two components. The Biplot (PC1 vs. PC2) observed how the variables contributed to the first two principal components (Figure 1c). The arrows indicated the direction and magnitude of each variable's contribution, highlighting that LACT, PROT, and NFS were highly associated with PC1, while density had an intermediate association. In contrast, ketones (KBD and KAC) showed minimal influence on the first components (PC1). Figure 1d, the K-means Clustering, displayed three main clusters in the space defined by PC1 and PC2. The colors distinguished the clusters, and the ellipses indicated the density areas of the groups. This clustering highlighted patterns in the data that would not be evident through univariate analyses, suggesting potential structural differences among the observations based on the evaluated variables.

DISCUSSION

The descriptive analysis and PCA provided insights into the variability and relationships within the studied dataset. The results revealed significant differences among the variables, emphasizing their relevance in evaluating the metabolic and productive status of the animals. The average age and BCS suggest a relatively uniform group. However, the large standard deviation observed in ketone levels, both KBC and post-KAC, underscores the significant metabolic heterogeneity among individuals. This variability aligns with findings from previous studies, which also report notable inter-individual differences in metabolic responses (Martens, 2020; Michalopoulou et al., 2024). It is important to note that factors like genetic predisposition, differences in energy metabolism, or variations in physiological states could influence such heterogeneity. The reduction in postpartum ketone levels suggested a shift in metabolic priorities, likely related to the stabilization of energy balance, as documented in previous studies (Parrettini et al., 2020; Wathes et al., 2021). Compositional variables such as FAT, PROT, and NFS exhibited patterns consistent with optimal nutritional management. However, the significant dispersion in protein levels suggested the influence of factors, such as lactation stage or genetics (Cheng et al., 2022; 2023). The PCA indicated that the first two principal components (PC1 and PC2) captured 49.8% of the total variability, with PC1 dominated by compositional variables (LACT, PROT, and NFS) and PC2 likely associated with metabolic indicators such as ketones. This finding underscored the value of PCA as a tool for identifying patterns in complex systems (Ellis et al., 2020). The negative correlations, although weak, suggest that higher levels of ketone bodies (KBC or KAC) could be inversely related to parameters such as lactose, fat, or milk density, reflecting potential adverse metabolic effects.

On the other hand, the graphical analysis of the figures provided additional insights into the relationships among variables and the structure of the dataset. The correlation matrix highlighted strong relationships, such as LACT with PROT (0.67) and NFS with density (0.62), underscoring the importance of these factors in milk composition. However, ketones showed weaker correlations with other variables, particularly before calving, where moderate negative correlations were observed with body condition (-0.15) and non-fat solids (-0.21). These relationships suggested that better metabolic status was associated with lower ketone levels, although their influence was limited (Torres et al., 2020; Hubner et al., 2022; Mohsin et al., 2022). The biplot provided a clear representation of how the variables contributed to the first principal components. The long arrows of LACT, PROT, and NFS toward PC1 confirmed their high influence, while the shorter arrows of ketones indicated a marginal contribution. These results aligned with studies that emphasized the importance of compositional components in milk quality and production efficiency (Magan et al., 2021; Timlin et al., 2021). Additionally, the K-means clustering analysis (Figure 1d) identified three main clusters in the space defined by PC1 and PC2, suggesting significant structural differences among the groups. These clusters could have been linked to genetic or management factors, as previous studies demonstrated that these variables were critical determinants in differentiating metabolic and productive patterns (Zhang et al., 2021; Adamik et al., 2022). The integration of these multivariate analyses with correlation data provided a comprehensive perspective, enabling a more precise characterization of the factors influencing animal productivity and metabolic status. This highlighted the potential of PCA and clustering analysis as essential tools for advanced studies in animal sciences.

CONCLUSION

The reduction in ketone levels after calving suggested metabolic stabilization associated with energy recovery during this stage, while differences in compositional variables such as protein and non-fat solids reflected the influence of factors such as diet, genetics, and physiological status. Although ketones showed weak to moderate correlations with the evaluated variables, the negative relationships with body condition and non-fat solids indicated that better nutritional and

metabolic status might be associated with lower ketone levels. The utility of multivariate analysis to simplify complex data with models that would otherwise be difficult to interpret proved crucial in explaining variability, allowing the identification of fundamental patterns that optimize the assessment of milk quality in cattle. Strategies to monitor and control ketone body levels during the peripartum period are recommended, such as improving nutritional management with diets balanced in energy and effective fiber, supplementing with glucogenic compounds to reduce the risk of ketosis, and regular monitoring of metabolic parameters. These measures could improve both cow metabolic health and milk quality.

DECLARATIONS

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Availability of data and materials

The original data presented in the study are included in the article. For inquiries, please contact the corresponding author.

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Authors' contributions

Fernando Arauco Villar was in charge of supervising the overall research; Noemí Mayorga Sánchez was in charge of programming and follow-up; Milk samples and laboratory analysis were performed by Leonor Guzman Estremadoyro and Yakelin Mauricio-Ramos, Blood sampling and laboratory analysis for ketone was performed by Ronald Damas Huaman and Carlos Arana de la Cruz; Ide Unchupaico Payano was in charge of data collection and cleaning; Jordan Ninahuanca Carhuas was in charge of the statistical analysis and writing of the article. All authors have read and approved the final version of the manuscript before publication in the present journal.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time. Additionally, all authors checked the originality of data and sentences via plagiarism checkers.

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Effects of Anti-inhibin Free α Immunization on Ovulation, *in Vitro* Fertilization, and Embryo Development in Mice

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ABSTRACT

Inhibin is a dimeric glycoprotein comprised of two subunits, α and β . Immunization against dimeric inhibin is mainly used in assisted reproductive technology to induce superovulation. However, the specific function of immunoreactive-free inhibin α subunit remains unclear. In this study, two main investigations were conducted (first on ovulation and the other on fertilization) using a novel monoclonal antibody targeting free inhibin α subunit (ProαN-αC). The ovulation study was conducted in 6 replicates, involving a total of 48 female CD1 mice aged 4-6 weeks. In each replicate, 4 control mice received PMSG/hCG treatment, and 4 treated mice received PMSG/hCG with mAb- Free α subunit. The fertilization study was conducted in 3 replicates, involving a total of 22 female CD1 mice. In each replicate, there were 4, 3, and 4 mice respectively for both control and treatment groups. In both investigations, female mice were injected intraperitoneally with 50 units/ml of Pregnant Mare Serum Gonadotropin (PMSG), alone or combined with 400ug of mAb- Free α subunit, followed by an injection of 50 units/ml of Human Chorionic Gonadotropin (hCG) 48 hours later. Seventeen hours post-injection, the females from all groups were sacrificed, and the ovulated oocytes were collected from the oviducts. For the fertilization study, in vitro fertilization was performed using fresh sperm from male CD1 mice. The results revealed that neutralization of the free inhibin α subunit significantly decreased the ovulation rate by 47.29% compared to the control group, while immunoneutralization significantly increased the fertilization rate by 55.68% and the blastocyst development by 43.85% compared to the control group. This study suggests that immunization against free inhibin α subunit decreases ovulation, in contrast to the effect of immunoneutralization of dimeric inhibin. The authors hypothesize that the free α subunit may function as an inhibin antagonist, competing with inhibin for binding to its co-receptor.

Keywords: Activin, Betaglycan, Fertilization, Immunoneutralization, Inhibin, Ovulation

INTRODUCTION

The use of genetically modified mice as models of human disease in life science research provides detailed insights into disease mechanisms and therapeutic strategies for rare diseases and pathological conditions (Kaushik et al., 2024; Zhong et al., 2024). To achieve efficient production and maintenance of genetically modified mice, various protocols based on superovulation are used (Guan et al., 2012). However, as the new transgenic technologies are improving, the number of mutant mice is increasing exponentially, and so is the number of female oocyte donors. The success of *in vitro* fertilization (IVF) is very much dependent on the quality of the oocyte/embryo and on a culture system that supports the development of healthy embryos capable of reaching their implantation potential (Sciorio et al., 2024). The production of large numbers of mature, high-quality oocytes is of great importance for assisted reproduction techniques (ARTs). Superovulation is a major component of embryo transfer and transgenic technologies; it facilitates the generation of genetically engineered mice/embryos and reduces the number of animals used. Superovulation techniques are based on the induction of follicle maturation and ovulation through the administration of hormones (Kaneko and Garrels, 2020).

Since the late 1980s, standard superovulation using a combination of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) has been introduced in the production of transgenic mice to induce follicular development and ovulation of a large number of oocytes from a limited number of female mice (Fleming and Yanagimachi, 1980). Ovulation rates have also been enhanced by vaccination against inhibin in many species including

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mice (Mochida, 2020; Hasegawa et al., 2022) rats (Mochida et al., 2024), guinea pigs (Shi et al., 2000), goats (Medan et al., 2003) and heifers (Bleach et al., 2001). Inhibin is a heterodimeric glycoprotein, mainly secreted by granulosa cells in response to follicle-stimulating hormone (FSH) release and to inhibit FSH secretion from the pituitary gland, thus regulating the number of developing follicles and ovulated oocytes (Makanji et al., 2014). The mature form of inhibin, with a molecular weight of 32-34 kDa, consists of an α C subunit (~20 kDa) disulfide-linked to either a β A or a β B subunit (~13 kDa), forming inhibin A or inhibin B, respectively (Vale et al., 1988; Makanji et al., 2014). However, immunoreactive free inhibin α subunits (~50-58 kDa) are also found in abundance, though their specific function remains unclear (Findlay et al., 2001; Laird et al., 2019). While some studies suggest that the free form of inhibin α subunit may have an antagonist effect on the mature form of inhibin (Drummond et al., 2004; Laird et al., 2019). there is no experimental evidence to support this hypothesis.

To date, no specific receptor has been identified for inhibin that associates with its signaling pathway (Robertson et al., 2000; Bernard et al., 2020). However, the physiological effects of inhibin are generated by a system of competition with activin receptors, leading to the inhibition of activin action (Bernard et al., 2020). Unlike inhibin, activins stimulate FSH secretion from the pituitary gland and are composed of two β-subunits, which are also shared with inhibin (Gray et al., 2005). Inhibin's antagonism of activin depends on interactions with betaglycan, a cell-surface proteoglycan coreceptor, also known as TGFBR3 (Lewis et al., 2000; Makanji et al., 2007). Betaglycan binds inhibin to form a stable complex with a high affinity for type II activin receptors, thereby antagonizing activin-mediated receptor activation (Lewis et al., 2000). In addition, a recent study indicates that inhibin B acts preferentially via another gonadotroph-specific co-receptor, called transforming growth factor-beta receptor 3 (TGFBR3L), to suppress FSH secretion (Brûlé et al., 2021).

A great majority of protocols used in assisted reproductive technology (ART) and superovulation are based on the immunoneutralization of endogenous inhibin (Mochida, 2020). However, given the limited understanding of the inhibin signaling pathway and the potential role of its immunoreactive-free α subunit, the success of superovulation protocols is not always guaranteed. In some studies, these protocols have surprisingly failed to induce ovulation (Drummond et al., 2004). In fact, evidence suggests that free α inhibin may have a biological activity distinct from that of inhibin in the female reproductive system (Mason et al., 1996; Drummond et al., 2004). Therefore, this study aimed to investigate the effects of immunoneutralizing the free inhibin α subunit (Pro- α N- α C) on mice ovulation, *in vitro* fertilization, and embryo development. The study employed a novel monoclonal antibody developed against 13-amino acid epitope; <⁶⁴HAVGGFMHRTSEPE>, within the mouse inhibin α N region. There is a lack of studies in this area to explore the physiological role of free inhibin α subunit and provide new insights to improve ART protocols and fertility treatments based on inhibin.

MATERIALS AND METHODS

Ethical approval

All experiments were approved by the University of California San Francisco guidelines under Institutional Animal Care and Use Program (IACUC) approved protocols (AN203067).

Animals

A total of 98 outbred CD1 mice strains (95 females and 3 males) were purchased from Charles River Laboratories, USA, for the entire study. The research was conducted at the University of California San Francisco, Cryopreservation Core. The female mice, used as oocyte donors, were 4-6 weeks old with an average weight of 18 grams, and the male mice, used as sperm donors, were 12 weeks old with an average weight of 40 grams. Throughout the study, the animals were housed under a monitored light/dark cycle, (light from 07:00 to 19:00), provided with water and fed *ad libitum*, and checked daily by the animal care staff.

Anti-Inhibin free α subunit monoclonal antibody

Bioinformatic analysis was initially conducted to select a suitable peptide antigen for antibody production. The sequence of mouse inhibin α (*Mus musculus*) was obtained from the NCBI database. The peptide was created through GenScript's Optimum Antigen Design Program, and was optimized using the industry's most advanced antigen design algorithm. The peptide was measured against several protein databases to confirm the desired epitope specificity. Benefits of using the Optimum Antigen Design Tool include avoidance of unexposed epitopes, ability to specify desired cross-reactivity, strong antigenicity of chosen peptide, identification of the best conjugation, and presentation options (GenScript, 2002). Using the GenScript Optimum Antigen design tool, the sequence with the highest immunogenicity within the Inhibin α Subunit Pro- α N was identified, specifically <HAVGGFMHRTSEPE>. The 13-amino-acid sequence

⁶⁴HAVGGFMHRTSEPE-C-KLH' was then chemically synthesized by GenScript (Lot No.: 95490490005/pe3623). To enhance its immunogenicity, the peptide was conjugated to keyhole limpet hemocyanin (KLH) via a cysteine residue. A monoclonal antibody was then produced using hybridoma technology. following the protocol described by Yokoyama et al. (2013) and Yokoyama et al. (2013).

A group of 5 female Balb/c mice (6 to 8 weeks old) were intraperitoneally injected with 50 μ g of the chemically synthesized peptide mixed with 1:1 Complete Freund's adjuvant (Sigma-Aldrich, Germany). A control group of 3 female Balb/c mice received normal saline only. After 15 days, a booster immunization was administered, consisting of 50 μ g of antigen mixed with 1:1 Incomplete Freund's adjuvant Sigma-Aldrich, Germany) and injected intraperitoneally. Three days after the booster, the animals were prepared for cell fusion. Hybridoma cells were generated by fusing spleen cells from immunized female Balb/c 6-week-old mice with SP2O myeloma cells. The supernatant from these cultures was initially screened using a homemade ELISA test, developed using the synthesized peptide antigen, according to the protocol described by Holzlöhne et al. (2017) and Holzlöhner and Hanack (2017). Following this, a stable antibody-secreting cell line was expanded, and the monoclonal antibody was then purified using a Sephadex G-200 column. The monoclonal antibody was characterized as IgG Kappa and was shown to be immunospecific only to the Free Inhibin α Subunit Pro- α N- α C (\sim 52 kDa) in Western blotting analysis (unpublished observations).

Reagents

All reagents used in this study were obtained from Sigma-Aldrich, Germany, or ThermoFisher Scientific, USA, unless otherwise mentioned.

Superovulation and oocyte collection

CD1 female mice were injected intraperitoneally with 0.1 ml PMSG (50 units/ml, Biovendor, Czech Republic) either alone or combined with mAb-Free α subunit (0.1 ml), followed by an injection of 0.1 ml of hCG (50 units/ml, Sigma-Aldrich, Germany) intraperitoneally 48 hours later. At 17 h after this, mice were sacrificed by CO2 asphyxiation followed by cervical dislocation. The sacrificed animals were dissected, and the oviducts were collected and transferred to the collection dish containing High Calcium HTF medium (Human Tubal Fluid) (Fisher Scientific, USA), preequilibrated at 37°C. The clutches of cumulus-oocyte complexes were collected from the oviducts and transferred to a 200- μ L drop of fertilization medium containing HTF with 1.25 mM of reduced glutathione (GSH) (Sigma-Aldrich, Germany), covered with paraffin oil (Fisher Scientific, USA), and then incubated at 37°C for 30 min before insemination (Behringer et al., 2014).

In vitro fertilization and embryo culture

Mature male mice were first sacrificed by CO2 asphyxiation and cervical dislocation. The sacrificed animals were dissected, and the cauda epididymis was then collected and placed in a petri dish containing HTF medium. The cauda was punctured using a 26-gauge disposable needle to collect the spermatozoa (Meniru et al., 1998; Nagy et al., 2003). The sperm were first preincubated at 37°C for 10 min to induce capacitation. Then, a total of 20-30µl sperm was slowly collected and expelled gently onto the egg clutches in the fertilization medium drop. The oocytes and sperm were co-cultured in the fertilization HTF-GSH drop for 3 to 4 hours at 37°C with 5% CO2, 5% O2 and 90% N2 incubator. Afterward, the oocytes were washed into three 100µl drops of HTF medium. The number of ovulated oocytes was first counted and then cultured overnight in an HTF medium. At 24h after insemination, the number of 2-cell embryos was counted and the fertilization rates were calculated (Nagy et al., 2003), and the oocytes presenting a fragmented or small ooplasm or an expanded zona pellucida were considered abnormal. The 2-cell embryos were transferred to 100ul drop of Potassium Simplex Optimized Medium (KSOM, Fisher Scientist, USA) for *in vitro* embryo development to the blastocyst stage (Takeo and Nakagata, 2011; Kidder, 2014).

Study design

In this study, the treated group consisted of female CD1 mice receiving PMSG/hCG along with the mAb-Free α subunit, while the control group received only PMSG/hCG.

Effect of immunoneutralization of inhibin-free subunit on ovulation rate

The following investigation was performed in order to examine the immunoneutralization of inhibin-free α subunit on mice ovulation rate. Six replicates were conducted, each consisting of two groups of four female CD1 mice. In each replicate, the treated group received PMSG/hCG with 400 µg of mAb-Free α subunit, while the control group received only PMSG/hCG. A total of 48 female mice were used across all replicates, equally divided into 24 in the treated group and 24 in the control group. Ovulation rates were determined by counting the total number of oocytes produced in each

group. Further investigation was also conducted using increased concentrations of mAb-Free α subunit. A total of five groups, each consisting of five female CD1 mice, were used. Four groups received PMSG/hCG in combination with different doses of mAb-Free α subunit: 200 μ g, 400 μ g, 800 μ g, and 1 mg, respectively. The fifth group, serving as the control, received only PMSG/hCG. Ovulation rates were assessed by counting the total number of oocytes produced in each group.

Effects of immunoneutralization of free inhibin α subunit on mice fertilization and embryo development

The following investigation was carried out to examine the effects of immunoneutralization of inhibin-free α subunit on *in vitro* fertilization and embryo development. The experiment was repeated three times, involving a total of 22 female CD1 mice and 3 male CD1 mice, with one male used as a sperm donor for each of the following experiments. In each experiment, the mice were divided into treated and control groups. In Experiment 1, 4 mice were treated with PMSG/hCG and 400 μ g of mAb-Free α subunit, while the 4 control mice received only PMSG/hCG. In Experiment 2, the treated and control groups consisted of 3 mice each. In Experiment 3, 4 mice received PMSG/hCG with the mAb, and 4 mice served as controls, receiving only PMSG/hCG. The oocytes generated from the groups were fertilized with fresh sperm, and *in vitro* embryo culture was then performed. The fertilization rates were calculated as the total number of 2-cell embryos divided by the total number of generated oocytes and multiplied by 100. The embryo development was assessed by the number of 2-cell embryos developed to the blastocyst stage *in vitro*.

Statistical analysis

Results are expressed as the mean \pm standard deviation (SD). The significance of the difference between the control groups and the treated groups in all experiments was determined by the Mann-Whitney nonparametric test and relative standard deviation values (RSD). A probability value (P) of less than 0.05 was considered to be significant. RSD values higher than 10% indicated considerable variability, suggesting a significant difference. All statistical analyses were performed using Mini tab software 17.1.0.

To assess the variability across the replicates of the same experiment and evaluate the potential influence of data collection time on ovulation, fertilization, and blastocyst rates, a mixed-effects linear model was employed to account for both fixed and random effects. Treatment and experiment (time) were included as fixed effects, while the random effect was set at the level of the experiment to control for repeated measures over time. The interaction between treatment and experiment was also assessed to determine if the treatment effect varied across different time points. The mixed model was fitted using Restricted Maximum Likelihood (REML) estimation. All statistical analyses were performed using the LME4 package in R (version 4.0.0), with significance evaluated at the 5% level.

RESULTS

The present study investigated the physiological effects of free inhibin α subunit on ovulation, *in vitro* fertilization, and embryo development in mice.

Ovulation rate

In the six experiments conducted, a total of 242 oocytes were collected from 24 donors following mAb- free α subunit/PMSG/hCG treatment, while a total of 459 oocytes were collected from 24 donors after PMSG/hCG treatment (Table 1, Figure 1). The mean number of oocytes per female obtained from mAb- Free α subunit/PMSG/HCG treatment (N=10.08) was lower compared to the PMSG/hCG group (N=19.12), with a statistically significant difference (p < 0.05). The results revealed that the neutralization of free inhibin α subunit significantly (p < 0.05) decreased the ovulation rate in all six experiments by 47.29% (p < 0.05) (Figure 2).

The mixed-effects linear model included treatment and experiment (time) as fixed effects, with the experiment also treated as a random effect. The treatment with mAb-free α subunit resulted in a significant decrease in the average number of oocytes per female (p < 0.05), while the effects of time (experiment number) and the interaction between treatment and time were not statistically significant (p > 0.05).

To further support these findings, a dose-effect study was conducted using increased doses of the mAb against the free inhibin α subunit and investigated their effects on the ovulation rate (Table 2). The results showed a significant negative correlation between the mAb concentration injected and the number of oocytes produced indicating that the ovulation rate decreased as a result of increasing mAb concentration (Figure 3, Figure 4), thus supporting the initial findings. According to these findings, it was hypothesized that free inhibin α subunit may interfere with the competition system between active and mature inhibin to bind the activin receptor, which involves the α subunit (Figure 5).

Table 1. Effect of treatment with PMSG/hCG + 400ug mAb- Free α subunit or PMSG/hCG alone on the ovulation rate of female CD1 aged between 4 and 6 weeks

No. of experiment Treatment		No. of animals	Total no. of oocyte donors **	Average no. of oocytes/female ± SD	
1	PMSG/hCG mAb- Free α subunit	4	33*	8.25 ± 0.94	
	PMSG/hCG	4	76	19.00 ± 0.80	
2	PMSG/hCG mAb- Free α subunit	4	45*	11.25 ± 0.94	
	PMSG/hCG	4	78	19.50 ± 1.28	
3	PMSG/hCG mAb- Free α subunit	4	34*	8.50 ± 1.28	
	PMSG/hCG	4	80	20.00 ± 0.00	
4	PMSG/hCG mAb- Free α subunit	4	42*	10.50 ± 0.56	
	PMSG/hCG	4	68	17.00 ± 1.14	
5	PMSG/hCG mAb- Free α subunit	4	45*	11.25 ± 0.50	
	PMSG/hCG	4	79	19.75 ± 0.64	
6	PMSG/hCG mAb- Free α subunit	4	43*	10.75 ± 1.28	
	PMSG/hCG	4	78	19.50 ± 2.08	

No: Number, SD: Standard deviation. Values are mean \pm SD (n = 4). *p < 0.05 versus control group. **The statistical measure of the dispersion of data points around the mean shows low variability between the six experiments for each treatment and high variability between the two groups of treatments.

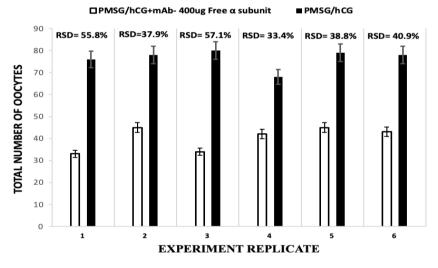
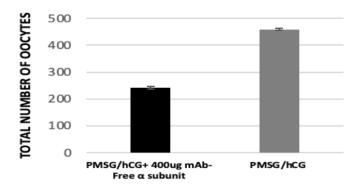


Figure 1. Effects of treatment with PMSG/hCG + 400ug mAb- Free α subunit or PMSG/hCG alone on the ovulation rate of female CD1 with 6 replicates. Number of animals per group was 4 (p < 0.05). RSD: Relative standard deviation.



TREATMENT ADMINISTERED

Figure 2. Total number of oocytes generated following two treatments, PMSG/hCG + 400ug mAb- Free α subunit (number of animals was 24) and PMSG/hCG alone (number of animals was 24) in CD1 mice (p < 0.05).

Table 2. A dose-effect study using four doses of mAb-Free α subunit administered to CD1 mice on ovulation rate compared with administration of PMSG/hCG alone (n = 5).

Treatment	mAb- Free α subunit concentration	Number of oocyte donors *	Average no. of oocytes/female
	200ug	59	11.80
PMSG/hCG mAb- Free α	400ug	43	8.6
subunit	800ug	30	6
	1mg	26	5.2
PMSG/hCG		85	17

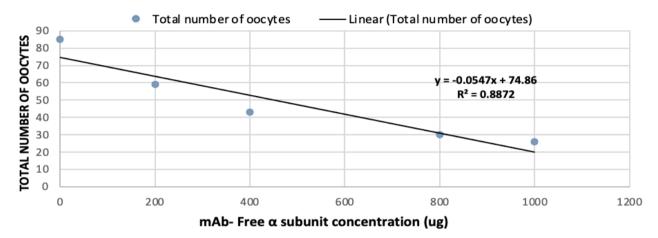


Figure 3. Dose-response regression curve of mAb- Free α subunit concentration on ovulation rate. Number of oocytes collected from CD1 females, One group treated with PMSG/hCG alone and four groups treated with PMSG/hCG combined with increasing concentrations of mAb-Free α subunit, each consisting of 5 animals.

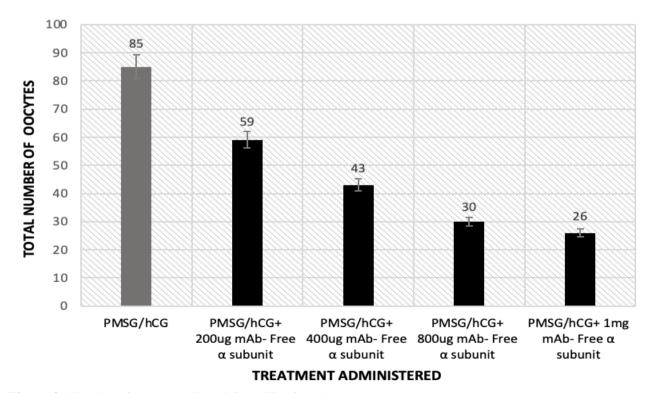


Figure 4. Number of oocytes collected from CD1 females. One group was treated with PMSG/hCG alone, and four groups were treated with PMSG/hCG combined with increasing concentrations of mAb-Free α subunit, each consisting of five animals.

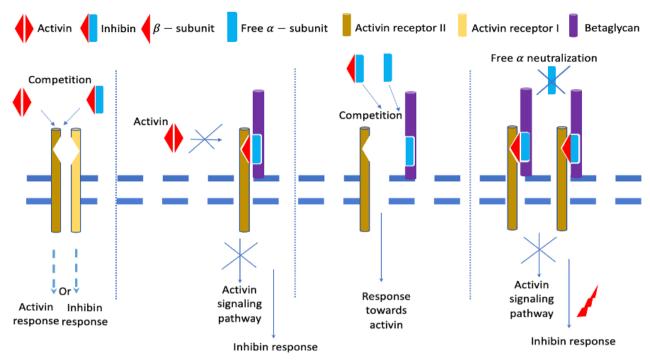


Figure 5. Competition between inhibin and activin β subunits for binding to the activin type II receptor. As a consequence of inhibin binding, activin type I receptor recruitment is inhibited thus blocking activin's action. There is also competition between the α subunit of inhibin and the free inhibin α subunit to bind betaglycan. As a result of the free α subunit binding, inhibin's affinity to the activin type II receptor decreases. Neutralization of the free inhibin α subunit increases the inhibin effect.

Fertilization and embryo development

Further investigation was conducted to examine the effect of the free inhibin α subunit on the fertilization rate of cleaved oocytes as well as the blastocyst development (Table 3). Considering the results of the three experiments, the fertilization rate of mice injected with mAb- free α subunit was 83.48%, as compared to 27.80% in the control group (PMSG/hCG) (Table 3, Figure 6), which represents a significant increase in fertilization of 55.68% (p < 0.05). A similar improvement was observed in embryo and blastocyst development following the administration of mAb-free α subunit (Table 3); indeed, in the group treated with mAb- free α subunit, 62.63% of two-cell embryos developed to the blastocyst stage. In the group treated with PMSG/hCG alone, however, only 15.78% of two-cell embryos developed to the blastocyst stage (Figure 7), resulting in a significant increase in the blastocyst rate of 43.85% (p < 0.05).

The mixed-effects analysis showed that the treatment with mAb-free subunit had a significant positive effect on both fertilization and blastocyst rates (p < 0.05), as compared to the PMSG/hCG treatment alone. However, there was no significant effect of time on either the fertilization rate (p = 0.593) or the blastocyst rate (p = 0.760), indicating that the timing of the experiments did not significantly influence the outcomes.

Table 3. Effect of treatment with PMSG/hCG + 400ug mAb- Free α subunit (n = 11) or PMSG/hCG alone (n = 11) on the fertilization rate and embryo development of female CD1 aged between 4 and 6 weeks

Number of experiments	Treatment	No of animals	No. of oocyte donors	Average no. of oocytes/female	No. of unfertilized oocytes	No. of two-cell embryos	Fertilization rate (%)	No. of blastocyst	Blastocyst (%)
1	PMSG/hCG mAb- Free <i>α</i> subunit	4	31	7.75	3	28	90.32	17	60.71
	PMSG/hCG	4	80	20	57	23	28.75	3	13.04
2	PMSG/hCG mAb- Free <i>α</i> subunit	3	22	7.33	5	17	77.27	12	70.58
	PMSG/hCG	3	45	15.33	34	11	24.44	2	18.18
3	PMSG/hCG mAb- Free <i>α</i> subunit	4	56	14	10	46	82.14	28	60.86
	PMSG/hCG	4	80	20	57	23	28.75	4	17.39

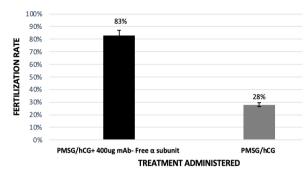


Figure 6. Effects of treatment with PMSG/hCG + 400ug mAb- Free α subunit or PMSG/hCG alone on the fertilization rate of female CD1 (Number of animals per group was 7 e; p < 0.05).

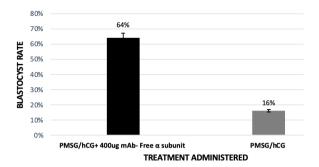


Figure 7. Effects of treatment with PMSG/HCG + 400ug Mab-anti INH α or PMSG/HCG alone on blastocyst and embryo development of female CD1 (Number of animals per group was 7; p < 0.05).

DISCUSSION

Inhibin and activin are structurally and functionally related. Unlike inhibin, activin stimulates FSH secretion, and its activity could be indirectly affected by changes in the levels of the free inhibin α subunit (Gray et al., 2005). Inhibin applies its biological effects by antagonizing activin's action (Massagué and Chen, 2000). Activin's binding to the ActRII receptor is critical to initiate a cascade of actions involved in its signaling pathway (Lebrun and Vale, 1997; Kawabata and Miyazono, 1999). Inhibin competes with activin to bind the ActRII receptor via the β subunit, thereby antagonizing activin's signaling (Lebrun and Vale, 1997; Pangas and Woodruff, 2000). The mechanism of action toward activin response or inhibin response depends on the amount of inhibin; at higher concentrations, inhibin is more likely to bind to the ActRII receptor than activin (Martens et al., 1997; Lebrun et al., 1999). Indeed, studies have shown that activin has a higher affinity for the ActRII receptor than inhibin because of avidity/cooperative binding effects, with activin binding two type II receptors at once, while inhibin binds only one (Thompson et al., 2003; Harrison et al., 2004). The study conducted by Lewis et al. (2000) proved the necessity of a co-receptor, betaglycan, to enhance inhibin's affinity to the ActRII receptor, to which inhibin binds via its α subunit (Lewis et al., 2000; Makanji et al., 2008). However, betaglycan has a high affinity for inhibin and can mediate inhibin reactivity to cells that are insensitive to inhibin (Lewis et al., 2000; Esparza-Lopez et al., 2001; Harrison et al., 2001; Brûlé et al., 2021).

The present study assessed the effect of immunoneutralizing the free inhibin α subunit using a novel monoclonal antibody raised against the amino acid epitope HIS⁶⁴ ALA⁶⁵ VAL⁶⁶ GLY⁶⁷ GLY⁶⁸ PHE⁶⁹ MET⁷⁰ HIS⁷¹ ARG⁷² THR⁷³ SER⁷⁴ GLU⁷⁵ PRO⁷⁶ GLU⁷⁷ within the α N region of the mouse inhibin α subunit.

Following the immunoneutralization of the monomeric free inhibin α subunit in mice, the results showed a 47.29% decrease in the ovulation rate compared to the control group (p < 0.05; Figure 2, Table 1). Additionally, the ovulation rate decreased further with higher concentrations of the mAb-free α subunit (Figures 3 and 4). These results unexpectedly demonstrated a positive effect of the free inhibin α subunit on follicular development and ovulation in mice, contrasting with the effect of mature inhibin. These findings support the hypothesis addressed by previous studies (Schneyer et al., 1991; Silva et al., 1999; Laird et al., 2019) suggesting that the free inhibin α subunit may have the ability to antagonize inhibin. The effects associated with the production of immunoreactive monomeric pro- α N- α C protein remain an area requiring further research. Evidence suggests that the free inhibin α subunit may possess intrinsic biological properties distinct from those of dimeric inhibins (Risbridger et al., 1989; Lambert-Messerlian et al., 1994; Mason et al., 1996; Drummond et al., 2004; Makanji et al., 2014). It may act as an additional local modulator of follicular function, potentially serving as an inhibin antagonist and thereby functioning as an activin agonist (Silva et al., 1999; Lewis et al., 2000; Chapman and Woodruff, 2003).

To date, experimental research has been insufficient to elucidate the effect of free α subunit on ovulation and embryo development. The monoclonal antibody generated in this study targets the free α subunit of inhibin (~52 kDa) and may influence ovulation and reproductive function through several indirect mechanisms. By neutralizing the free α subunit, it might alter the overall balance and availability of inhibin and activin.

Most superovulation protocols based on inhibin neutralization target inhibin α because it is the common component shared by both inhibin A and B, thereby potentially neutralizing both forms by preventing their proper assembly or receptor interaction. In this study, neutralizing the monomeric free α subunit could potentially alter the processing or availability of mature inhibin forms, which would be expected to enhance, rather than inhibit, ovulation. However, in the presence of its co-receptor betaglycan, inhibin can interact with the activin receptor even at equimolar or lower

concentrations than activin (Carroll et al., 1989; Rivier and Vale, 1991, Weiss et al., 1993; Gray et al., 2005; Brûlé et al., 2021), suggesting that the enhancement of its affinity by betaglycan is more critical for inhibin antagonism.

From another perspective, based on the present results, it can be hypothesized that the free α subunit competes with mature inhibin to bind betaglycan, making ActRII more available to bind activin's β subunit. The neutralization of the free α subunit increases the amount of inhibin binding to betaglycan, thereby increasing activin antagonism (Figure 5). The findings of the present study strongly support this mode of action. Furthermore, a previous study demonstrated that transgenic mice with knockdown of the inhibin α subunit exhibited a 35.28% reduction in litter size, which was associated with a decreased ovulation rate (Kadariya et al., 2015).

Overall, there is widespread agreement that neutralizing endogenous inhibin could enhance folliculogenesis and fertility in females. However, previous research has yielded conflicting results. Numerous studies in both rodents and different farm mammals have clearly demonstrated that active or passive immunization against inhibin or the inhibin α subunit led to an increase in ovulation rate (Wheaton et al., 1996; Mao et al., 2016; Jia et al., 2021; Hasegawa et al., 2022; Mochida et al., 2024). However, other studies did not observe such an increase (Findlay et al., 1989; Ireland et al., 1992; King et al., 1995; Terhaar et al., 1997; Dhar et al., 1998; Lu et al., 2020). According to the findings of the present study, this inconsistency among studies may largely stem from the influence of the free inhibin α subunit, suggesting that in these studies, the antibodies generated after inhibin immunization were likely targeting the free inhibin α rather than the mature α subunit.

In addition to the unique effects of the free α subunit, the mature forms of inhibin A and B have been shown to operate through different mechanisms. They might act through distinct co-receptors to impair activin signaling and suppress FSH secretion and synthesis. A significant amount of data evaluates the effect of immunizing animals against inhibin on serum FSH levels (Drummond et al., 2004). Numerous studies have shown an increase in oocyte numbers following the disruption of inhibin feedback, resulting in elevated FSH levels. However, other studies unexpectedly showed no effect on FSH levels despite stimulated follicular growth and enhanced ovulation. The reasons behind these discrepancies remain unclear. A recent review has analyzed existing models of inhibin action, highlighting how recent discoveries in both mice and humans have posed challenges to these models (Bernard et al., 2020). It has been noted that inhibin A and B elicit distinct reactions within the reproductive system. Despite sharing a common α subunit, they display differing affinities towards betaglycan. Specifically, inhibin A binds to betaglycan with a higher affinity compared to inhibin B. Notably, inhibin B is the form responsible for regulating FSH release during the follicular phase of the menstrual cycle in primates and during metestrus/diestrus in rodents (Bernard and Woodruff, 2001; Chapman and Woodruff, 2003; Yding Andersen, 2017).

Another interesting finding of the present study is the negative effect of the inhibin free α on oocyte maturation and embryo development. This study demonstrates that the neuralization of the free α subunit increases the fertilization rate by 55.68% and the blastocyst development by 43.85%, as compared to the control group (p < 0.05, figures 6 and 7), indicating that the free inhibin α subunit may have a negative impact on the quality of the mature oocyte and the competence of the fertilized oocyte to attend the blastocyst stage. The substantial secretion of inhibin by multiple developing follicles may adversely affect follicular development, oocyte function, and quality during follicular atresia and selection (Jimenez-Krassel et al., 2003). Immunoneutralizing inhibin could mitigate the negative impacts of this hormone on maturing oocytes and the resulting embryos. The present study shows a consistent improvement in both embryo yield and development as a result of neutralizing the inhibin free α subunit.

There is substantial evidence to support this finding; a previous study has reported that the mAb against inhibin free α subunit co-cultured with the bovine cumulus oocyte complex enhances the blastocyst yield by 77% (Silva et al., 1999). Furthermore, it has been proved that at higher concentrations, inhibin α subunit is deleterious to embryo development (Fujiwara et al., 2000). In a previous study investigating the influence of progesterone on *in vitro* maturation of bovine oocytes, it was found that the negative impact of progesterone on blastocyst yield was associated with a significantly higher concentration of total inhibin α subunit. They reported that the adverse effect of progesterone on blastocyst yield may be mediated by increased expression of inhibin α subunit by cumulus cells (Silva and Knight, 2000). Furthermore, it has been reported that the presence of free inhibin α subunit during oocyte maturation reduces oocyte development after cleavage (Silva et al., 1999). Results from another study by Li et al. (2011) also demonstrated that the addition of an increased concentration of inhibin antibody decreased the embryo development to blastocyst stage, stating that blastocyst rate was not further enhanced after pretreatment with antibody against inhibin (Li et al., 2011). Together, the present findings revealed that the free inhibin α subunit may have an opposite effect on inhibin at both ovulation and blastocyst rates.

In addition, several studies have investigated the impact of inhibin immunoneutralization as a superovulation protocol on oocyte quality and subsequent blastocyst formation after *in vitro* fertilization, studies revealing varying outcomes. Some have demonstrated that superovulation treatment can negatively affect both fertilization rates and

embryo development (Takeo and Nakagata, 2015; Hasegawa et al., 2016), while others have found no significant differences compared to the control groups (Wang et al., 2001; Ishigame et al., 2004). Further insights were gained in studies that administered increased doses of anti-inhibin treatment, which revealed a greater decrease in blastocyst development, suggesting a negative correlation (Wang et al., 2001; et al., 2004; Li et al., 2011).

CONCLUSION

In summary, the present study demonstrated that the free inhibin α subunit has a positive effect on ovulation rate in contrast to the effect of mature inhibin. However, the free inhibin α subunit was shown to harm embryo competence. These findings provided experimental evidence to support the hypotheses and questions raised by various studies regarding the potential antagonist effect of inhibin-free α on inhibin itself. Together, these results indicated that free inhibin α subunit and inhibin protein may play an opposite effect in normal ovarian physiology. In so far as the present study was conducted only on mice, further investigation should be conducted to validate these observations in other mammalian species. Furthermore, the findings of the present study may open a new area of investigation in understanding the physiological effects of inhibin and its unique signaling pathways.

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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

Kaoutar Aalilouch, Ouafaa Fassi Fihiri, Khalida Sabeur, Faouzi Kichou, and Ikhlass El Berbri designed and coordinated the work. Najet Safini and Mehdi Elharrak conducted the epitope design and supervised the antigen immunization for antibody production. Kaoutar Aalilouch performed the cell fusion and monoclonal antibody production and purification under the supervision of Ouafaa Fassi Fihri. Kaoutar Aalilouch and Khalida Sabeur evaluated the *in vitro* and *in vivo* activity of the produced antibody, conducting laboratory work on mice ovulation tests, IVF, and embryo development. The manuscript was written by Kaoutar Aalilouch. Kaoutar Aalilouch conducted the presented statistical analysis of the experimental data, with contributions from Khalida Sabeur and Ikhlass El Berbri. All authors participated in the overall interpretation of the data and confirmed the final draft of the manuscript for submission to the journal.

Competing interests

The authors declare that they have no competing interests

Ethical considerations

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

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Effect of Artificial Insemination Timing on Conception Rate in Lactating Holstein-Friesian Cows

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ABSTRACT

Successful pregnancy in dairy cows requires accurate timing of artificial insemination (AI). Artificial insemination conducted in the morning, midday, and afternoon exposes cows to different environmental temperatures with high ambient temperatures can potentially reduce the viability of spermatozoa, thus disrupting the fertilization process and increasing the possibility risk of pregnancy failure. The purpose of the present study was to ascertain and determine how the effect of various AI timings (during the morning, midday, and afternoon) affected the pregnancy success of the pregnancies in Holstein-Friesian cows. The purposive sampling was used to select a total of 191 Holstein-Friesian cows based on the following specific criteria; cows showing symptoms of estrus, having healthy reproductive organs, having one parturition at least once, aged 2-6 years old, having body condition score of 2.5-3.5 (on a 1-5 scale). The cows were divided into 3 three treatment groups including T1 cows inseminated from 06.00 am to 10.59 am (n=38 cows), T2 cows inseminated from 11.00 am to 03.59 pm (n=82), and T3 cows inseminated from 04.00 pm to 08.59 pm (n=71). The non-return rate was monitored at 19-22 days post-insemination (NRR-1) and 39-42 days post-insemination (NRR-2) was monitored, while the conception rate (CR) was assessed on day 60 post-insemination. Artificial insemination was performed 8 hours after estrus. NRR-1 values for T1, T2, and T3 were 82%, 80%, and 89%, respectively, The NRR-2 values were 71% in T1, 66% in T2, and 79% in T3. The CR for T1, T2, and T3 were 50%, 48%, and 54%, respectively. Although AI timing did not yield conception rates based on AI timing did not show a significant difference in conception rates, artificial insemination performed in the morning insemination is recommended due to its higher likelihood of successful pregnancy compared to other times.

Keywords: Artificial insemination, Conception rate, Dairy cow, Insemination timing, Lactating dairy cows, Non-return rate

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INTRODUCTION

In dairy cows, achieving successful pregnancy requires artificial insemination (AI) at the right time. Accurate timing is crucial since AI conducted too early or too late can decrease the probability of pregnancy. Early AI decreases the chance of fertilization (Fernandez-Novo et al., 2020). Spermatozoa will age, and if ovulation occurs during this period, spermatozoa will not be able to fertilize the egg (Hawk, 1987). Spermatozoa that spend too long in the female reproductive tract will experience decreased motility due to excessive capacitation (Aitken et al., 2015). Capacitation is a physiological change that enables spermatozoa to fertilize the egg, but if this process is prolonged without fertilization, spermatozoa may lose their energy and ability to move actively (Mahdavinezhad et al., 2021). Sperm aging plays a critical role in reducing fertility in mammals, as it leads to alterations in membrane integrity and an increase in lipid peroxidation, ultimately decreasing the proportion of viable sperm capable of successful fertilization (Am-in et al., 2011; Donnellan et al., 2022). On the contrary, late AI can lead to the aging of the egg and disrupt the fertilization process (Hunter and Greve, 1997).

According to Roelofs et al. (2006), AI conducted 12-24 hours before ovulation has a high success rate. However, the absence of a definite sign of ovulation is an obstacle for small-scale farmers in determining the optimal AI time. Therefore, AI based on the onset of estrus is an option that can be used by farmers in deciding on an appropriate AI time. The optimal AI time is 8 hours after the onset of estrus (Marques et al., 2024). Therefore, AI conducted at 8 hours after estrus could lead to the possibility of AI in the morning, midday, and afternoon. Artificial insemination conducted during the midday is potentially detrimental to pregnancy success because daytime temperatures are higher than morning and afternoon temperatures (Hamid et al., 2018; Szenci et al., 2018). Elevated temperatures negatively impact sperm quality, potentially reducing pregnancy success (Hansen et al., 2001).

Properly timed AI is essential to maximize the probability of pregnancy on the first insemination attempt. Artificial insemination failure leads to various disadvantages due to longer feed costs, extended time required for conception, and a decrease in the number of offspring that can be born during the lifetime (Tadesse et al., 2022). Therefore, the objective of this study was to investigate whether the morning, midday, and afternoon AI times influence the success of conceptions among Holstein-Friesian cows.

MATERIALS AND METHODS

Ethical approval

Ethical approval was given by the ethics committee of the Institute of Biosciences, Universitas Brawijaya, Malang, East Java, Indonesia, in compliance with ethical guidelines regarding responsible behavior in the use of Holstein-Friesian Friesian Holstein cows in experimental animal research (Ethical clearance number: 47/EC/KEPK/02/2024).

Animals and treatment groups

The cows used in this study consisted of 191 Friesian Holstein-Friesian cows owned by members of Sinau Andandani Ekonomi (SAE) Cooperative Pujon, a private dairy farm cooperative. In this study, the cows were divided into 3 three treatment groups: Treatment 1 (T1) with 38 cows inseminated from 06.00 am to 10.59 am, treatment 2 (T2) with 82 cows were inseminated from 11.00 am to 03.59 pm, and treatment 3 (T3) with 71 cows were inseminated from 04.00 pm to 08.59 pm. The difference in the number of cows per treatment is due to variations in the availability of cows in each period. The cows included in this study met the following criteria first, they had given birth at least once. Cows that have given birth indicate that the cow had normal reproductive organs capable of pregnancy and giving birth, as evidenced by the cow being able to get pregnant and give birth normally (Diskin, 2014). Second, cows had normal reproductive organs, as evidenced by rectal palpation before artificial insemination (AI). Third, cows were between 2 and 6 years old. According to the report of Susilawati (2014) cows older than six years tend to exhibit lower fertility rates compared to their younger counterpart. Fourth, cows had a Body Condition Score (BCS) of 2.5 to 3.25 (on a 1-5 scale). According to Yamada et al. (2003), BCS of 2.75-3.25 is associated with better AI outcomes. Fifth, the cows exhibited clear signs of estrus.

Artificial insemination

Artificial insemination was carried out by experienced, nationally certified local inseminators who are experienced and nationally certified. To minimize stress during the AI process, cows were handled gently using low-stress handling techniques, ensuring that no force or rough movements were applied. The environment was kept calm and quiet to create a stress-free atmosphere. Furthermore, cows were only inseminated when they showed clear signs of estrus, and the process was conducted swiftly to avoid prolonged handling, ensuring that the cows remained comfortable throughout the process. Artificial insemination was conducted in the 8th hour after the first appearance of estrus. The semen was deposited at the corpus uteri during AI. The frozen semen used in this study was supplied by the Singosari Artificial Insemination Center, and its quality has been previously documented by Yekti et al. (2023). Farmers reporting their cows exhibiting estrous behavior prompted the use of artificial insemination. Insemination was performed eight hours after the onset of estrus signals. Eight hours following the onset of estrus signals, the inseminator inseminated the cows. NNR-1 monitoring was carried out on days 19-22 following AI. Days 39-42 after AI, observation was followed by NRR-2 observation if the cow did not show estrus. Cows that did not show signs of estrus during NRR-1 and NRR-2 observations were confirmed pregnant using the rectal palpation method on day 60 after AI. Rectal palpation, an old long-used, and cost-effective method for diagnosis of pregnancy in cattle, involves manual examination of the uterus to detect the presence of a fetus. Although it provides rapid results, it requires a skilled practitioner and carries the risk of fetal damage (Jaśkowski et al., 2019).

Non-return rate

Non-return rate 1 (NRR-1) was a metric used to evaluate the proportion of cows displaying signs of estrus during the first estrous cycle, specifically between days 19 and 22 post-AI. Non-return rate 2 (NRR-2) measured the percentage of cows that did not exhibit estrus during the second cycle, which occurred between days 39 and 42 after AI. According to Syah et al. (2024), NRR-1 and NRR-2 values were calculated using the following formulas.

NRR1 = (Total inseminated cows – total cows showing signs of estrus on days 19 to 22 / Total inseminated cows) $\times 100$ NRR2 = (Total inseminated cows – total cows showing signs of estrus on days 39 to 42 / Total inseminated cows) $\times 100$

Cows that did not show any signs of estrus during the NRR observations were assumed to be pregnant and were subsequently examined followed by a pregnancy examination using the rectal palpation method on day 60.

Conception rate

The conception rate (CR) represents the proportion of cows that became pregnant after the first insemination (Souames and Berrama, 2020). This parameter was used to assess the success of pregnancy after cows were considered pregnant based on NRR-1 and NRR-2 observations. Cows not showing signs of estrus at either observation time were subsequently checked for pregnancy using the rectal palpation method, performed on day 60 after AI. According to

Jainudeen and Hafez (2000), CR was calculated as follows: the number of pregnant cows from the first insemination is divided by the total number of cows used in the study multiplied by 100.

CR (%) = (Total cows pregnant at first insemination / Total inseminated cows) $\times 100$

Statistical analysis

Data were tested statistically using R Studio version 4.3.3. This study used a chi-square test to compare the pregnancy success of the three treatments. Statistical significance was expressed at $p \le 0.05$.

RESULTS AND DISCUSSION

The effect of different AI timings on NRR-1 and NRR-2 is shown in Table 1. No significant differences were found in NRR-1 and NRR-2 values between AI timings (p > 0.05). Although there was no significant difference, T2 showed the lowest NRR-1 and NRR-2 values, which were 80% and 66%, respectively. In the present study, a decrease in NRR-1 to NRR-2 values was observed across all treatments, with the largest drop in T2 (14%), indicating that the cows in T2 showed the most estrus signs over the two estrus cycles. In contrast, decreases in NRR-1 to NRR-2 in T1 and T3 were 11% and 10%, respectively.

The NRR-1 values for T1, T2, and T3 were 82%, 80%, and 89%, respectively. An imperfect NRR-1 value means that some cows exhibited signs of estrus in the first estrus cycle after AI. Furthermore, this is explained by the failure of fertilization between spermatozoa and ovum. Moreover, the failure of fertilization might arise from poor semen quality so spermatozoa penetration is not optimal. Alternatively, cows might experience stress, which might lead to reduced quality of the ovum (Walsh et al., 2011). For NRR-2, values were 71%, 66%, and 79%, respectively. There was a decrease in all treatments in the NRR-2 value, attributable to early embryonic death or silent heat (Syah et al., 2024). Early embryonic death can occur if the cow experiences a deficiency of progesterone hormone (Smith et al., 2022), as this hormone functions to maintain pregnancy, and insufficient levels can increase early embryonic mortality (Baruselli et al., 2022). Silent heat, where estrus signs are minimal or absent despite ovulation, can also affect NRR-2 (Sammad et al., 2020). The reduced NRR1 and NRR2 values observed in T2 could be attributed to elevated ambient temperatures. Temperatures in AI at midday (T2) can reach up to 29°C (Figure 1). High ambient temperatures increase the potential for cows to suffer from heat stress (Herbut et al., 2021). Heat stress is a condition in which certain mechanisms are triggered to maintain the body temperature of cattle when exposed to uncomfortably high temperatures (Dash et al., 2016). Heat stress conditions cause a decrease in the quality of spermatozoa in the female reproductive organs during penetration into the fallopian tubes. Spermatozoa exposed to high temperatures in the female reproductive tract can reduce spermatozoa viability, therefore disrupting fertilization and embryo development (Hansen et al., 2001). Furthermore, while high ambient temperatures may induce stress in cattle, studies have shown that their direct impact on ovulation is less pronounced compared to other reproductive processes (Roth, 2020). Nonetheless, heat stress significantly affects fertility rates and increases early embryonic death risks (Wolfenson and Roth, 2019).

The effects of different AI timings on CR are presented in Table 1. In this study, AI timing did not show a statistically significant difference in pregnancy success (p > 0.05). Although the difference between treatments indicated no significant difference, T2 showed the lowest pregnancy success rate, while T3 showed the highest CR value. Furthermore, there was a decrease in the percentage of NRR-2 to CR in all treatment groups. While no significant differences were observed, T2 had the lowest NRR-1, NRR-2, and CR values, indicating that insemination from 11:00 am to 3:59 pm may be associated with higher ambient temperatures compared to the other timings. Research on the effects of AI timing on conception rates in lactating Holstein-Friesian cows remains limited. This lack of research is likely due to several factors, including the historical focus on other reproductive technologies and the assumption that optimal insemination timing has been adequately addressed in standardized protocols. However, the timing of AI can significantly affect conception rates due to physiological variations in the estrous cycles of cows, influenced by factors such as hormonal changes, ambient temperature, and stress levels at different times of the day. Further research into AI timing is essential as understanding the optimal timing of insemination can improve conception rates, especially in lactating cows where reproductive efficiency is critical to maintaining milk production and fertility. Addressing these shortcomings will contribute to refining AI practices and increasing reproductive success in dairy farming operations, especially in areas with variable environmental conditions.

In this study, AI was conducted at the 8th hour after the onset of estrus. Therefore, the time of insemination was matched with the onset of estrus. Cows inseminated at 11:00 am-3:59 pm had a lower conception rate, although there was no significant difference between T1 and T3 (p > 0.05). Artificial insemination conducted during the midday (T2) showed no significant difference with AI in the morning (T1) and in the afternoon (T3), so AI can be conducted at any

time as long as the cows are still in the estrus phase. Previous studies have shown that the optimal time is 4–16 hours after estrus (López-Gatius, 2022; Udin et al., 2022).

Artificial insemination conducted more than 16 hours after the onset of estrus tends to have lower conception rates. However, this could be due to the time of ovulation being too close to the time of insemination, as estrus length is generally 24 to 33 hours and spermatozoa need 6-8 hours to capacitate (López-Gatius, 2022; De Rensis et al., 2024). Capacitation, a complicated process occurring in the reproductive organs of females, allows spermatozoa to bind to and penetrate the zona pellucida, facilitating fertilization (Mostek et al., 2021).

These results support previous studies suggesting that the timing between AI and ovulation is crucial for pregnancy success (Lauber et al., 2020). This study also confirms that AI can be done with the "a.m.-p.m. insemination rule", where cows showing estrus symptoms in the morning can be inseminated in the afternoon of the same day (Foote et al., 1979).

Table 1. Non-return rate and conception rate values in Holstein-Friesian cows at Sinau Andandani Ekonomi Cooperative Pujon, Malang, East Java, Indonesia

\$7	T1 (n=	=38)	T2 (n=82)		T3 (n=71)		D1
Variable	Not estrus	Pregnant	Not estrus	Pregnant	Not estrus	Pregnant	P-value
NRR1	31 (82%)	-	66 (80%)	-	63 (89%)	-	0.35
NRR2	27 (71%)	-	54 (66%)	-	56 (79%)	-	0.20
CR	-	19 (50%)	-	39 (48%)	-	38 (54%)	0.76

T1: Artificial insemination in the morning (06.00 am - 10.59 am), T2: Artificial insemination in the midday (11.00 am - 03.59 pm), T3: Artificial insemination in the afternoon (04.00 pm - 08.59 pm), NRR1: Non-return rate 1, NRR2: Non-return rate 2, CR: Conception rate

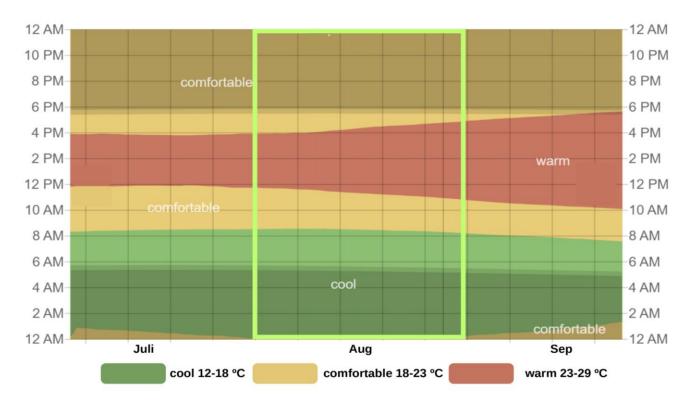


Figure 1. The hourly temperature at Sinau Andandani Ekonomi Cooperative Pujon, Malang, East Java, Indonesia, as recorded by weathers park

CONCLUSION

The timing of AI (morning, midday, and afternoon) in Holstein- Friesian cows did not affect pregnancy success. Although the difference in AI time did not show a significant effect, AI conducted in the midday had the lowest pregnancy success, while AI conducted in the afternoon showed the highest pregnancy success. While AI timing alone (morning, midday, or afternoon) did not significantly affect pregnancy success, further studies are recommended to incorporate additional environmental factors, such as temperature, humidity, and heat stress levels.

DECLARATIONS

Authors' contributions

Habib Asshidiq Syah wrote the manuscript, collected data, analyzed data, and reviewed the final version of the manuscript. Putri Utami reviewed the final version of the manuscript. Aulia Puspita Anugra Yekti, Nurul Isnaini, and Trinil Susilawati designed the study, supervised the study, and reviewed the final version of the manuscript. All authors have approved the final edition of the manuscript manuscript.

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Ethical considerations

All authors have reviewed and confirmed the original content of the article before submission to this journal.

Availability of data and materials

The original contributions of this study are available within the article and its supplementary materials. For further information, please reach out to the corresponding authors.

Competing interests

The authors have not declared any conflict of interest.

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Gastrointestinal Parasitic Infections of Ruminants in Pastoral Communities of Ondo State, Nigeria

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ABSTRACT

Livestock are important for food production and agricultural systems; however, helminth infections are a major constraint on their productivity and value. The present study aimed to investigate the prevalence and associated risk factors for helminth infections in four pastoral communities in Ondo State, Nigeria. A total of 1,165 fecal samples from livestock in four pastoral rural/peri-urban communities (Akungba, Ikare, Oka, and Supare) in the Akoko area of Ondo State were screened for gastrointestinal parasitic infections via the sedimentation technique. The overall prevalence of parasitic infections was 67.7%, with sheep having the highest prevalence of infection (72.8%). The prevalence of parasitic infection was significantly associated with the breed of livestock. Important parasites of public health, including *Fasciola* species, *Strongyloides* species, *Trichuris* species, hookworms, *Schistosoma* species, *Trichostrongylus* species, and *Fasciola* species, which are the most common, were isolated in the current study. Two-thirds (75.5%) of the observed prevalence was due to infection with a single helminth species, whereas the remaining one-third (24.5%) comprised infections with two or three species of helminths. Among multiple infections, *Fasciola* species plus *Strongyloides* species coinfection had the highest prevalence (46%). The present findings suggest the need for regular epidemiological surveillance and treatment of infected ruminants with gastrointestinal helminths.

Keywords: Helminth, Infection, Livestock, Prevalence, Ruminant

1: September 24, 202 October 18, 2024 1: November 23, 202

INTRODUCTION

Livestock farming and trading are sources of income in rural communities across Nigeria, especially among subsistence farmers and pastoralists, contributing approximately 1.7% of the national gross domestic product (GDP) and approximately 9% of the agricultural value added (FAO, 2019). Over the years, parasitic infections have remained a major challenge to productivity in livestock farming in Nigeria, with helminth infections accounting for up to 79.92% of infections (Karshima et al., 2018; Ola-Fadunsin et al., 2020).

The extensive system of animal husbandry commonly practiced across the country contributes to the incidence of parasitic infections. Although the effects of these infections are difficult to measure, they result in economic losses reflected in poor growth rates and reduced production of meat, milk, wool, and carcass quality (Strydom et al., 2023). The intestinal parasites of ruminants and their prevalence have been documented in studies from different parts of Nigeria; however, the majority of the studies are from northern Nigeria, as livestock farming is a predominant occupation in this region (Karshima et al., 2018).

Numerous factors, including host-related factors (host species, the animal's sex, age, bodily condition, and breed/genotype), and environmental factors (the degree of infection across different strata), influence the abundance and distribution of parasitic infections in livestock ruminants (Kołodziej-Sobocińska, 2019). As the global population increases continuously, livestock production needs to be more efficient to sustain food security, especially meat and dairy products (Morgan et al., 2013). Therefore, there is a need for constant epidemiological surveys to understand the pattern of infections and the various risk factors in different regions. However, previous studies on parasitic infections among livestock in Nigeria were conducted in abattoirs, possibly due to the ease of sample collection compared with the challenges of locating herders in rural areas (Elelu and Eisler, 2018; Karshima et al., 2018). Despite being well known for having several pastoral communities, studies on gastrointestinal diseases in cattle in the Akoko area of Ondo State are rare (Akinmoladun and Olafare, 2014).

Thus, the present study aimed to provide baseline epidemiological data on parasitic infection in cattle, sheep, and goats in four pastoral communities in the Akoko area of Ondo State, Nigeria.

MATERIALS AND METHODS

Ethical approval

Ethical approval was obtained from the ethical review board of Adekunle Ajasin University, Akungba Akoko, and oral consent was secured from individual traders and herdsmen. All applicable national and institutional guidelines for the care and use of animals were followed.

Study location

The Akoko subgroup comprises forty small rural to peri-urban settlements spread across four local government areas of Ondo State, Nigeria. The present study was carried out in four of the major settlements: Ikare (7.5248° N, 5.7669° E), Akungba (7.4740° N, 5.7379° E), Oka (7.4570° N, 5.8011° E), and Supare (7.3812° N, 5.6248° E) (Figure 1). The area has rocky terrain characterized by rainforest vegetation with an annual rainfall of 1500 mm, a relative humidity of 75% to 95%, and an average temperature of 24°C. The population is mixed, comprising people from different tribes involved in various occupations, of which farming and/or pastoralism is predominant. The area hosts various agricultural markets and is a collection point for crops and animals.

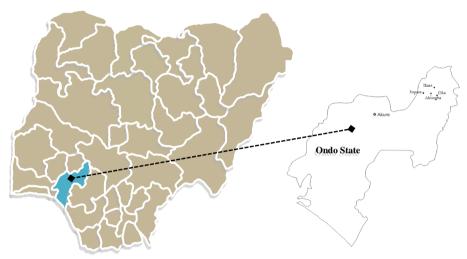


Figure 1. Map of Nigeria showing the study location

Sample collection and processing

The sample size (n = 369) was determined based on the 60% regional prevalence of helminths in ruminants in Nigeria (Karshima et al., 2018), 95% confidence level, Z score = 1.96, and 5% absolute precision. At the four study sites (three rural and one peri-urban), 1165 livestock, comprising 391 cattle, 384 goats, and 390 sheep, from 2 months to 8 years were selected via simple random sampling. Fresh fecal samples were collected directly from the rectums of the animals or ground immediately after they were voided into properly well-labeled sterile containers and were taken to the laboratory for microscopic examination. The fluke finder, a commercially available tool, was used for the isolation of parasite eggs from fecal samples. This kit has a single unit made of two 2-inch-wide sieves of approximately 125 nm and 30 nm mesh, and parasite ova/cysts are isolated via differential filtration followed by differential sedimentation (Zárate-Rendón et al., 2019). Approximately 2 g of feces was mixed with water and poured into the upper part of the fluke finder unit. This mixture was washed several times with water, and the larger material that was retained was discarded. The material that was left in the small diameter sieve was drained into a beaker, where it settled for a few minutes before the supernatant was removed. The process was repeated until the residue was clear. The sediment was then poured into a 2inch Petri dish. A pipette was used to place a small amount of sample on the slide, which was stained with three drops of methylene blue and covered with a cover glass. The slides were examined systematically under x10 and x40 magnifications via a light microscope (Olympus, Japan) for larvae, helminth eggs, and cysts. The ova and larvae of the parasites were identified based on their morphological characteristics according to the methods of Cheesbrough (2005) and WHO (2019).

Statistical analysis

The data were analyzed via Microsoft Excel, version 2407 (2024), and R software, version 4.4.1 (2024). The variables (sex, age, breed, and location) were examined for their associations with the incidence of parasites via chi-square (χ 2) tests. The p-value was regarded as statistically significant if it was less than 0.05 at the 95% confidence level.

RESULTS

Among the 1,165 livestock examined for gastrointestinal infections, the overall prevalence of infection was 67.7%, with sheep having the highest prevalence of infection (72.8%), followed by goats (66.7%) and cattle (63.7%, Table 1). In the populations that were sampled, there were more female animals (51.9%) than male animals (48.1%), and most of the animals were either under one-year-old (38.4%) or more than two years old (40.6%). The West African dwarf breed made up a relatively large portion of the animals. Except for the Dalgae breed, all age groups were represented in the samples of male and female animals throughout the breeds. There were slightly more infected female animals (68.6%) and animals more than two years old (69.8%) than male animals (66.8%) and animals younger than two years of age (67.6% and 64.1%), despite the proportion of infection based on the sex and age of the animals being similar (Table 2). The age- and sex-related variations were statistically insignificant (p = 0.302, p = 0.509). Among the breeds, the Gbokono and West African breeds had higher infection rates (70.5% and 69.8%), whereas the Dalgae and Yakana breeds had lower infection rates (44% and 65.4%, respectively). The difference in infection rates among the breeds was statistically significant (p = 0.002). The prevalence of infection among the study sites was similar, ranging between 65.5% and 68.9%, and statistically insignificant (p = 0.762). Multiple regression analysis of risk factors revealed a significant relationship between infection rates and breeds of livestock (p = 0.001).

Table 1. The overall prevalence of gastrointestinal helminths in livestock in the Akoko area of Ondo State, Nigeria, in 2021

Animal	Total	No positive	Percentage
Cattle	391	249	63.7
Goat	384	256	66.7
Sheep	390	284	72.8
Total	1165	789	67.7

Table 2. Demographic characteristics of infected livestock and odds ratios of the risk of infection in the Akoko area of Ondo State, Nigeria, in 2021

Characteristic	octeristic $N = 1,165^1$ Number positive (%), $N = 789^1$		OR ²	95% CI ²	P value
Sex					
Female	605	415 (68.6%) ^a	Ref.	Ref.	
Male	560	374 (66.8%) ^a	18	0.83, 1.41	0.6
Age group					
<1	447	302 (67.6%) ^a	Ref.	Ref.	
1-2	245	157 (64.1%) ^a	0.96	0.68, 1.35	0.8
>2	473	330 (69.8%) ^a	1.16	0.86, 1.57	0.3
Breed					
Dalgae	50	22 (44.0%) ^a	Ref.	Ref.	
Gbokono	78	55 (70.5%) ^b	3.51	1.54, 8.11	0.003
West African Dwarf	774	540 (69.8%) ^c	3.34	1.72, 6.58	< 0.001
Yakana	263	$172 (65.4\%)^{d}$	2.58	1.31, 5.12	0.006
Location					
Akungba	380	262 (68.9%) ^a	Ref.	Ref.	
Ikare	410	281 (68.5%) ^a	0.98	0.72, 1.33	0.9
Oka	123	81 (65.9%) ^a	0.82	0.52, 1.28	0.4
Supare	252	165 (65.5%) ^a	18	0.74, 1.58	0.7

^Tn (%), ² OR: Odds ratio, CI: Confidence interval, Ref: Reference. ^{a-d} Different superscript letters in the same row indicate statistical significance at p < 0.05.

Among the 789 animals infected, 24.5% had multiple gastrointestinal helminth infections, while the remaining animals (75.5%) were infected with a single species. Among the livestock, goats (30.9%) had the highest rate of multiple infections, followed by sheep (22.2%) and cattle (20.5%). However, for single infections, cattle (79.5%) had the highest rate of infection, followed by sheep (77.8%) and goats (69.1%). Concerning the sex of the animals, both sexes had similar infection rates of single and multiple infections (p = 0.400). The prevalence of single and multiple infections also

varied insignificantly across the different age categories of livestock (p = 0.700). A higher rate of multiple infections was observed in Gbokono (29.1%), followed by the West African Dwarf breed (26.3%) and Yakana (20.3%), whereas the Dalgae breed had no multiple infections (Table 3). There were significant differences between the type of infection and the species and breed of the animals (p = 0.016). The frequencies of single (71.9%-79.8%) and multiple (20.3%-29.1%) infections were comparable across the different study sites (p = 0.200).

The eggs of the different parasites identified in the present study were Fasciola species (77%), Strongyloides species (14%), Trichuris species (6.6%), hookworms (1.5%), Schistosoma species (0.9%), and Trichostrongylus species (0.4%, Figure 2). Multiple infections were observed, with Fasciola species plus Strongyloides species (46%) having the highest prevalence among all the samples. In multiple infections with three helminths, coinfections with hookworm plus Fasciola species plus Strongyloides species (2.7%) and Fasciola species plus Strongyloides species plus Trichostrongylus species (1.4%) were observed (Figure 3).

Table 3. Types of infections according to the demographic characteristics of livestock in the Akoko area of Ondo State, Nigeria, in 2021

Characteristic	acteristic $N = 789^1$ Multiple infections, $N = 193^1$ (%)		Single infection, N = 596 ¹ (%)	P value ²
Animal				0.014
Cattle	249	51 (20.5%) ^a	198 (79.5%)	
Goat	256	79 (30.9%) ^a	177 (69.1%) ^a	
Sheep	284	63 (22.2%) ^a	221 (77.8%) ^a	
Sex				0.4
Female	415	96 (23.1%) ^a	319 (76.9%) ^a	
Male	374	97 (25.9%) ^a	277 (74.1%) ^a	
Age group				07
<1	302	83 (27.5%) ^a	219 (72.5%) ^a	
1-2	157	43 (27.4%) ^a	114 (72.6%) ^a	
>2	330	67 (20.3%) ^a	263 (79.7%) ^a	
Breed				0.016
Dalgae	22	$0 (0\%)^{a}$	22 (100.0%) ^a	
Gbokono	55	16 (29.1%) ^b	39 (70.9%) ^b	
West African dwarf	540	142 (26.3%) ^c	398 (73.7%) ^c	
Yakana	172	35 (20.3%) ^d	137 (79.7%) ^d	
Location				0.2
Akungba	262	53 (20.2%) ^a	209 (79.8%) ^a	
Ikare	281	79 (28.1%) ^a	202 (71.9%) ^a	
Oka	81	20 (24.7%) ^a	61 (75.3%) ^a	
Supare	165	41 (24.8%) ^a	124 (75.2%) ^a	

¹ n (%): Number and percentages in brackets. ² Pearson's chi-square test; ^{a-d} Different superscript letters in the same row indicate statistical significance at p < 0.05.

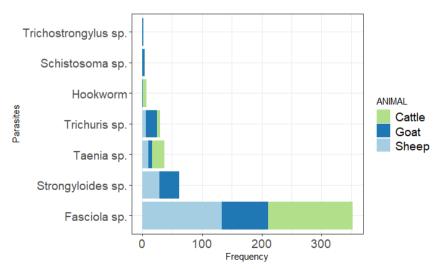


Figure 2. Frequency of single parasitic infections in livestock in the Akoko area of Ondo State, Nigeria, in 2021.

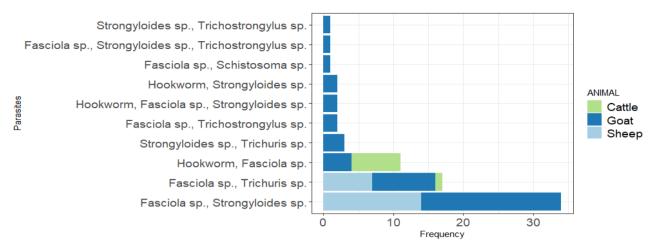


Figure 3. Frequency of multiple parasitic infections in livestock in the Akoko area of Ondo State, Nigeria, in 2021.

DISCUSSION

The high overall prevalence (67.7%) observed in the current study is suggestive of high transmission of gastrointestinal helminths among livestock in the area, with potential impacts on livestock productivity and human health (Table 1). The high rates of infection recorded among the animals exceeded the Southwest regional prevalence of 49.18% reported in a study by Karshima et al. (2018). The high prevalence is consistent with previous studies within the southwestern part of the country (Unigwe et al., 2016; Bolaji et al., 2023; Jonathan et al., 2023).

The high rate of infection recorded in cattle (63.7%) and goats (66.7%) in the present study exceeded the prevalence reported in previous studies in Ondo State from abattoir surveys (Omoleye et al., 2013; Afolabi and Olususi, 2016; Dada and Jegede, 2019; Simon-Oke and Awosolu, 2021).

The West African dwarf is a native goat and sheep breed that predominates in the southwestern region of Nigeria, while the White Fulani is the most common breed of cattle (Kubkomawa, 2017). In the present study, three breeds of cattle from the White Fulani group, as well as goats and sheep from the West African dwarf type, were examined. The age and sex patterns of infections observed in the present study are similar to those reported by Sylvia et al. (2015), Jegede et al. (2015), and Yuguda et al. (2018), with females and older animals being more infected. A commonly reported pattern of increased infection in young animals was similarly observed in the present study; however, a contrasting pattern was observed in older animals. This observation suggests that the interplay of different host and management factors, such as immunity, diet, grazing system, and veterinary care, might have played a role in the observed pattern of infection across the different age groups. The differences in sex prevalence have been attributed to possible variations in immunology and adaptivity to infection between sexes, and reproduction in female animals is also believed to play a role in infection, as it lowers the resistance of female animals to infection (Jegede et al., 2015). Although the susceptibility of animals to gastrointestinal helminth infection could be influenced by a variety of host factors, including age, sex, and breed, only the breed of the animal was significantly associated with the rate of infection in the present study, similar to the findings of Ola-Fadunsin (2017) and Ola-Fadunsin et al. (2020). Breeding has been reported to be an important factor in the epidemiology of parasitic infections in livestock, with local cattle breeds at greater risk of infection than imported cattle breeds (Ola-Fadunsin et al., 2020). The odds of infection with helminths were two times greater in the Yakana breed (OR = 2.58, p = 0.006) and three times greater in the Gbokono and West African Dwarf breeds (OR = 3.51, p = 0.003, OR = 3.34, p < 0.05) than in the Dalgae breed (Table 2). On the other hand, some other studies have found no appreciable variations in the relationship between animal breed and infection (Adedipe et al., 2014; Jegede et al., 2015; Sylvia et al., 2015). Several factors, including the animal rearing system, the frequency of treatment, and the physiological and nutritional health of the animals, are important contributors to the prevalence of helminth infections in various breeds of ruminants (Ola-Fadunsin et al., 2020). There was no significant difference in the pattern of infection across the different study sites. This is expected, as the settlements are close to each other and share the same ecological and environmental factors.

Approximately one-third of the infections reported in the present study were multiple infections comprising two to three helminths (Table 3 and Figure 3). This observation is similar to that of Yuguda et al. (2018) and Ola-Fadunsin et al. (2020), who reported multiple infections in livestock in Bauchi and Ilorin, respectively. The occurrence of multiple infections in livestock in the current study could be attributed to the nomadic system of management, which allows the

animals to access a wide range of habitats and possibly encounter diverse parasites with a relatively high risk of infection. Host-ranging patterns have been proposed to have a major influence on parasite diversity (Bordes et al., 2009). The presence of mixed infections has been linked to morbidity and poor productivity in cattle (Kumsa et al., 2011). Multiple infections suppress the immune system of hosts and increase their vulnerability to other illnesses or parasites (Hananeh et al., 2022).

The different parasites encountered in the present study (Figure 2) have been commonly reported across the country, as described in a review by Karshima et al. (2018). Some of the species encountered are emerging as important zoonoses of great public health concern, especially in developing countries (Robinson and Dalton, 2009; Mathison and Pritt, 2018; Majewska et al., 2021). Fasciolosis, formerly regarded as a livestock disease, has now been recognized as an emerging zoonotic disease with an increasing number of human infections (Robinson and Dalton, 2009). The impact of schistosomiasis on livestock has received less attention than that of human infections; however, current reports on hybridization between human and animal species with evidence of possible transmission of zoonotic schistosomiasis have resulted in renewed attention because of its potential impact on public health (Webster et al., 2013). Similarly, hybridization has also been reported in other helminths, such as Fasciola (Webster et al., 2016; Easton et al., 2020). Recently, the subtle impact of hookworm infection has been recognized, with the disease being linked to severe iron deficiency anemia (IDA) and potential effects on cognitive development in children (Brooker et al., 2004). Helminth infection, in addition to being transmitted to humans, sometimes results in unquantifiable direct and indirect economic losses due to reduced productivity in livestock. Increasing interactions between humans and animals, climate change, migration, and changes in the human diet are some of the factors that have been linked to the increasing emergence of zoonotic potential in parasites of animal origin (McCarthy and Moore, 2000; Keiser and Utzinger, 2005; Majewska et al., 2021).

CONCLUSION

The high prevalence of gastrointestinal helminth infection recorded in the study areas suggests ongoing transmission of parasites between different animal species and the environment, possibly due to the congregation of livestock in the region. The high rates of gastrointestinal infection in livestock in the present study cannot be ignored because of its possible impacts on livestock productivity as well as the potential risk to human health through food consumption and environmental contamination. There is a need for improvements in the animal management and treatment of infected animals in the study area.

DECLARATIONS

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Availability of data and materials

This manuscript includes all the data generated or analyzed during the study.

Authors' contributions

Ajakaye Oluwaremilekun Grace conceptualized the study and conducted fieldwork, laboratory experiments, and data analysis. Adejuyigbe Aderotimi carried out the fieldwork and laboratory experiments. Ajakaye Oluwaremilekun Grace drafted the manuscript, and both authors read and approved the final manuscript.

Competing interests

The authors declare that they have no conflicts of interest.

Ethical considerations

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

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Effects of Hygiene on Milk Quality and Milking Practices of Small Andean Herds during the Rainy Season

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ABSTRACT

Dairy production in the Peruvian Andes is mainly based on small herds. However, there is little information on how hygiene affects milk quality during the rainy season. The study's objective was to evaluate the hygienic factors of milk and milking practices during the rainy season in small herds located at two high Andean altitudinal levels. The characteristics of the factors related to the milking process were recorded using an observation guide, and 108 raw milk samples were collected from 18 herds with Brown Swiss cows at two altitude levels. Samples were obtained from the milk collection containers and milk. The results showed that 56.5% of the samples analyzed were unhygienic, with no significant differences in bacterial counts between altitudinal levels. However, a strong correlation was identified between precipitation and the presence of coliforms (0.726) and mesophilic aerobes (0.861). Factors such as milking location, hand washing, and use of cleaning agents were associated with microbial contamination, acquiring odds ratios (OR) of 4.04, 5.26, and 4.71, respectively, during the months of heavy rain. The study concludes that the hygienic quality of milk in small high Andean herds significantly deteriorates during the rainy season, with counts of total coliform bacteria and mesophilic aerobes exceeding recommended levels, particularly during peak rainfalls. This finding highlights a direct relationship between the intensity of rainfall and the quality of milk, underscoring the need for improved milking practices in the rainy season to ensure the safety of the products.

Keywords: Andean ecosystem, Milk quality, Rainy season, Small farmer

INTRODUCTION

The hygienic quality of milk is a fundamental aspect of production, as milk is an essential component of the population's daily diet. Ensuring that milk is safe is crucial for meeting basic nutritional needs and ensuring the consumers' overall well-being. Milk provides essential nutrients such as proteins, calcium, and vitamins, which are vital for development and health at all stages of life. However, its susceptibility to microbial contamination can compromise its quality and safety (Boor et al., 2017). It is essential to understand that milk, as a biological product, can be a vehicle for pathogens if not handled under strict hygienic standards (Owusu-Kwarteng et al., 2020).

Internationally, various studies have identified the main factors influencing the hygienic quality of raw milk. Research in Latin America, Africa, and Asia has shown that milking hygiene, udder cleanliness, and milker hygiene are critical factors in reducing contamination by total coliform bacteria and mesophilic aerobic bacteria (Múnera-Bedoya et al., 2017; Bereda et al., 2018; Kazeminia et al., 2023). Additionally, the presence of zoonotic pathogens such as *Escherichia coli* and *Salmonella* in raw milk has been correlated with inadequate milking practices, highlighting the importance of improving sanitary conditions in dairy farms (Geletu et al., 2022). However, in countries like Canada, small farms employ good milking practices, conducting monthly sampling to detect pathogenic microorganisms and indicator bacteria to ensure milk safety and quality (Berge and Baars, 2020). In contrast, in developing countries, noncompliance with quality standards often forces dairy producers into the unofficial market (Candiotto et al., 2020).

However, some studies on milk quality in intensive and semi-extensive production systems have been conducted, such as those by Fuentes et al. (2014) and Chagray et al. (2023). Notwithstanding, small dairy producers in high Andean areas have received less scientific attention. This lack of focus is concerning, as adverse weather conditions—such as wind, rain, and fog—affect the hygienic conditions of animals, milkers, and the milking process, thereby increasing the

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chances of contamination. Prolonged rainfall may promote the proliferation of microorganisms in corrals and during milking, increasing the risk of cross-contamination between the environment, animals, and the final product (Candiotto et al., 2020).

The scientific gap lies in the lack of research that directly links milking practices and the hygienic quality of milk in small dairy herds, especially in the Peruvian highlands. This study assesses the hygienic quality of milk by measuring the total coliform bacteria (TCB) and mesophilic aerobic bacteria (MBB) through the Compact Dry methods (Nissui Pharmaceutical, 2019), alongside an examination of the factors affecting manual milking during the rainy season in small dairy herds located in the high Andean region of central-southern Peru. Therefore, the study aimed to evaluate the hygienic quality of milk and milking practices during the rainy season in small herds located at two high Andean altitudinal levels.

MATERIALS AND METHODS

Ethical approval

The study received approval from the Ethics Committee of the National University of San Cristóbal de Huamanga (Peru) with Letter No. 01-CE-VRI-UNSCH-2024. The research was carried out taking into consideration the Code of Ethics of the Peruvian Veterinary Medical College (s.f.), on the promotion and protection of animal health and well-being, public health, and environmental conservation (Art. 10) and on conducting research with living animals, trying to avoid physical suffering and stress (Art. 85).

Study area

The study was conducted in the Ayacucho region of Peru, where two ecological levels were identified based on altitude (Aybar and Lavado, 2017), the upper montane level (UMZ), between 3800 and 3920 meters above sea level (masl) in the Socos district, and the lower montane level (LMZ), ranging from 3350 to 3500 masl in the Chiara district. In this area, 58 small dairy herds of Brown Swiss cows were identified, with 27 herds in the upper part and 31 in the lower part. From these, 9 herds were selected from each level. The inclusion criteria were accessibility and having at least 4 cows in production (Figure 1). The fieldwork was conducted between October 2023 and March 2024, corresponding with the start and end of the rainy season (Table 1).

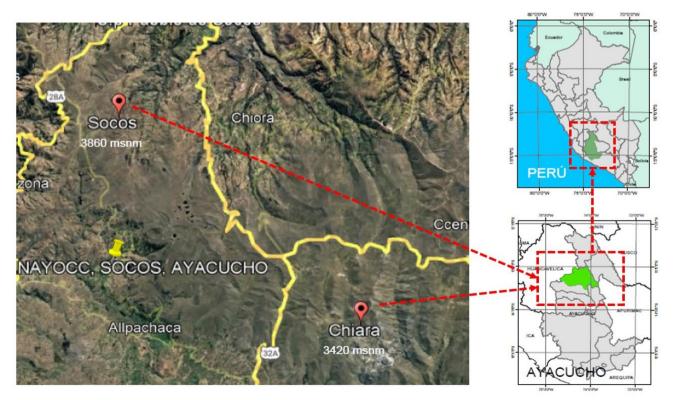


Figure 1. The location of the study in Peru, Ayacucho region, Socos, and Chiara districts

Table 1. Rainfall during the study period in Huamanga, Ayacucho, Peru

Weather conditions		2023			2024			
(average)	October	November	December	January	February	March		
Precipitation (mm)	70	89	126	140	130	94		
Temperature (°C)	15	15	15	15	14	14		
Humidity (%)	78	81	86	88	89	86		

Sources: Weather-Atlas (2023-2024) and Senamhi (2023).

Observation of milking factors

The characteristics of the manual milking process in the absence of infrastructure were recorded using an observation guide. The guide converged on the sex of the milkers, milking location, hand washing, use of a mask, udder cleaning, and cleaning of utensils. The use of cleaning agents such as soap for hand washing and udder cleaning, as well as detergents for cleaning utensils, was also observed.

Sampling

Milk samples were collected from the collection containers, and the product of the day's milking was stored in the herd between 8:00 and 9:00 a.m. Approximately 500 ml of milk was obtained for each sample using sterile polypropylene containers (Pantoja et al., 2011). Subsequently, the samples were refrigerated at 4°C and transported to the laboratory within 6 hours of collection (Kiambi et al., 2022).

Laboratory analysis

The analyses were performed at the Microbiology Laboratory of the National University of San Cristóbal de Huamanga (Ayacucho, Peru). The samples were analyzed to determine the presence of total coliform bacteria (TCB) and aerobic mesophilic bacteria (AMB), using the rapid colony count method with Compact DryTM plates (Nissui Fharmaceutical, 2019). The procedure included the preparation of a 0.1% peptone broth, the subsequent preparation of serial dilutions (10⁻³ dilutions for total coliforms and 10⁻⁴ for mesophilic aerobes), followed by the inoculation of the milk samples on Compact Dry EC plates for total coliforms and on TC plates for mesophilic aerobes (2 replicates per sample), and finally the incubation of Compact Dry EC plates at 37°C for 24 hours and TC plates at 37°C for 48 hours. The results were expressed in CFU/ml according to the manufacturer's recommendations (Nissui Pharmaceutical, 2019) and evaluated according to NTP.202.001 (Peruvian Technical Standard, 2016).

Statistical analysis

Data were tabulated and statistically analyzed to calculate measures of central tendency and dispersion for total coliform bacteria (TCB) and aerobic mesophilic bacteria (AMB) counts. Spearman correlation analysis was used to assess relationships while Analysis of Variance (ANOVA) and Tukey's significance test (p < 0.05) were used to identify statistical differences. In addition, logistic regression was applied to determine odds ratios. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 24.

RESULTS AND DISCUSSION

Total coliform bacteria

Total coliform bacteria count (CFU/ml) varied significantly, reaching their highest value in January, both in the lower montane and upper montane altitudinal levels (p < 0.05). In both altitudinal levels, the variation in the bacterial counts followed a similar trajectory; at the beginning of the study in October, the total coliform counts were low, with a progressive increase until January, followed by a gradual decrease until March. Statistical comparison between the two altitudinal levels showed no significant differences in coliform counts, indicating a consistent pattern in both zones (p > 0.05). A high percentage of raw milk samples in both herds, located in the lower montane and the upper montane altitudinal levels, showed contamination with total coliform bacteria. According to the Peruvian technical standard, on average, only 43.5 % of the samples met the safety standards showing no contamination (PTS, 2016).

A correlation analysis was conducted to assess the association between variations in total coliform bacteria counts (Table 2) and the intensity of monthly rainfall (Table 1), which indicated a significant relationship (r = 0.726, p < 0.05). The analysis showed a strong and statistically significant positive correlation between rainfall and total coliform bacteria counts (p < 0.05). The findings indicate that increased humidity levels resulting from rainfall may promote the growth of

coliform bacteria within livestock populations, highlighting the critical need for effective sanitary management during the rainy season. Additionally, the data reveal a correlation between the total coliform bacteria counts and the intensity of rainfall. The results are consistent with earlier studies that demonstrate seasonal fluctuations in coliform levels in hand-milked herds, showing elevated counts during colder and wetter seasons (De Garnica et al., 2013). Other studies have noted positive correlations between temperature, humidity, and somatic cell counts (Bertocchi et al., 2014) as well as seasonal patterns, in which total coliform bacteria counts are typically lowest in winter (Bokharaeian et al., 2023).

Total coliform bacteria, once released from cow feces, tend to disseminate within the environment, thereby establishing a transmission cycle (Perdomo and Calle, 2024). These bacteria not only compromise the hygienic quality of milk but also pose a significant risk to public health (Rahman et al., 2020). The lack of variation in total coliform bacteria counts across different altitudinal levels indicates uniform conditions regarding bacterial load. Furthermore, the identified positive correlation between rainfall and coliform counts is consistent with the observations made by Bokharaeian et al. (2023), who also reported a relationship between humidity levels and microbial load that can adversely affect milk quality.

Table 2. The total coliform bacteria (CFU/ml) count, according to altitudinal level and months of the year

Months	Lower montane altitudinal level	Upper montane altitudinal level	Overall
Withins	Mean	Mean	Overan
October	1.83×10 ³ b	1.11×10^3 b	1.47×10^3
November	1.67×10^3 ab	3.11×10^3 b	2.39×10^{3}
December	3.28×10^3 ab	9.17×10^3 ab	6.22×10^3
January	3.07×10^4 a	4.24×10^4 a	3.66×10^4
February	8.51×10^3 ab	1.16×10^4 ab	1.01×10^4
March	5.06×10^3 ab	2.22×10^3 b	3.64×10^{3}
p-value ^f	0.008	0.001	
p-value ^g	0.	198	
The percentage of unhygienic samples that do not follow PTS	55.5 %	57.5 %	56.5 %

Relationship between TCB and rainfall: r = 0.726; confidence interval = 0.616 - 0.999; Bilateral sig. = 0.042

Aerobic mesophilic bacteria

The measurements of mesophilic aerobic bacteria (CFU/ml) displayed a trend consistent with that of total coliform bacteria, showing considerable monthly variations and attaining a maximum in January at both lower and upper montane altitudinal levels (p < 0.05). At both altitudinal levels, the variation in counts followed a comparable trajectory, in which mesophilic aerobic bacteria counts were initially low at the beginning of the study in October, progressively increased until January, and then gradually declined until March. A statistical comparison between the altitudinal levels indicated no significant differences, suggesting that the bacterial counts were similar across both levels (p > 0.05). A large proportion of raw milk samples in both regions showed contamination by mesophilic aerobic bacteria, with 56.3% meeting safety standards as indicated by Peruvian technical standards (PTS, 2016).

A correlation analysis was performed to determine if the variation in mesophilic aerobic bacteria counts (Table 3) is related to the intensity of monthly rainfall (Table 1), which indicated a strong and significant relationship (r = 0.861, p < 0.05). The results indicate a strong and statistically significant positive correlation between rainfall and mesophilic aerobic bacteria counts. The findings indicate that increased humidity levels resulting from rainfall may promote the growth of mesophilic aerobic bacteria in livestock populations, which is significant for health management practices during the rainy season. The observed aerobic mesophilic bacteria count (CFU/ml) aligns with rainfall intensity. Other studies report that temperature and humidity are positively associated with somatic cell count (Bertocchi et al., 2014), that mesophilic colonies were lower in winter and higher in summer (Petróczki et al., 2020), and that changes in seasons, months, and temperature and humidity indices affect both milk production and quality (Bokharaeian et al., 2023).

The techniques used during milking are linked to aerobic mesophilic bacteria counts (Ngolombe et al., 2024), with lower counts reported in colder seasons (Kazeminia et al., 2023); however, poor farm hygiene remains a significant factor compromising milk quality and safety (Nyokabi et al., 2021). The similarity in CFU/ml counts across altitudinal levels suggests the existence of comparable conditions for bacterial load across both regions. In addition, the positive correlation between rainfall and aerobic mesophilic bacteria counts corroborates findings by Bokharaeian et al. (2023),

a,b Different superscript letters in the same column differ significantly at 95%. PTS: Peruvian technical standard (PTS: 202.001). TCB: Total coliform bacteria, Sig: Significant; ^f Comparison of the total coliform bacteria in each altitudinal floor, ^g Comparison of the total coliform bacteria between the altitudinal floors

which also pointed to a close relationship between humidity indices and microbial load. Improved milking practices can play a vital role in enhancing milk's hygienic quality (Lemma et al., 2018).

Table 3. The aerobic mesophilic bacteria (CFU/ml) count, according to altitudinal level and months of the year

Months	Lower montane altitudinal level	Upper montane altitudinal level	Overall
Withins	Mean	Mean	Overall
October	4.96×10^5 °	5.98×10^5 °	5.47×10^5
November	6.04×10^5 bc	7.73×10^5 bc	6.89×10^{5}
December	1.65×10^6 ab	1.20×10^6 ab	1.42×10^6
January	3.05×10^6 a	1.92×10^6 a	2.48×10^{6}
February	1.09×10^6 b	8.71×10^5 bc	1.22×10^6
March	1.38×10^6 bc	1.07×10^6 bc	9.79×10^{5}
p-value ^f	0.001	0.001	
p-value ^g	0.9	982	
The percentage of unhygienic samples that do not follow PTS	43.1 %	44.3 %	43.7 %

Relationship between MAB and rainfall: r = 0.861; confidence interval = 0.731 - 0.999; Bilateral sig. = 0.028

Table 4. Different factors associated with milking, in months with low and high precipitation, according to altitudinal levels

		In months of less rainfall			In months of greater rainfall					
Factor	Variable	LMF UN		MF	LMF		UMF		p-value	
	-	n	(%)	n	(%)	n	(%)	n	(%)	=
Gender	Woman	24	88.9	23	85.2	24	88.9	22	81.5	0.698
Gender	Man	3	11.1	4	14.8	3	11.1	5	18.5	
Milking place	Clean	18	66.7	10	37.0	19	70.4	10	37.0	0.941
Wilking place	No clean	9	33.3	17	63.0	8	29.6	17	63.0	
Handwashina	Yes	21	77.8	15	55.6	25	92.6	19	70.4	0.445
Handwashing	No	6	22.2	12	44.4	2	7.4	8	29.6	
Hand washing	Water	24	88.9	21	77.8	24	88.9	17	63.0	0.652
cleaning agent	Water and soap	3	11.1	6	22.2	3	11.1	10	37.0	
Use of mask	Yes	16	59.3	13	48.1	17	63.0	10	40.7	0.895
Use of mask	No	11	40.7	14	51.9	10	37.0	16	59.3	
Classina uddana	Yes	17	63.0	15	55.6	18	66.7	12	44.4	0.782
Cleaning udders	No	10	37.0	12	44.4	9	33.3	15	55.6	
Udder cleaning	Water	21	77.8	20	74.1	23	85.2	18	66.7	0.938
agent	Water and soap	6	22.2	7	25.9	4	14.8	9	33.3	
Cl. :	Yes	18	66.7	17	63.0	19	70.4	13	48.1	0.843
Cleaning utensils	No	9	33.3	10	37.0	8	29.6	14	51.9	
Cleaning agent	Water	24	88.9	23	85.2	21	77.8	14	51.9	0.420
for utensils	Water and detergent	3	11.1	4	14.8	6	22.2	13	48.1	

^{*} LMF: Lower montane altitudinal floor; UMF: Upper montane altitudinal floor. N: Number

Factors associated with milking

All the factors associated with milking observed in herds on both the lower and upper montane altitudinal floors as well as in the months with the lowest and highest rainfall (Table 4) showed similar characteristics (p > 0.05). Logistic regression analysis revealed specific risk factors that may serve as indicators for milk contamination, as evidenced by their elevated odds ratio (OR) values. In the months characterized by minimal rainfall (October-November), the factors of milking location, hand washing practices, and the application of cleaning agents for hand washing exhibited ORs of

abc Different superscript letters in the same column differ significantly at 95%. PTS: Peruvian technical standard (PTS: 202.001). MAB: mesophilic aerobic bacteria, Sig: Significant; ^f comparison of the mesophilic aerobic bacteria in each altitudinal floor, ^g comparison of the mesophilic aerobic bacteria between the altitudinal floors

3.40, 2.80, and 2.29, respectively; however, these values did not achieve statistical significance (p > 0.05). Conversely, during the months with the highest levels of rainfall (December-January), these factors demonstrated a notable increase in their OR values, reaching 4.04, 5.26, and 4.71 (p < 0.05), indicating an elevated risk. Furthermore, the utilization of cleaning agents for milking utensils emerged as an additional concern during these months, with an OR of 3.25 (p < 0.05), highlighting an increased risk associated with higher rainfall conditions (Table 5).

The increase in ORs for several milking-associated factors during high-rainfall months suggests a direct impact of rainfall on suitable conditions for milking during the rainy season. This finding is consistent with the results of studies by Paraffin et al. (2018), Alaru et al. (2022), and Deddefo et al. (2023), which have documented how high rainfall increases microbiological contamination due to the difficulty of maintaining hygienic practices in humid and unhygienic environments. Likewise, studies by Nyokabi et al. (2021) and Xulu and Naidoo (2023) highlight the need for adequate cleaning and disinfection of utensils as a fundamental practice to ensure the microbiological safety of milk.

Table 5. Logistic regression of different factors associated with milking in cows

Factor	Ir	months of less rain	nfall	In months of greater rainfall			
ractor	OR	CI	p-value	OR	CI	p-value	
Gender	1.04	0.19 - 5.71	0.687	1.45	0.29 - 7.24	0.478	
Milking place	3.40	1.11 - 10.40	0.280	4.04	1.30 - 12.59	0.014	
Handwashing	2.80	0.86 - 9.14	0.074	5.26	1.00 - 27.69	0.038	
Hand washing cleaning agent	2.29	0.51 - 10.29	0.234	4.71	1.12 - 19.70	0.027	
Use of mask	1.57	0.53 - 4.60	0.290	2.47	0.83 - 7.39	0.086	
Cleaning udders	1.36	0.46 - 4.04	0.391	2.50	0.83 - 7.53	0.085	
Udder cleaning agent	1.23	0.35 - 4.28	0.500	2.88	0.76 - 10.87	0.101	
Cleaning utensils	1.18	0.39 - 3.60	0.500	2.56	0.84 - 7.83	0.083	
Cleaning agent for utensils	1.39	0.28 - 6.91	0.500	3.25	1.00 - 10.58	0.043	

OR: Odds ratio; CI: confidence interval.

CONCLUSION

The rainy season significantly affects the hygienic quality of milk produced by small herds in the high Andes. During this period, total coliform and mesophilic aerobic counts surpass the recommended limits, especially in December and January, which coincide with the highest levels of rainfall. This observation highlights the direct correlation between the intensity of rainfall and the quality of milk. Three key factors related to the milking process were identified as impacting the hygienic quality of the milk including the location of milking, the practices of handwashing, and the application of cleaning agents for both handwashing and utensils. Addressing these elements is essential for maintaining adequate hygienic standards, indicating the priority attention they require. The findings highlight the urgency of implementing programs to improve hygiene practices during the rainy season. Such programs include focusing on training producers, providing adequate cleaning supplies, and improving milking infrastructure.

DECLARATIONS

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Availability of data and materials

Data of the present study is available upon reasonable request.

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Authors' contributions

Gloria Adrianzen, Julio Ruiz, and Nelly Umppiri planned the experiment and performed the fieldwork. Francisco Espinoza interpreted and analyzed the data to draft the manuscripts. All authors reviewed and approved the final edition of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

Before submitting their study to the journal, all authors thoroughly checked and confirmed ethical concerns regarding the originality of the data collected and analyzed.

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Antibacterial Effect of *Allium sativum* L. and *Allium cepa* L. Extracts against Multidrug-Resistant *Escherichia coli* Strains Isolated from Broiler Chickens

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ABSTRACT

Over the past decades, the incidence of avian colibacillosis caused by multidrug-resistant *Escherichia coli* (*E. coli*) has increased dramatically worldwide. The present *in vitro* study focused on evaluating the antibacterial properties of *Allium sativum* L. and *Allium cepa* L. extracts against multidrug-resistant *E. coli* strains isolated from broiler chickens suffering from colibacillosis. The confirmation of *E. coli* isolates and their antibiotic resistance was performed using conventional methods. Furthermore, the antimicrobial activity of both extracts was assessed through the disk diffusion method, along with the determination of the minimum inhibitory concentration (MIC) via liquid macrodilution and the minimum bactericidal concentration (MBC) using solid media. The obtained results showed that the multidrug-resistant *E. coli* strains were extremely sensitive to garlic extract with a MIC of 41.5 mg/mL and CMB of 166 mg/mL and very sensitive to the combination of garlic and onion extracts. However, onion extract was ineffective against the resistant *E. coli* strains. The findings of the present study suggested the possibility of using garlic as an alternative to antibiotics in the treatment of colibacillosis caused by resistant *E. coli* strains.

Keywords: Allium cepa L., Allium sativum L., Broiler chicken, Colibacillosis, Escherichia coli, Multidrugresistance

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INTRODUCTION

Avian colibacillosis is an infectious disease caused by the bacterium *Escherichia coli (E. coli)*. This prevalent disease is a primary concern in the poultry industry worldwide, frequently leading to condemnation at slaughterhouses (Nolan et al., 2013; Naoufal et al., 2017). The disease significantly affects the health and welfare of poultry populations, presenting in various clinical forms ranging from acute septicemia to chronic respiratory and genital problems (Joseph et al., 2023). This bacterial infection causes substantial economic losses due to reduced productivity, higher mortality rates, and the need for extensive therapeutic treatments. *E. coli* is commonly found in the avian digestive system as a commensal organism, with most strains displaying non-pathogenic characteristics. However, certain strains, identified as Avian Pathogenic *E. coli* and belonging to specific serotypes, particularly O1, O2, and O78, are associated with the manifestation of the disease (Apostolakos et al., 2021; Joseph et al., 2023).

The management of this disease relies mainly on antibiotics. Although this approach has helped in combating harmful bacteria, it has also led to the development of antibiotic-resistant strains (Merati et al., 2020; Abdel-Rahman et al., 2023). The rise of antibiotic-resistant strains of *E. coli* makes treatment more challenging and raises concerns about the ongoing effectiveness of antimicrobial measures in poultry farming. Excessive antibiotic use has resulted in the development of multi-drug-resistant strains, highlighting the necessity of alternative methods to mitigate the effects of colibacillosis on both poultry health and industry viability (Bhattarai et al., 2024).

The emergence of antibiotic resistance poses a significant challenge in humans and veterinary medicine, often leading to treatment failures. Health authorities are particularly concerned about the rise of multi-resistant bacteria in community infections. Consequently, the scientific community has been actively researching alternative strategies to replace antibiotics and effectively manage colibacillosis in broiler farms (Koutsianos et al., 2021; Eid et al., 2022; Song et al., 2023).

Medicinal plants currently represent an interesting alternative to antibiotics; some plant extracts exhibit potent antimicrobial, fungicidal, and antiviral effects (Bhardwaj et al., 2016; Reiter et al., 2020; Abdallah et al., 2023). Allium sativum L., commonly known as garlic, and Allium cepa L., commonly known as onion, are bulbous herbaceous plants of the Alliaceae family. They are known to have a wide range of pharmacological properties, including antimicrobial, anticancer, and antioxidant effects. These plants have been utilized for centuries to treat various health conditions (Krstin

et al., 2018; Raj et al., 2021; Oyawoye et al., 2022). Recent studies have highlighted the significant potential of garlic and onion as antimicrobial agents, emphasizing the importance of further research into their effectiveness against various multidrug-resistant clinical bacteria. The present study was conducted to evaluate the antibacterial activity of *Allium sativum* L. and *Allium cepa* L. extracts against multidrug-resistant *Escherichia coli* strains isolated from broiler chickens affected by colibacillosis.

MATERIALS AND METHODS

Ethical approval

This research was conducted *in vitro* by the guidelines of the Veterinary Sciences Institute, University of Tiaret, Algeria.

Plant material

The plants used in this study were purchased in October 2022 from a local market in Tiaret Province, Algeria. The white garlic variety was chosen for *Allium sativum* L., and the red onion variety was selected for *Allium cepa* L. The fresh plants were placed in bags and transported to the Laboratory of Hygiene and Animal Pathology, Tiaret, Algeria, within 24 hours.

Bacterial isolates

The antibacterial assays were carried out on five clinical multidrug-resistant *E. coli* strains, isolated by Merati et al. (2020) from the liver and spleen of broiler chickens exhibiting gross lesions suspected to be colibacillosis. The bacterial isolates were stored at -20°C in the Laboratory of Hygiene and Animal Pathology, Tiaret, Algeria.

Preparation of crude garlic (Allium sativum L.) and onion (Allium cepa L.) extracts

Fresh garlic and onion bulbs were washed and peeled, and then 50 g of each plant was weighed before being crushed in a sterile mortar and pestle under aseptic conditions. The resulting homogenized mixtures were centrifuged at 3000 rpm for 15 minutes, and the supernatants were filtered through Whatman 125 mm filter paper. The obtained extracts were designated as 100% concentration. The concentration was determined based on the total weight of the plant material per milliliter of extract. From 50 g of raw garlic, 12 ml of extract was obtained, corresponding to a concentration of 4.16 g/ml. Similarly, 50 g of raw onion produced 15 ml of extract, with a concentration of 3.33 g/ml. Before use, the extracts were inoculated onto nutrient agar (Biokar, France) and incubated at 37°C for 24 hours to ensure sterility (Yadav et al., 2015).

Revival of Escherichia coli isolates

The conserved bacterial isolates were inoculated into a tube containing peptone broth (Biokar, France) and incubated aerobically at 37°C for 24 hours to check their viability. These suspensions were then plated onto eosin methylene bleu (EMB) agar (Biokar, France) and incubated aerobically at 37°C for 24 hours. Colonies with a greenish metallic sheen were subcultured and plated onto EMB agar for purification. Pure colonies were stored at 4°C for confirmation of the bacterial isolates (Quinn et al., 2002).

Confirmation of Escherichia coli isolates

After purification of the colonies on EMB agar, they were confirmed as *E. coli* by various tests, including Gram staining, catalase test, oxidase test, and analysis of biochemical characteristics using Analytic Profile Index (API) 20 E test strips (BioMerieux, France; Quinn et al., 2002).

Susceptibility to antibiotics of Escherichia coli isolates

The antibiotic sensitivity test was performed using the standard disk diffusion method on solid media according to the recommendations of CASFM/EUCAST (2020). Mueller Hinton agar (MH; Biokar, France) and a range of antibiotics from different classes, in the form of discs impregnated with each molecule, were used. The present study included the following antibiotics Amoxicillin/Clavulanic acid (AMC: 20 µg; CYPRESS DIAGNOSTICS, Belgium); Tetracycline (TE: 30 µg; CYPRESS DIAGNOSTICS, Belgium); Nalidixic acid (NA: 30 µg; Liofilchem, Italy); Erythromycin (E: 15 µg; Liofilchem, Italy); and Ampicillin (AM: 10 µg; Bio-analyse, Turkey).

Assessment of the antibacterial activity of garlic and onion extracts The agar diffusion technique

From a pure bacterial culture, four to five colonies were collected using a wire loop and emulsified in 5 mL of physiological saline. The resulting suspension was then adjusted to achieve a turbidity equivalent to 0.5 McFarland units (10^8 CFU/mL) . The culture was streaked on MH agar plates using a sterile cotton swab and allowed to dry for about 5 minutes. On the surface of each plate, sterile Whatman paper discs, 6 mm in diameter, containing 10 μ l of garlic extract, 10 μ l of onion extract, and 10 μ l of a 50/50 mixture of the two plant extracts were applied. During the process, two controls were performed: A negative control with a disc containing 10 μ L of sterile distilled water, and a positive control with an antibiotic disc containing Amikacin (AMK, 30 μ g; CYPRESS DIAGNOSTICS, Belgium). The plates were left

for one hour at room temperature for proper diffusion and then incubated in an upright position at 37°C for 24 hours. The test was performed in triplicate under the same experimental conditions, and mean of the diameter of inhibition zones was calculated (CASFM/EUCAST, 2020).

Determination of minimal inhibitory concentration

The determination of minimal inhibitory concentration was carried out by adding 400 µL of the crude plant extracts to be tested into a sterile tube containing 4.6 mL of MH Broth (MHB) medium (Biokar, France). A serial dilution was conducted in MHB medium to achieve concentrations of garlic ranging from 332 mg/mL to 1.29 mg/mL and concentrations of onion ranging from 266 mg/mL to 1.03 mg/mL. To each tube, 13 µL of bacterial inoculum, corresponding to a density equivalent to the 0.5 McFarland standard, was added. A bacterial growth control, in which 13 µL of standardized inoculum was added to the MHB medium, was also performed. After 48 hours of incubation at 37°C, the tubes were centrifuged at 5000 rpm for 5 minutes, and the minimal inhibitory concentration (MIC) was determined from the first tube in the series in which no bacterial growth was observed (Forbes et al., 1998; NCCLS, 2003).

Determination of minimal bactericidal concentration

The same concentration range utilized in the liquid macrodilution technique was applied to determine the minimal bactericidal concentration (MBC) of the plant extracts. A loopful of culture was taken from the control tube and from each tube where no growth was observed after 48 hours of incubation at 37 °C, and it was inoculated onto the surface of MH agar. The plates were then incubated for 24 hours at 37 °C, and the MBC was identified as the plate showing no colony formation (Forbes et al., 1998; NCCLS, 2003).

Statistical analysis

Descriptive statistics were performed to compare results. The data were analyzed using Microsoft Excel 2016 (USA). The data were expressed as mean \pm standard deviation.

RESULTS

Revival and confirmation of Escherichia coli isolates

The confirmation of the *E coli* isolates was determined based on morphological and biochemical characteristics. The isolates produced typical deep purple colonies with a greenish metallic sheen. Biochemical characterization using the API 20 E system revealed that all isolates tested positive for o-nitrophenyl-β-D-galactopyranoside, lysine decarboxylase, ornithine decarboxylase, indole, Voges-Proskauer, mannose, glucose, sorbitol, rhamnose, melibiose, and arabinose. Conversely, the isolates tested negative for arginine dihydrolase, citrate, hydrogen sulfide H₂S production, urease, tryptophan deaminase, gelatinase, inositol, saccharose, and amygdalin.

Antibiotic susceptibility of Escherichia coli isolates

This test aimed to evaluate the antibiotic resistance of the *E. coli* isolates. The results of the antibiotic susceptibility test are shown in Table 1. According to the results of the antibiogram presented in Table 1, a complete absence of inhibition zones was observed around the five antibiotic discs tested involving amoxicillin + clavulanic acid, ampicillin, erythromycin, nalidixic acid, and tetracycline. These findings confirmed the resistance of the tested *E. coli* isolates in the present study.

Antibacterial activity of garlic and onion extracts

The antibacterial activity of the studied extracts was evaluated using the disc diffusion method on an MH agar medium (Figure 1). It was a qualitative technique based on determining the diameter of inhibition zones around discs loaded with the tested extracts. Table 2 presents the diameters of inhibition zones for garlic extracts, onion extracts, and their combination. According to the results presented in Table 2, the resistant strains of E. coli tested were sensitive to both garlic extract and the combination of garlic and onion extracts, with inhibition zones of 20.5 mm \pm 1.29 and 16 mm \pm 0.57, respectively. However, there was a complete absence of an inhibition zone for the onion extract, indicating that there was no antibacterial activity against the tested E. coli strains. The positive control confirmed that the bacteria were susceptible to amikacin, with an inhibition zone of 24.33 mm \pm 0.47. The MIC was a quantitative technique that involved exposing a microorganism to progressively diluted concentrations of the test substance to determine its inhibitory effect. In the present study, only garlic exhibited antibacterial effects against the multidrug-resistant E. coli isolates tested, therefore, the MIC and MBC of the crude extract of $Allium\ sativum\ L$. were determined (Figure 2), and the results indicated that the MIC was 41.5 mg/mL, while the MBC was 166 mg/mL.

Table 1. Antibiotic sensitivity of isolated Escherichia coli from broiler chickens affected by colibacillosis

Antibiotics	Concentration of the disc	Inhibition zone	Results*
Amoxicillin + clavulanic acid (AMC)	20 μg	Absence	R
Ampicillin (AM)	10 μg	Absence	R
Erythromycin (E)	15 μg	Absence	R
Nalidixic acid (N)	30 μg	Absence	R
Tétracycline (TE)	30 μg	Absence	R

R: Resistant; * Recommendations of CASFM/EUCAST (2020).

Table 2. Antibacterial activity of the *Allium sativum L*. and *Allium cepa L*. extracts against isolated *Escherichia coli* from broiler chickens affected by colibacillosis

Tested extracts/antibiotic	Inhibition diameter (mm)	Sensitivity
Allium sativum	20.5 ± 1.29	S
Allium cepa	Absent	R
Allium sativum + Allium cepa	16.5 ± 0.57	S
Amikacin (AMK: 30 μg)	$24,33 \pm 0.47$	S

R: Resistant; S: Sensitive; *Recommendations of CASFM/EUCAST (2020).

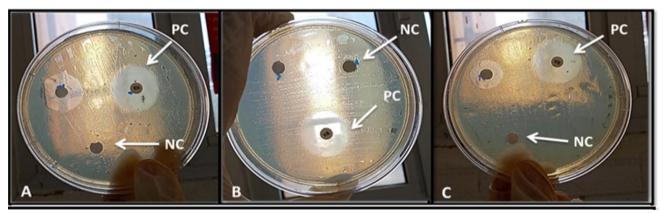


Figure 1. Antibacterial activity (inhibition zone) of *Allium sativum L.* and *Allium cepa L.* extracts against isolated *Escherichia coli* from broiler chickens affected by colibacillosis. **A**: *Allium sativum*; **B**: *Allium cepa*; **C**: *Allium sativum* + *Allium cepa*; PC: Positive control; NC: Negative control.

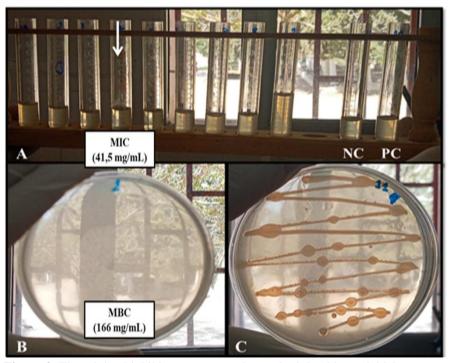


Figure 2. The minimal inhibitory concentration and minimal bactericidal concentration of *Allium sativum* extract against *Escherichia coli* isolated from broiler chickens affected by colibacillosis. **A**: MIC of *Allium sativum*; **B**: MBC of *Allium sativum*; **C**: Positive control for MBC.

DISCUSSION

The potential of *Allium sativum* L. and *Allium cepa* L. in combating multidrug-resistant bacteria has attracted considerable interest as an alternative therapeutic option. Previous studies have investigated the antibacterial effects of garlic and onion, particularly their efficacy against bacteria resistant to multiple drugs (Bhardwaj et al., 2016; Reiter et al., 2020). The current study aimed to assess the antibacterial properties of garlic and onion extracts against strains of *E. coli* that were resistant, and isolated from broiler chickens suffering from colibacillosis.

The obtained results revealed that $E.\ coli$ resistant strains were extremely sensitive to the whole Allium sativum L. extract with an inhibition zone of 20.5 ± 1.29 mm, MIC of 41.5 mg/mL, and MBC of 166 mg/mL. These findings align partially with those of Magryś et al. (2021), who evaluated the antimicrobial activity of Allium sativum extract against various antibiotic-resistant bacterial strains. Their study demonstrated the extract's strong efficacy, particularly against multidrug-resistant $E.\ coli$ and methicillin-resistant $E.\ coli$ and methicillin-resistant $E.\ coli$ by Farrag et al. (2019), and reported that the MIC value was found to be 16 mg/ml. However, Noman et al. (2023) evaluated the antibacterial potential of garlic against different resistant bacteria isolated from chickens in Bangladesh and reported an inhibition zone of 14.03 ± 0.15 mm and a MIC of 0.625 mg/mL against $E.\ coli$. These variations could be due to the plant used or the bacterial strains used. Conditions related to the plant include genetic diversity, concentration of active compounds, climatic conditions, and farming techniques. Conditions related to bacterial strains include genetic background diversity, variations in drug resistance, and various methodologies used for isolation (Noman et al., 2023).

The positive antibacterial effect of garlic extracts on multidrug-resistant *E. coli* observed in the present study suggested that the antibiotic resistance mechanisms of *E. coli* do not influence its susceptibility to garlic. This was particularly attributed to its abundance of organosulfur compounds. The main organosulfur antibacterial compounds found in garlic were allicin, ajoenes, and various aliphatic sulfides (Bhatwalkar et al., 2021). Allicin was widely recognized as the most potent antibacterial agent found in crushed garlic extracts. Previous research has indicated that allicin effectively inhibits a wide range of infectious agents, including those that have developed resistance to conventional antibiotics (Bayan et al., 2014; Bhardwaj et al., 2016; Reiter et al., 2020). It offers a significant advantage over most antibiotics by not being limited to a specific protein within the bacterial cell. Consequently, the probability of resistance originating from alterations to the target site within the bacterial cell was reduced (Magryś et al., 2021).

According to the current study, a complete absence of the inhibition zone for *Allium cepa* L. extract was noticed, indicating a lack of antibacterial activity against the tested resistant *E. coli* strains. These findings align with those reported by Omotola et al. (2018), who tested onion aqueous extract against several pathogenic bacteria and found a total absence of antibacterial activity against *E. coli*, even at high concentrations. Furthermore, the same authors concluded that aqueous garlic extracts exhibited greater antimicrobial potential compared with onion extract. These researchers suggested that the reduced antibacterial activity of the onion extract could be attributed to the extraction method employed for the plant. This explains the results of the present study following the use of fresh onion juice.

Several studies have reported strong antibacterial activities of onion against numerous pathogenic bacteria using organic solvents for extraction. Bakht et al. (2013) reported that onion extracts in petroleum ether, ethyl acetate, and chloroform inhibited the growth of certain bacteria at both low and high concentrations. Eltaweel (2013) documented better antibacterial activity of methanolic onion extracts compared to aqueous extracts. Organic solvents were known to better dissolve organic compounds, thereby releasing the active component necessary for antimicrobial activity (Ekwenye and Elegalam, 2005).

CONCLUSION

The present study indicated that fresh *Allium sativum* juice exhibited a significant antibacterial effect on the resistant E. coli strains, with an inhibition zone diameter of 20.5 ± 1.29 mm, a MIC of 41.5 mg/mL, and an MBC of 166 mg/mL. Conversely, fresh *Allium cepa* juice was ineffective against the tested bacterial strain. Furthermore, the antibacterial effect of the combination of both extracts was inferior to that of garlic alone, with an inhibition zone diameter of 16.5 ± 0.57 mm. These findings suggested the potential use of garlic as an alternative to antibiotics in treating colibacillosis caused by a resistant strain of E. coli. It's essential to note that while the antimicrobial properties of *Allium sativum* showed promise, further research was needed to understand the specific mechanisms of action and to explore the potential development of garlic-based treatments or supplements for combating multidrug-resistant bacterial infections. Additionally, considering the dosage, formulation, and potential side effects should be thoroughly investigated for the practical application of *Allium sativum* in clinical aspects.

DECLARATIONS

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Authors' contributions

Rachid Merati contributed to the conception, design, data collection, analysis, interpretation, and writing. Abdellatif Boudra contributed to the data collection, analysis, editing, and writing the final draft of the manuscript. All authors approved the analyzed data and the last revised article.

Competing interests

The authors confirm that the data presented do not represent any conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethical considerations

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

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Phenotypic Variability of Native Guinea Pig (*Cavia porcellus*) Lines Associated with Productive and Reproductive Variables in the Traditional Production Systems of the Pastos Indigenous Reserve

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ABSTRACT

Genetic improvement seeks to meet human needs, resulting in a loss of genetic variability, affecting indigenous communities' biodiversity and food sovereignty. Therefore, this study aimed to determine the phenotypic variability of native guinea pig (Cavia porcellus) lines associated with productive and reproductive variables in the traditional production systems of the Pastos Indigenous Reserve in the Department of Nariño, southwestern Colombia. A total of 2007 guinea pigs older than 3 months were divided into 2 batches. 1934 individuals (batch 1) were randomly selected for phenotypic characterization, including hair length, leg size, body size, and behavior. Seventy-three individuals (batch 2) were used to evaluate productive and reproductive variables, and the lines with the highest similarity were clustered using the UPGMA method. In addition, ethnoveterinary information obtained through interviews within the production systems was described. As a result, nine traditionally known phenotypes were described including Shinhuzo, Pelochon, Zambo, Guarico, Chocolate, Peruvian, Coral, Piño, and Moro. The lines were clustered into 4 groups, highlighted by lines with high production and reproductive potential (group 1), lines with low progeny mortality (group 2), a line with low reproductive potential (group 3) a line that presented a unique coat and high productive potential as Group 1 (Group 4). As a result, local knowledge was shown to be crucial for the conservation of native guinea pig lines, as it includes traditional feeding techniques and disease treatment. The native lines Shinhuzo and Coral, with morphological differences between them, showed the potential to reach productive and reproductive parameters similar to the improved Peruvian line, according to the UPGMA dendrogram. However, a detailed analysis of the specific nutritional requirements of each guinea pig line is necessary to improve the traditional breeding of guinea pigs, enhancing the production of all native lines already adapted to the indigenous territory, maintaining the important genetic variability that, in the context of climate change, is relevant to promoting research on sustainable production strategies using resilient native species adapted to local conditions for the future exploration of differentiated markets.

Keywords: Animal conservation, Ethno-veterinary, Genetic variability, Traditional knowledge

INTRODUCTION

Domestication of animals and improving productive characteristics, such as weight gain and muscle strength have been developed to meet human needs (Alves et al., 2018; Lord et al., 2020). However, selection and inbreeding in populations of the same lineage bring significant problems, mainly the loss of genetic variability (Lacy, 1997). This is the case of native lines of guinea pigs (*Cavia porcellus*), which were affected by advances in intensive production and the adoption of improved varieties/breeds (Avilés et al., 2014; Díaz et al., 2016).

Native to the high Andean region of countries such as Colombia, Peru, and Ecuador, the domestic guinea pig belongs to the order Rodentia and is classified as *Cavia porcellus*, derived from its ancestor, the wild guinea pig (*Cavia tschudii*; Díaz et al., 2016; Lord et al., 2020). In Colombia, guinea pig production is associated with a traditional or indigenous management agroecosystem called "shagra" by indigenous communities (Rosero-Alpala et al., 2020) and is limited to the high Andean region near the border with Ecuador, where more than half a million guinea pigs are raised (Dalle-Zotte and Cullere, 2019). In this region, the guinea pig is economically and culturally significant, being the main animal that feeds on the bioproducts of the shagra system (Rosero-Alpala et al., 2020). Although guinea pigs are a fundamental basis for food sovereignty and a source of income for rural populations (Dalle-Zotte and Cullere, 2019; Benavides-Benavides et al., 2021), their low cost makes them an excellent alternative for sustainable production (Salvo et al., 2023). However, genetic improvement to obtain more efficient commercial populations for meat production is

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reducing the diversity of native lines, as they are considered low production lines due to their small size (Spotorno et al., 2004; Dalle-Zotte and Cullere, 2019; Lord et al., 2020).

For decades, guinea pigs have been produced in a traditional system that has been beneficial due to the herbivorous habits and reproductive parameters of guinea pigs (Rosales-Jaramillo et al., 2021). Commonly, the guinea pigs are often fed on kitchen waste when reared in rural smallholder houses (Dalle-Zotte and Cullere, 2019) and are reared from juveniles (0-150 days) to adults at 4-5 months, weighing 500-1500 g, at temperatures between 15-22°C (Ayagirwe et al., 2018; Grada et al., 2018). Although sexual maturity is reached at 2 months of age, the fecundity of females (up to 5 births per year) and the number of live offspring per birth (up to 6) are breed-dependent (Ayagirwe et al., 2018). The versatility of guinea pigs has allowed them to be distributed worldwide as companion, laboratory, or production animals.

The conservation of local animal sources of genetic variability has become a current area of research (Ayagirwe et al., 2017; Lord et al., 2020; Rosales-Jaramillo et al., 2021) because phenotypic features vary between populations, regions, and countries and depend on both the breed and the reproductive management system (Ayagirwe et al., 2019). For example, in Latin America, phenotypic characterization showed a significant difference in guinea pig morphology between Ecuador's two departments (Azuay and Cañar) (Rosales-Jaramillo et al., 2021). Similarly, in the Democratic Republic of Congo (DRC), the trichromic color pattern was the most dominant, while in Cameroon, it was more dichromic (Ayagirwe et al., 2017). Moreover, hair rosettes are known in South America but have not been reported in Africa (Ayagirwe et al., 2019). However, in Africa, data on guinea pig production is only available from 1968 onwards (Dorothy et al., 2014), as information on how and when the species was introduced into Africa is still unknown (Ayagirwe et al., 2017).

Molecular analyses suggest that the improved lines share a recent common ancestor with the European guinea

pig, which is not the ancestor of the native Latin American lines (Spotorno et al., 2004). In addition, an independent domestication center of Cavia has been identified in Colombia. However, it is not related to modern Colombian guinea pigs (from markets), promotes the study of genetic diversity in specimens of Cavia species (Díaz et al., 2016), and the verification of a possible independent domestication process (Lord et al., 2020). Based on the above information, this study aimed to identify the different lines of guinea pigs present in the Gran Cumbal Indigenous Reserve, evaluating the morphological characteristics related to productive and reproductive development in the traditional production systems of the Gran Cumbal Indigenous Reserve, municipality of Cumbal, department of Nariño, Colombia. In addition, the use of traditional knowledge and ethnoveterinary medicine was described.

MATERIAL AND METHODS

Ethical approval

The experiments with Cavia porcellus from the indigenous reserve of Gran Cumbal (Cumbal–Nariño, Colombia) were conducted following the Animal Ethics Committee from the Universidad Católica de Oriente (Agreement #D-007/2024-05-31).

Location and experimental design

This research was carried out in the indigenous reserve of Gran Cumbal, 0°55' north latitude and 77°49' west longitude, in the department of Nariño, southwestern Colombia, in the large mountain massif called "Nudo de los Pastos", and only 3 districts were evaluated and chosen for high agricultural production (Cuaspud), Páramo with low agricultural and livestock production (Cuetial) and high livestock production (Boyera). The three sectors have different altitudes (Cuetial at 3400 m, Boyera at 3200 m, and Cuaspud at 3000 m), to evaluate possible differences in the productive and reproductive performance of guinea pigs between regions. Temperature and seasonal variation were not evaluated during data collection. All evaluations were conducted between the periods of high production between June-August (corresponding to the beginning of the summer period) and December-February (during holidays) between 2017 and 2020. Data collection was developed over three years to ensure that all guinea pig lines were characterized.

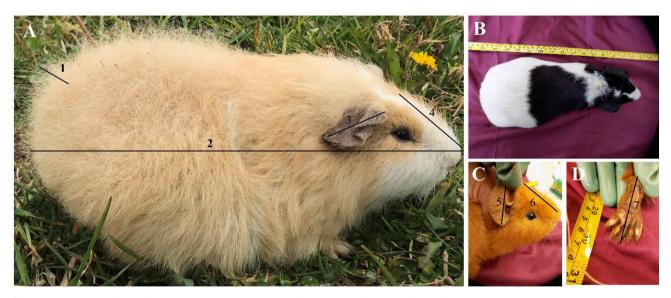
A total of 20 agroecological sheds were selected to determine the prevalence of native guinea pig lines. Only agroecological farms where traditional knowledge was practiced and/or animals were fed without supplements were included. Interviews, workshops, and surveys were conducted in the Boyera, Cuetial, and Cuaspud sheds. Activities such as traditional management, feeding, and health care (biosecurity) were considered influential factors in the production and reproduction parameters of the Indigenous community's production systems.

The procedures developed in this study are shown in the graphical summary. A total of 2007 guinea pigs of different ages (older than 4 months) were selected in the 20 agroecological sheds. Most agroecological systems (n = 16) used a wire cage housing system for guinea pig production. Another part (n = 4) had an unrestricted housing system inside the sheds, and the animals were reared freely on the ground. The animals were divided into batch 1 (n = 1934) for

morphological evaluation and batch 2 (n = 73) to assess productive and reproductive parameters. Animals with breeding records were used for reproductive and productive evaluation, and animals without breeding records were used for morphological evaluation.

Evaluation of morphological characteristics in situ

An experimental group (n = 1934) was used for morphological characterization, according to the Ayagirwe et al. (2019) protocol. Briefly, 9 phenotypic variables were described, such as relative body size (small, medium, large), behavior (shy or docile), coat color pattern (monochromatic, dichromatic, trichromatic), coat length (< 2 cm short, 2-3 cm medium, > 3 cm large), relative leg size (small or large), eye color (red or black), ear orientation (erect, drooping), head profile (elongated or rounded), in addition to their frequency of presentation and use (medicinal, ritual, consumption, or sale) in traditional production systems was evaluated. The Peruvian line was used as a parameter to assess behavior, relative body size, relative leg size, and head shape since the line has a large body size (~34 cm), short coat (~1 cm), large legs (~2.5 cm), docile behavior and drooping ear (Chauca, 2023). According to the classification of Ayagirwe et al. (2019), the line showed an elongated head profile. The length of the legs (long or short) was described as "Yes = long legs", if the legs of the native line were equal to or longer than those of the control (\sim 2.5 cm), "Not = short legs" if the legs of the native line were shorter than those of the control. This concept was applied to relative body size (small, medium, large) and coat length. The usual size of the Peruvian line (~34 cm) was categorized as large, medium if the animal had less than 5 cm to reach the reference value, and small if the animal had more than 5 cm to reach the reference value. Coat length was categorized as short (~1 cm), large (~4 cm), and medium (>1 cm and <4 cm). Numerical values were not presented as absolute values but only considered classification variables. The coat color, and eye color, were described without the use of the control characteristics. An example of this classification is shown in the supplementary Figure 1. All images were taken with a camera Nikon (Coolpix L330, Nikon, Japan).



Supplementary Figure 1. Phenotypic evaluation of male and female guinea pigs older than 4 months belonging to 9 lines found in traditional indigenous systems in the community of Pastos, Nariño, Colombia.

A: Determination of coat length (1), relative body size (2), ear with dropping orientation (3), and head with elongated profile (4). The values are presented as qualitative variables, using the standards of the Peruvian line. **B-D:** Manipulation of animals during data collection. Ear orientation (5), head with a rounded profile (6), and leg size (7). The standard values obtained in the Peruvian line were used such as control to some features. The Peruvian line has a large body size (\sim 34 cm), short coat (\sim 1 cm), large legs (\sim 2.5 cm), docile behavior, and drooping ears. Statistical analysis was used to determine the length of the legs (long or short). Where Yes = long legs, if the legs of the native line were equal to or longer than those of the control (\sim 2.5 cm), Not = short legs if the legs of the native line were shorter than those of the control. This concept was applied to relative body size (small, medium, large) and coat length. The usual size of the Peruvian line (\sim 34 cm) was categorized as large, medium if the animal had less than 5 cm to reach the reference value, and small if the animal had more than 5 cm to reach the reference value. Coat length was categorized as short (\sim 1 cm), large (\sim 4 cm), and medium (>1 cm and <4 cm). Numerical values were not presented as absolute values but only considered classification variables. The coat color, and eye color, were described without the use of the control characteristics.

Assessment of productive and reproductive parameters in situ

Batch 2 (n = 73 animals) comprises the nine lines identified in the previous evaluation of morphological characteristics (Evaluation of morphological characteristics in-situ topic). The selected animals, aged 4-6 months, including males and females, were obtained only from agroecological sheds (n =16) that used a wire cage housing system for guinea pig production. The animals were identified and divided into couples (male and female) by native line

in a single wire cage and placed in the same shed, in the Cuaspud district. The owner recorded and shared the reproductive records obtained over six months.

Productive variables

Body weight (BW) was determined by individual weighing (n = 73) using a mechanical hanging scale (Salter, #235-6S, New York, USA) in all the sheds. Mean weights were compared between lines, sheds, and districts.

Reproductive variables

A retrospective analysis was performed using breeding records (obtained in the results of the Productive variables topic) collected from adult females (n = 38). The variables assessed were mean weight (kg) obtained by individual weighing, number of live-born pups (NBA), stillbirths, number of pups per year (NPY), and mortality of pups to 3 months of age (M). The NPY value was obtained by multiplying the NBA by the number of annual reproductions (3.7 and 3.4 to improved and native lines, respectively) defined by Andean guinea pigs (Patiño-Burbano et al., 2019).

Dendrogram construction using the Unweighted Pair Group Method using Arithmetic averages (UPGMA)

The phenotypic, productive, and reproductive variables of the nine lines identified (Evaluation of morphological characteristics *in situ* and assessment of productive and reproductive parameters in situ topics) were used to construct a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA) to cluster the lines with the highest similarity using Euclidean distances.

Ethno-veterinary application in traditional production systems

The assessment was carried out through oral communication and the "dialogue of knowledge" based on active participatory research with the guinea pig farmer of each shed (n = 20), following the recommendations of Rosero-Alpala et al. (2015). Information on each plant species was collected using the botanical and ethnobotanical format proposed by Rosero-Alpala et al. (2015). During the dialogue of knowledge, family members of each guinea pig farmer attended meetings to express their opinions.

Information on ethno-veterinary practices was verified by observing the practices *in situ*, noting how local communities use traditional methods and remedies, and through interviews providing first-hand accounts and insights into practices and their efficacy. In addition, the information was compared with written records of ethno-veterinary practices in this and other indigenous communities to provide context and support for current methods.

Assessment of biosafety and health in traditional guinea pig husbandry systems

The management and safety of the traditional guinea pig systems (sheds, n = 20) were self-assessed by the participating families using the evaluation form. An analysis of the main problems of this production was identified through technique visits (agronomist and veterinarian student) to each shed. The format was divided into 5 sections, with different topics evaluated on a scale of 1 to 10, where higher numbers indicate better performance or alignment with criteria according to Artica (2020). In the breeding environment section (20 points) was evaluated the luminosity (natural lighting) and window protection (mesh or curtains). In the materials and equipment (20 points) section was evaluated the constant cleaning of the shed and application of a disinfection program. In the infrastructure (40 points) section was evaluated the isolation method of guinea pigs (cages), the implementation of records: production, reproduction, and sales, the method of selection of guinea pigs (classified by size, line, and sex), and dead animal's management. In the animal feeding (20 points) section was evaluated the protection place for airing of forage and disinfection of forage before consumption. In the management of traditional knowledge (30 points) was evaluated the practices during animal feeding, animal care, and for disease treatment. The total score was 130 points. Each section applied 3 categories including values less than 75 of the reference score was considered productive imbalance, between 76 % to 85 % indicating a production that could be improved in some aspects, and greater than 86 % points indicating a good biosecurity plan with a balance of the productive system. a scale was 1 to 10, where higher numbers indicate better performance or alignment with criteria.

Statistical analysis

Qualitative variables are presented in frequency tables. Quantitative data were analyzed by ANOVA, followed by the Tukey test to evaluate the differences between lines. The data are represented as mean \pm standard error. Analysis was performed using the software Rstudio version. 4.1 (Rstudio, Boston, U.S.A.), and a significant level was set at p < 0.05. The phenotypic and productive variables were used to construct a dendrogram (UPGMA) with the groups of most significant similarity using Euclidean distances.

RESULTS

Location and experimental design

The 20 sheds distributed across the three districts presented a variety of diets used in traditional production systems. Fifty-six percent (n = 9) of the systems had a diet based on forage consumption similar to that of cattle, such as ryegrass (*Lolium perenne*), alfalfa (*Medicago sativa*), oats (*Avena sativa*), clover (*Trifolium pratense L*). The 25 % (n = 4) feeding with other types of grasses such as Sonchus oleraceus or Taraxacum officinale, and 19 % (n = 3) were fed with crop residues, corn (*Zea mays*), broad bean (*Vicia faba*), cabbage (*Brassica oleracea*) and other vegetables.

Evaluation of morphological characteristics in situ

The study showed that in traditional management systems, there is a conservation of genetic resources, expressed in 9 phenotypes traditionally known as Shinhuzo, Pelochon-chilpudo, Zambo, Guarico, Chocolate, Peruvian (including Perú and Inti improved breeds), Coral, Piño and Moro. The frequency and specific description of each line are shown in Table 1. 35% of guinea pigs evaluated belonged to the Peruvian line (n = 676), and 65% were native lines (n = 1256). Within the native lines, Shinhuzo was easily found and represented 17% (n = 330) of all animals. Lines such as Chocolate and Piño were very little known, representing 1.7% (n = 32) and 4% (n = 78) respectively.

According to the producers, the relative size of the legs is considered a breed selection standard in the communities because it is related to productivity and reproductive efficiency. According to the results presented in this study, 7% of the indigenous community population uses the guinea pig as a medicinal source to treat some health problems, such as anemia and weakness after childbirth, preferring lines with black color, such as the Moro. Three percent use guinea pigs in rituals and festivals such as Inti Raymi (summer solstice) to ward off evil spirits. Most of the population (80%) use guinea pigs as a source of protein in their diet, and 10% of the population produces guinea pigs for sale. Most people who eat guinea pigs prefer roasted (90%), broth (6%), or stewed (4%).

Assessment of productive and reproductive parameters in situ

There was a significant difference (p < 0.05) between females' and male's weight, with a weighted mean of 0.95 ± 0.06 kg for males (n = 20) and 1.29 ± 0.10 kg for females (n = 53; Supplementary Table 1). In addition, there was no significant difference (p > 0.05) between lines between sheds (p > 0.05) and between districts (p > 0.05) evaluated (Supplementary Table 2). This study described feed consumption as the weight of feed offered but did not show the exact consumption value (Table 2). There was no significant difference in feed consumption among districts (p > 0.05, Supplementary Table 2), and the quality of feed used in the production systems by the district varied. Cuetial district is located in the highlands of the indigenous reserve, resulting in the lowest level of dairy cattle production, so there are lower quality pastures for guinea pigs, which can lead to lower average animal weight and higher feed consumption compared to the other districts. Boyera and Cuaspud are located in the more lowland region of the reserve, which favors agricultural production and directly affects the feed quality for guinea pig raising.

Reproductive variables

The results of the reproductive variables are shown in Table 3. No significant difference was found among the lines in the percentages of stillbirth (p > 0.05) and mortality up to 3 months (p > 0.05). The number of puppies born alive (BA) and the number of pupp per year (NPY) showed a significant difference between the lines (p < 0.05).

Dendrogram construction using the Unweighted Pair Group Method using Arithmetic averages (UPGMA)

According to the UPGMA, the 9 lines were grouped into 4 groups: Group 1: Peruvian, Shinhuzo, and Coral;

Group 2: Moro, Guarico, Zambo, and Piño; Group 3: Pelochon Chilpudo; Group 4: Chocolate (Figure 1). The last two groups were described individually due to their high divergence in terms of their morphological and reproductive characteristics. Group 1 (Coral, Shihuzo, and Peruvian) showed high productive characteristics (weigh between 1.20 ± 0.14 to 1.23 ± 0.09) and reproductive potential. The reproductive variables such as high NBA (4.50 ± 0.34 to 4.70 ± 0.15), high NPY (15.75 ± 1.20 to 17.63 ± 0.57), and low stillbirth percentage (4.76 ± 4.19 to 6.15 ± 3.25), making these lines preferred for production and commercialization. Group 2 (Piño, Moro, Zambo, and Guarico) showed a high stillbirth percentage (15 ± 5.93 to 20 ± 5.13) with low mortality of pups (0 to 1.7%). Group 3 (Pelochon-chilpudo) showed low reproductive potentials, such as low NBA (2.50 ± 0.50), high stillbirth (12.50 ± 7.26), and mortality (6.3 ± 0.50) percentage. The productive variables were similar to the other groups. Group 4 (Chocolate) was different in coat color (unique with gray and white colors) and coat color pattern, but it has a similar productive and reproductive potential.

Table 1. Phenotypic description of 9 lines of guinea pigs in the indigenous traditional systems of Cumbal, Nariño, Colombia

Lines of guinea pigs

Character	Moro	Zambo	Shinhuzo	Chocolate	Peruvian	Pelochon	Guarico	Coral	Piño
Coat length	Short	Medium	Long	Short	Short	Long	Long	Short	Short
Size	Small	Small	Large	Large	Large	Large	Medium	Medium	Small
Behavior	Shy	Shy	Docile	Docile	Docile	Docile	Shy	Docile	Shy
Eye colour	Red	Black	Red	Red	Black	Black	Black	Black	Black
Ears orientation	Erect	Dropping	Erect	Dropping	Dropping	Dropping	Dropping	Dropping	Erect
Head profile	Rounded	Rounded	Rounded	Rounded	Elongated	Rounded	Elongated	Rounded	Elongated
Leg size	Medium	Small	Large	Medium	Large	Large	Medium	Small	Small
Coat color pattern	Dichromic	Trichromic	Trichromic	Dichromic	Monochromic	Trichromic	Trichromic	Dichromic	Dichromic
Coat colour	black and white	brown, beige, white	brown, beige, white, black	Gray, white	brown, beige, white, black	beige, white, black	brown, beige, white	brown, beige, white	beige, white
Use	S	C	S	C	S	C	C	C	C
n	183	225	330	32	676	210	20	180	78

The use of each line was determined to sell (S) or self-consumption (C). The data was obtained from 1934 individuals belonging to 20 agroecological sheds. n: Number.

Supplementary Table 1.

Group 2						
Sex	N	Weight	p-value			
Male	20	0.95 ± 0.10^{a}	P = 0.04			
Female	53	1.29 ± 0.06^{b}	P = 0.04			
Total	73					

Data means average \pm standard error. Identical superscript letters indicate non-significant differences as determined by the Tukey multiple-range test (P > 0.05).

Supplementary Table. 2.

Shed	District	Total Animals	Feed (kg)/ day/ all animals	Feed (Kg)/ Day/Unit	Weight Mean	Weight Mean/ districts
1		8	10	1.25	1.2	
2		33	30	0.90	1.1	1.14 ± 0.09^{a}
3		30	50	1.66	1.52	111. = 0.09
4	Cuetial	15	10	0.66	0.97	
5		32	20	0.62	0.83	
6		25	20	0.8	1.28	
7		10	10	1	1.23	
8		10	10	1	1.08	1.25 ± 0.09^{a}
9	D	70	50	0.71	1.25	
10	Boyera	24	20	0.83	0.87	
11		25	30	1.2	1.38	
12		50	40	0.8	1.65	
13	Cuaspud	20	30	1.5	1.4	
14		7	8	1.14	0.93	1.18 ± 0.12^{a}
15		30	20	0.66	1.17	
16		30	25	0.83	1.17	
Total		419	383			73

Data means average \pm standard error. Identical superscript letters indicate non-significant differences as determined by the Tukey multiple-range test (P > 0.05).

Table 2. The production variable of 9 lines of 4 - 6 months guinea pigs in the indigenous traditional systems of the Pastos community, Nariño, Colombia

Line	N	Weight (kg)	Feed consumption (kg)
Moro	7	1.25 ± 0.18	1.00
Zambo	3	0.87 ± 0.26	1.04
Shinhuzo	13	1.20 ± 0.14	0.99
Chocolate	2	1.10 ± 0.32	0.96
Peruvian	27	1.23 ± 0.09	0.97
Pelochon	9	1.33 ± 0.17	0.99
Guarico	1	1.40	0.80
Coral	7	1.23 ± 0.18	0.80
Piño	4	0.85 ± 0.23	0.92
TOTAL	73		

Table 3. The reproduction variables of 9 lines of 3 - 6 months female guinea pigs in the indigenous traditional systems of the Pastos community, Nariño, Colombia

Line	NBA	Stillbirth (%)	NPY	M (%)
Moro	3.50 ± 0.29^{abcd}	20.0 ± 5.13	12.25 ± 1.01^{abcd}	1.7 ± 0
Zambo	3.00 ± 0.58^{bcd}	15.0 ± 5.93	10.50 ± 2.02^{bcd}	0.0 ± 0
Shinhuzo	4.67 ± 0.21^{ab}	4.76 ± 4.19	16.33 ± 0.74^{ab}	1.4 ± 0
Chocolate	3.50 ± 0.34^{abcd}	7.94 ± 4.19	12.25 ± 1.20^{abcd}	1.7 ± 0
Peruvian	4.70 ± 0.15^{a}	6.15 ± 3.25	17.63 ± 0.57^{a}	1.6 ± 0
Pelochon	2.50 ± 0.50^{d}	12.5 ± 7.26	8.75 ± 1.75^{d}	6.3 ± 0
Guarico	2.75 ± 0.48^{cd}	14.2 ± 0.0	9.63 ± 1.68^{cd}	0.8 ± 0
Coral	4.50 ± 0.34^{ab}	5.16 ± 4.19	15.75 ± 1.20^{ab}	0.8 ± 0
Piño	4.25 ± 0.48^{abc}	16.67 ± 5.13	14.88 ± 1.68^{abc}	0.0 ± 0

The number of pups born alive (NBA), number of pups per year (NPY), and mortality (M) of pups until they reach 3 months old. Data means average \pm standard error. Identical superscript letters indicate non-significant differences determined by the Tukey multiple-range test (P > 0.05).

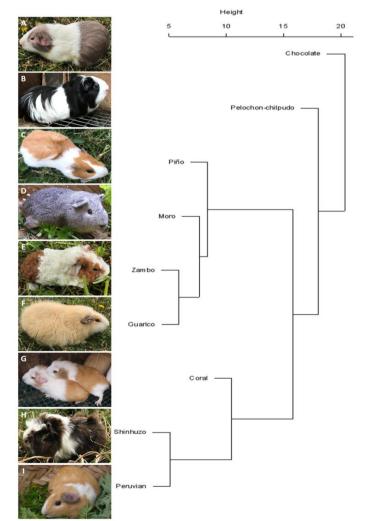


Figure 1. The dendrogram construction using UPGMA and reproduction and production variables of genetic resources of male and female guinea pigs, expressed in 9 phenotypes in the indigenous traditional systems of Pastos community, Nariño, Colombia. All phenotypes were described using the traditional name. A: the "chocolate". B: the "pelochon" with a long and straight coat. C: the "piño" with a hair swirl on the back, a specific characteristic of this line. D: the "Moro" with the dichromic coat (black and white). E: the "Zambo" with a coiled coat. F: the "guarico" with a long and curly coat. G: the "coral" with a hair swirl on the head, a specific characteristic of this line. H: the "shinhuzo" with a wavy coat. I: the Peruvian line. The lines were grouped into 4 groups, group 1: Peruvian, shihuzo, and coral; group 2: piño, moro, guarico, and zambo; group 3: pelochon-chilpudo; group 4: chocolate.

Ethnoveterinary application in traditional production systems

The information obtained through interviews, workshops, and surveys carried out in the sheds of Boyera, Cuetial, and Cuaspud showed a brief classification of the medicinal plants used in the traditional production of guinea pigs. The diseases found in the production systems were mainly related to fungi, internal parasites (traditionally known as "alicuya"), and external parasites ("piojera") that affect young animals during the first months of life. Among the main diseases disclosed were "pepa" (Yersinia infection), respiratory and gastrointestinal diseases, and ectoparasites (Table 4). In traditional production systems, several plants are used to treat different diseases by oral or topical application and improve the environment (cages). The "gallinazo" and the eucalyptus were the first options for cleaning the cages. The plants were placed inside the cages for one or two days, and their efficacy was related to the pungent odor that characterized each. The herbal extracts offered for oral use were prepared by maceration of fresh green leaves of each of the plants until a slurry was obtained, which was mixed with water to facilitate oral administration; thus, this procedure was performed on animals in serious conditions that could not eat the leaves placed in the cages. When the extracts were for external use, fresh early leaves and stems were used, then placed in water and boiled for 30 minutes until a change in the color of the water was obtained (the ratio of leaves/water used was 10 leaves with stem/5L of water). The water had to be cooled and the animals were immersed in the warm water for a maximum of 2 minutes.

Table 4. The treatment of principal diseases that affect 9 lines of guinea pigs in the indigenous traditional systems of Cumbal, Nariño, Colombia

Disease	Organic system	Symptoms	Medicinal plant	Administration
Yersinia infection	Gastrointestinal Liver Linfatic system	Hepatic or splenic necrosis, abscess formation, mesenteric lymphadenopathy	Marco (Ambrosia arborescens Mill) Chilca negra (Baccharis floribunda) Gallinazo (Tagetes zipaquirensis) Verbena (Verbena littoralis) Guanto (Brugmansia sanguínea)	Fresh leaves* Fresh leaves* Fresh flowers and leaves* Leaves extract* Leaves-
Pneumonia	Respiratory Gastrointestinal	Nasal, ocular, or oral discharge, labored or rapid breathing, lethargy, emaciation, diarrhea, and bristly fur	Eucalyptus globulus Labi Marco (Ambrosia arborescens Mill). Baccharis latifólia Wormwood (Artemisia absinthium L)	Leaves extract* Leaves extract* Leaves extract* Leaves extract*
Parasites and fungus	External integuments	Loss of fur and peeling and itching in the affected area, hair loss, dermatitis, and crusts	Chilca negra (Baccharis floribunda) Eucalyptus globulus Labi Gallinazo (Tagetes zipaquirensis)	Leaves extract+ Leaves extract+ Leaves and flowers extract

The information was collected through verbal communication in the "Dialogue of Knowledge" based on active participatory research with the community. Rout administration: * oral, + external, -housing disinfection

Assessment of biosecurity and health in traditional guinea pig farming systems

A self-assessment was conducted by a family member, and the strengths and weaknesses of their guinea pig production system were identified. Significant differences in biosecurity scores were observed between sheds and between districts (p < 0.05). In Boyera district, the average score was 108.67 ± 2.28 , 90.25 ± 1.59 , and 72.33 ± 3.4 for Cuaspud and Cuetial, respectively. The average score of all the farms was 89.4 points, indicating weaknesses in the traditional production system. In the average analysis of the sheds, it was found that their main weakness was the materials of equipment for cleaning (13.69) in contrast to animal feeding (18.06; Supplementary Table 3) where a higher score was obtained. Biosecurity and ethnoveterinary were important for producers to avoid using costly pharmaceutical treatments, opting for recovery, and traditional disease treatment techniques. However, the lack of cleaning facilities had a direct impact on the health of traditional productions since the prevalence of diseases was linked to sudden changes in temperature, with variations in humidity and air currents, typical of the transition from the dry season (summer) to the rainy season (winter). One of the main factors that favored the appearance of various respiratory and gastrointestinal symptoms was the dirty litter associated with abrupt changes in feeding, generating more food waste. In addition, the inefficient diagnosis of diseases in the early stages led to an increase in the proliferation of vectors including flies, and, therefore, an increase in the transmission of diseases between cages, triggering outbreaks of different diseases (Ethnoveterinary application in the traditional production systems section). Consequently, it was essential for the producers to establish an annual sanitary calendar to consider the season to prevent the outbreak of these diseases.

Supplementary Table 3.

supplementary rank 3.					
Section	Reference score	Category*	Score of sheds		
Breeding environment	20	Imbalance < 15, improve 16-17, balance > 17	13.69 ± 4.4		
Materials and equipment	20	Imbalance < 15, improve 16-17, balance > 17	12.63 ± 2.61		
Infrastructure	40	Imbalance < 30, improve 31-34, balance > 34	24.38 ± 6.55		
Animal feeding	20	Imbalance < 15, improve 16-17, balance > 17	18.06 ± 6.60		
Management of traditional knowledge	30	Imbalance < 22, improve 23-25, balance > 25	21.69 ± 11.83		
Total	130		89.44 ± 19.56		

^{*} For each section was applied 3 categories: values less than 75% of reference score (imbalance) between 76% to 85% (production that can improve in some aspects), and greater than 86% (good biosecurity plan with a balance of the productive system).

DISCUSSION

In the context of climate change, it is important to promote research into sustainable production strategies using resilient native species adapted to local conditions. The sheds were selected in three different production areas, which are sectors with high agricultural production (Cuaspud), Páramo (Cuetial), and high livestock production (Boyera). Furthermore, the vegetation management in these systems considers the different climatic factors present in the different altitudinal bands of the indigenous reserve (Rosero-Alpala et al., 2015). The production and biosecurity of guinea pigs were lower in the intervened areas of the Páramo due to the scarcity of feed, in contrast to the agricultural and livestock production districts. This situation corresponds to the environmental and vegetation peculiarities of the Andean ecosystem, where the availability of forage varies according to the altitudinal gradient. According to studies by Rosero-Alpala et al. (2020), in páramo areas, guinea pigs feed on the foliage of native shrubs and by-products of Andean tuber crops. Among the species with forage use are: Black elderberry (Sambucus nigra), sauco (Sambucus peruviana), chilca (Baccharis latifolia), and carrizo (Genus chusquea), these species have characteristics of high adaptability, biomass production, and excellent nutritional quality (Cardona et al., 2020).

In the traditional production systems, the native lines of guinea pigs predominate, with a prevalence of 65%, compared to the improved lines, such as the Peruvian, which represent 35%. Among the native lines, the Shinhuzo has been highlighted for its large size and resistance to disease. However, in intensive production, 85.5% is based on improved lines, while only 14.5% corresponds to native lines (Chávez-Tapia and Avilés-Esquivel, 2022). In addition, in the municipalities of Nariño, there are approximately 20000 small guinea pig farms with artisanal production, which have the production to support the family economy (Barco-Jiménez et al., 2021). According to Avilés et al. (2014), this panorama shows a typology of production and transition in guinea pig farming due to the economic factor of subsistence and family income. However, the development of the commercial production system has allowed the introduction of improved lines in the Andean region, reducing the production of native guinea pig lines. The results of the present study showed that 93% of the activities in guinea pig farming were carried out by women, revealing a notable gender predominance in the breeding, production, and conservation of indigenous lines of guinea pigs. Similarly, in intensive production, production management is also developed by women, with 76.6% of activities carried out by women (Chávez-Tapia and Avilés-Esquivel, 2022). Moreover, for several decades, small livestock species such as pigs, guinea pigs, and chickens have been reared by women in 57.1% of cases, while men alone carry out this activity in 4.8% of cases, reflecting the integration of women in the productive activities of rural families (Giraldo, 2008). The rural women in Nariño implement resilient actions in front of food and nutrition security through agroecological production practices. The women's gardens, which include vegetables, legumes, aromatic herbs, and medicinal plants, have integrated a livestock system with the raising of guinea pigs and chickens, reducing dependence on external inputs such as fertilizer for crops and feed for animals and facilitating the development of short marketing chains through agroecological markets (Bacca-Acosta et al., 2024).

This study showed the low reproductive potential of the native guinea pig lines compared to the improved lines. Only the Shinhuzo and Coral lines exhibited high reproductive potential. The limited performance of the native lines is attributed to their innate characteristics of resistance to disease, hardiness, and adaptation to the environment (Quispe et al., 2021). Similar studies developed by Patiño-Burbano et al. (2019), who evaluated the behavior of productive and reproductive variables of improved lines and several native lines in Nariño and Putumayo, showed that the improved lines had a lower age at first mating (months) (Peruvian line (5.1) and Andean (4.5) than the native lines (5.3). The number of births/female/year in the Peruvian and Andean lines was 3.7 and 3.4 in the native lines. The average weights at birth, weaning, and slaughter were higher in the improved lines than in the native lines, and the age at weaning was lower in the improved lines. Similarly, the study of David-Martínez et al. (2016) showed that the best averages of weights, litter sizes, fertility, birth rates, and calving intervals were obtained by the Peruvian breed in the context of genetic crossbreeding between purebred populations of native lines from Nariño and Peruvian origin. During the evaluation of reproductive performance, the animals were placed in the same shed, in the Cuaspud district, to avoid biases in the results related to the variety of feed quality between districts. These results indicated that low performance was line-dependent. Therefore, the specific factors (hormonal, genetic, or environmental) involved in the lower reproductive performance of the native lines should be studied.

Another aspect related to low reproductive potential in indigenous lines may be determined by diet (Cardonas et al. 2020; Vargas, 2022). According to Cardona et al. (2020), nutritional factors also play a fundamental role in the growth and reproduction of guinea pigs; low and high levels of protein and carbohydrates reduce the body's fat and weight loss, in addition to low fertility and reproductive performance. Although in the present study, the relative feed consumption did not show a difference between consumption and weight between native and Peruvian lines, the biometric growth of guinea pigs depends largely on the diet, which should be mediated by a diet rich in vitamins, proteins, fats, minerals, and

carbohydrates, so it is important to consider implementing a feeding system that generates a balanced consumption between forages and other food supplements. Since feed represents 70% of the cost of raising guinea pigs, it is necessary to determine the chemical composition and energy intake of the feed according to the requirements of each guinea pig line (NRC, 1995; Castro-Bedriñana and Chirinos-Peinado, 2021). Some studies have shown differences in the productivity of guinea pig lines under the same diet (Vargas, 2022). Reynaga-Roja et al. (2018) found differences in the productive behavior of guinea pigs of improved breeds, such as Peruvian, Inti, and Andean, where in a diet based on formulated feed plus chala corn, the Peruvian breed obtained higher slaughter weights, while for the Inti line it was less efficient.

On the other hand, the traditional knowledge of diseases, breeding techniques, and preservation of the native guinea pig is characterized by its focus on sustainable production based on native agro-food systems. According to Lastra-Bravo (2020), indigenous food systems emerge from an ancestral response with a significant component of resilience and environmental management. Therefore, a key element in the fight against hunger and poverty and promoters of sustainable agricultural practices that guarantee food sovereignty for indigenous communities. In traditional guinea pig production, animal health diagnosis is based on characteristic macroscopic findings. According to the findings of the present study in the indigenous community of Pastos, the disease with the highest prevalence, identified by reported symptoms, was Yersinia infection, commonly known as "Pepa". The infection affects the gastrointestinal tract and causes sepsis with secondary involvement of the liver, lymph nodes, and spleen (Mansfield and Fox, 2018). Although the prevalence of this disease may be related to the characteristics of guinea pig production systems (Benavides-Benavides et al., 2021), the type of housing system, such as unrestricted housing system or wire cages, does not appear to significantly affect growth performance, feed consumption, carcass characteristics, mortality, or fattening of guinea pigs (Mínguez Balaguer et al., 2019). Thus, in the context of production systems for food and nutrition, in the indigenous community of Pastos, aspects of local use of medicinal plants are considered for the treatment of internal and external diseases in domestic species such as guinea pigs, which are used in human food. According to Muyuy-Ojeda (2019), the communities of Inga and Kamentsa also use plant species for the care of guinea pigs. Among them, lemon balm (Melissa officinalis), vervain (Verbena officinalis), and garlic (Allium sativum) are used to treat gastrointestinal and respiratory diseases; marigold (Calendula officinalis) and chamomile (Chamaemelum nobile) are used to combat skin fungus; pumpkin (Cucurbita ficifolia) and paico (Chenopodium ambrosioides) for deworming; and botoncillo (Sphylantes sp) to control external parasites such as lice.

CONCLUSION

The native lines of guinea pigs showed the potential to achieve productive and reproductive parameters similar to those of the improved Peruvian line. In addition, the Indigenous families involved in this activity could benefit by strengthening their capacity to manage guinea pig farming sustainably and efficiently. However, further studies are needed to determine the values of specific consumption rates to obtain a dietary formulation for each indigenous line and to achieve productive efficiency. In addition, local knowledge was crucial for the effective conservation of native guinea pig lines, as it includes traditional feeding techniques, treatment of diseases with medicinal plants, and the use of sustainable practices such as composted substrates and biofertilizers in the "Shagra" farming system. It generates perspectives for the conservation and rescue of this species and the territories where it is found and within the current social challenges. Therefore, it is appropriate to promote research on mitigation strategies with sustainable production alternatives within agricultural systems that use resilient native species adapted to local conditions and the exploration of differentiated markets.

DECLARATIONS

Availability of data and materials

All data generated or analyzed during the current study are included in this article.

Authors' contributions

Jenyffer Rosero conceptualized, investigated, performed the data analysis, and wrote the original draft. Maria Gladis Rosero Alpala performed, validated, and visualized the methodology. Deisy Rosero performed the data curation and methodology. Alicia Rosero performed the methodology. William Armando Tapie: conceptualized, supervised, validated, reviewed, and edited the draft. All authors have read and approved the final version of the manuscript before publishing it in the present journal.

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Competing interests

The authors declare that they have no competing interests.

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Ethical considerations

The authors considered ethical concerns and Cabildo Indigena del Gran Cumbal consent to conducting and publication of this research. This article has not been published elsewhere and is copyrighted by the authors. The authors have checked the article for plagiarism index and confirmed that the article is based on original scientific work.

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Radiological Evaluation of Regenerative Growth Plate Defect Treated with Platelet-Rich Fibrin Membrane in Rabbits

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ABSTRACT

Bony bar formation after growth plate injuries leads to shortening and angulation of the long bone, which is considered one of the most critical sequelae affecting animals' and humans' lives in adulthood. The objective of the present study was to evaluate radiographically the role of using an autologous platelet-rich fibrin membrane in regenerating growth plate defects to prevent the formation of bony bars. A total of 20 kit rabbits, aged between 6-12 weeks and weighing 500-1100 g, were included in the current study. They were experimentally exposed to approximately $5\times5\times1$ mm growth plate defects, which were filled with an autologous platelet-rich fibrin membrane previously prepared at the time of the surgery. A radiological follow-up was conducted weekly at the first, second, third, fourth, sixth, and eighth weeks post-surgery to examine the growth plate defect area. The tibial length and angulation were measured during this period of the study and compared to the contralateral limb of the same animal. The radiological results showed no bony bar formation in most cases and the presence of the growth plate up to the end of the study (week 8 post-surgery) in the injured area. In addition, no significant differences were identified in the tibial length and angulation of the affected limb in comparison to the contralateral limb of the same animal throughout the study. In conclusion, treating serious growth plate injuries by PRF membrane may prevent angular deformity and length discrepancy in limbs.

Keywords: Angulation, Bony bar, Growth plate, Platelet-rich fibrin membrane, Shortening

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INTRODUCTION

Bone elongation is the main function of the growth plate located at the proximal and distal ends of long bones (Yu et al., 2019), and it is considered a weak area due to its composition compared to rigid bone, which renders it more prone to injuries, accounting for about 15-30% of the total skeletal system injuries in humans (Sabharwal and Sabharwal, 2018; Shaw et al., 2018; Shen et al., 2020). These injuries may lead to the formation of a bony bridge. A bony bar (bony bridge) is considered one of the most concerning issues in orthopedics since it can tighten both epiphyseal and metaphyseal bones, leading to various deformities such as the angulation and/or shortening of the affected limbs (Shaw et al., 2018; Ibrahim and Indra, 2022). These bony bars act as tethers that prevent certain areas of the growth plate from expanding (Khoshhal and Kiefer, 2005). Partial damage to the growth plate can result in shortening and progressive angular deformation of the bone, and more severe damage can lead to the complete arrest of longitudinal bone growth (Zhou et al., 2004). The early signs of bony bar formation are the structural disorganization and development of vertical septa, which eventually form the bony bridge, particularly in Salter's types III and IV (Wattenbarger et al., 2002; Xian et al., 2004). Radiological evaluation is used to identify bony and tendentious defects (Majeed and Hussein, 2017; Nazht, 2019; Hashim and Nazht, 2021) as well as cartilaginous defects (Yu et al., 2019). The growth plate is visible in radiographs of immature animals as a radiolucent area between the metaphysis and epiphysis due to its mostly hyaline cartilage composition (Kealy et al., 2010; Kazemi and Williams, 2021), and its defects may be seen on radiographs as a bony bar, which appears as a radiopaque area at the defect site (Gigante and Martinez, 2020). One of the long bones commonly affected by growth plate injuries during adolescence is the tibia, which is clinically evaluated via radiography (Tobita et al., 2002; Gültekin et al., 2020). An anteroposterior view of the hind limb best shows the formation of the bony bar and helps assess the tibial length and any resulting angulation (Sh et al., 2001; Wang et al., 2023). Various materials have been used to prevent bony bar formation in growth plate defects, such as scaffold materials (chitin and agarose) (Chen et al., 2003; Li et al., 2004; Azarpira et al., 2015), poly (lactic-co-glycolic acid) (Sundararaj et al., 2015), 3D-printed materials with or without autologous fat (Cheon et al., 2003; Yu et al., 2022), and autologous grafts (Alhusseni, 2008), with varying degrees of success. Other materials have also been used to treat cartilage defects, including ovine bone marrow-derived mesenchymal stem cells (Al-Mutheffer et al., 2023). However, the above methods have

many limitations. These *in vitro* tissue engineering methods require the harvesting of cells from the patient and thus necessitate multiple procedures. Recent studies have attempted to treat growth plate defects by developing biomaterial scaffolds incorporating growth factors and stem cells, which may aid in regenerating growth plate defects.

Platelet-rich fibrin (PRF) is a simple and easily prepared material (Dohan et al., 2006a). PRF is a second-generation platelet concentration composed of cytokines, leukocytes, stem cells, and platelets, which acts as a scaffold, supports micro-vascularization, and serves as a transport medium for carrying cells, all important for tissue regeneration (Temmerman et al., 2018; Dirja et al., 2023). PRF functions as an autologous fibrin matrix used to enhance bone regeneration (Salih and Al-Falahi, 2018; Thanoon et al., 2019), improve tendon repair (Al-Falahi, 2016), and enhance the viability, differentiation, and migration of chondrocytes, demonstrating significant potential in cartilage repair (Wong et al., 2020; Dirja et al., 2023). A PRF clot is also defined as a fibrin network that traps platelets and leukocytes, and its matrix allows the slower and more elongated release of growth factors (Ehrenfest et al., 2009). The growth factors present in PRF include the platelet-derived growth factor (PDGF), the insulin growth factor (IGF), the basic fibroblastic GF (bFGF), the epidermal GF (EGF), and the transforming growth factor beta (TGF-β). It also contains the stem cells. The growth factors may enhance the differentiation and proliferation of chondroblasts (Barbon et al., 2019; Pavlovic et al., 2021).

The regeneration of growth plate defects is still one of the most challenging issues for researchers, as it may affect the quality of life for both animals and humans if not properly treated. Therefore, the objective of the current study was to radiographically evaluate the effect of employing an autologous PRF membrane in regenerating growth plate defects in rabbit models.

MATERIALS AND METHODS

Ethical approval

Ethical approval was granted before starting the study by the local committee for animal care and use in research at the College of Veterinary Medicine, University of Baghdad, Iraq (P-G/2558 dated Nov 11, 2023).

Study design

The present study included twenty healthy male and female rabbit kits (White New Zealand; aged 6-12 weeks, body weight: 500-1100 g). According to Yoshida et al. (2012), their mothers were allocated to private animal houses to calculate their ages properly. The reason for using immature animals was to ensure the presence of active growth plates, which become ossified upon reaching maturity. All the kits were housed under controlled temperature ($22 \pm 2C^{\circ}$) conditions, relative humidity (60-65%), 12-hour light-dark cycle, and were fed fresh vegetables (carrots, and lettuce), hay, and Alfalfa grass during the entire experiment. Each animal of the total number was considered for the treated and control groups: the left limb of each rabbit was exposed to a growth plate defect and then treated with a PRF membrane, while the contralateral (right limb) of the same animal served as the control and remained untreated.

Anesthetic protocol

Rabbit kits were initially anesthetized by intramuscular injection of 2% xylazine hydrochloride at the dosage of 5 mg/kg. After 10 minutes, ketamine hydrochloride (10%) was administered intramuscularly at a dosage of 35 mg/kg (Eesa, 2010). The heart rate was examined by stethoscope, and oxygen saturation was monitored with an oximeter.

Surgical procedure

The PRF was prepared according to Dohan et al. (2006b) by collecting 3 ml of blood sample via cardiac collection during the operation. The results were then transformed into plain glass tubes (glass tubes without anticoagulants). Immediate centrifugation at 3000 rotation per minute (rpm) for 10 minutes yielded three separated layers: red blood corpuscles (lower), PRF (middle), and acellular platelet-poor plasma (upper). The PRF was withdrawn with sterile forceps from the tube, cutting off the red blood corpuscles and squeezed between two sterile compresses to remove excess fluid (Huang et al., 2010). A 10×5 mm of the produced PRF membrane was used to fill the defect in the left limb of each kit (Figure 1A). The left limb was aseptically prepared for the surgery, and then an anteromedial longitudinal incision of about 3 cm was performed. The medial collateral ligament was identified and served as a landmark, and the growth plate of the proximal tibia was clearly distinguished as a white line, later confirmed by radiography. A drill bit with a diameter of 1 mm was perpendicularly directed to the growth plate and parallel to the joint to induce a defect of about $(5 \times 5 \times 1)$ mm (width, depth, and length, respectively) according to Yu et al. (2019) (Figures 1B and C). Saline was irrigated through the drill track to cool and rinse out debris during drilling. The contralateral limb (right limb) of the same animal served as the control and was left untreated. Meanwhile, the PRF membrane (10 × 5 mm) (length and width, respectively) was inserted into the growth plate defect of the left limb (Figure 1D). Routine closure was performed, and antibiotics (Penicillin, 800,000 units administered intramuscularly twice a day for three days postsurgery) were administered until complete recovery was achieved. Stitches were removed 7-10 days post-surgery.

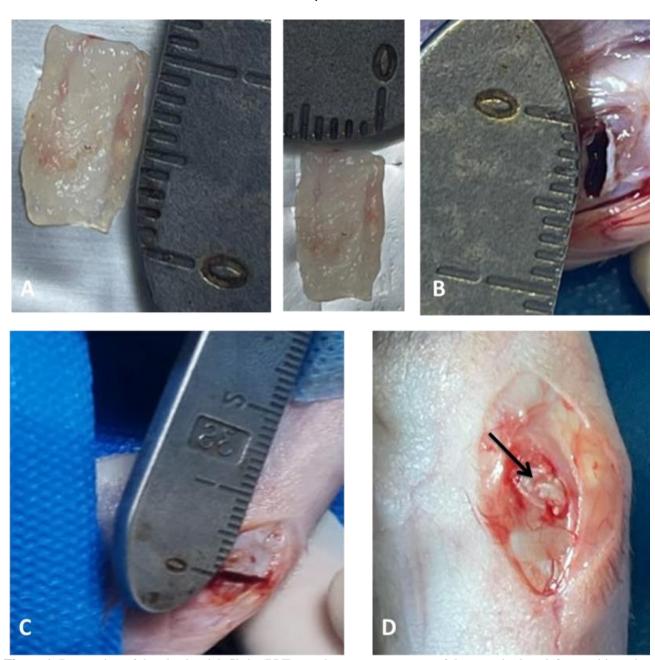


Figure 1. Preparation of the platelet-rich fibrin (PRF) membrane, measurement of the growth plate defect, and insertion of the PRF membrane into the growth plate defect in an immature White New Zealand rabbit. **A:** Platelet-rich fibrin membrane used in the defects. **B:** The length of the growth plate defect is 5mm. **C:** The width of the growth plate defect is 1 mm. **D:** The 10×5 mm area of the PRF membrane is inserted (black arrow) into the growth plate defect.

Radiological examination

An anteroposterior view of both hind limbs was examined after anesthetizing the animal with a DR machine (50 kV and 40 mA, Beam Limiting Device [Eco Ray] X-ray machine, Korea). The radiological examinations were conducted weekly up to eight weeks post-surgery. The opacity within the growth plate defect (ranging from a clear defect with radiolucent opacity to mild, moderate, and complete ossification), the growth plate area (normal, narrower, or wider than the adjacent unaffected growth plate), as well as a bony bar, shortening, and angulation of the affected limb (Figure 2) were evaluated weekly during the first, second, third, fourth, sixth, and eighth weeks and compared to the contralateral limb of the same kits within the population.

To diminish the effect of rabbit kit sizes on the tibial length measurement, images were calibrated before analyzing the tibial length and angle for both the affected and control limbs of each animal individually using ImageJ software (1.47v with Java 1.8.0-201). The tibial length was measured as the distance between the tibial distal growth plate and the proximal tibial plateau at 50% of the full width, while the tibial angle was assessed as the angle between the leg length measure and the average angle across the entire plateau. Tibial length and angle changes were assessed against the contralateral limb of the same animal within the whole population in line with Yu et al. (2022).

Statistical analysis

GraphPad Prism 5 (for Windows-Version 5.03) was used for data analysis with descriptive statistics including mean, standard deviation, and range as well as paired t-test at the level of $p \le 0.05$. The data passed the normality test using the Gaussian distribution (Kolmogorov-Smirmov test with Dallal-Wikinson-Liliefor P value), and all data were confirmed to follow a normal distribution ($\alpha = 0.05$).



Figure 2. An anteroposterior (AP) radiograph view of an immature male White New Zealand Rabbit. Demonstrating the calculation of tibial length (L), identified as the distance between the tibial distal growth plate and the proximal tibial plateau at 50% of the full width. The tibial angle is measured as the angle formed between the length of the limb and the entire plateau (A).

RESULTS AND DISCUSSION

Rabbit kits were gradually returned to their feeding and drinking water a few hours after full recovery from anesthesia with normal movement and posture. The degree of the growth plate defect induced in this study has been previously classified as Salter's types III and/or IV. For this reason, leaving the defect without treatment would lead to bony bar formation as both epiphysis and metaphysis areas are affected (Xian et al., 2004; Jaimes et al., 2014; Yu et al., 2019).

In the current study, when the left affected limbs were radiographically examined via anteroposterior view presurgery, images showed normal growth plate shape and opacity within the proximal tibial growth plate. However, immediately post-surgery, the images showed an obvious defect in all subjects, with a mild increase in its opacity and a clear defect area within the proximal tibial growth plate, located medially after applying the PRF membrane (Figure 3). The anteroposterior view was also considered for identifying the growth plate defect area in the rabbit model by others for determining the changes within the defect (Yu et al., 2022) or the posteroanterior view in humans (Nguyen et al., 2017). In addition, Salter's second-degree classification requires an extra view, the lateral view, as the anteroposterior or posteroanterior views may not capture miner defects (Chen et al., 2015).

At the end of the first week, the defect area was visible and widened at the medial aspect of the affected limb in 73% of the subjects. Opacity within the growth plate defect remained unchanged at 45.5%, increased mildly at another 45.5%, and was significantly higher in 9% of cases treated with the PRF membrane (Figure 4A). Widening of the growth plate after trauma has been identified as indicative of a growth plate defect (Nguyen et al., 2017). Moreover, others have noted that irregularities in growth plate edges might also be associated with the widening of the growth plate after injury, indicating a growth plate defect, which is often diagnosed through more accurate techniques such as magnetic resonance imaging (MRI) (Jawetz et al., 2015) since radiography is less precise in detecting minor or early defects, such as fibrous

bar (Ecklund and Jaramillo, 2001; Jawetz et al., 2015). Furthermore, the anti-compression pad effect of the PRF membrane observed in this study might initially result in high opacity within the defected area.

By the second week post-surgery, 29% of cases of defect treated with PRF membrane retained visible defect borders, while 71% exhibited slight narrowing of the defect compared to the contralateral limb of the same animal. Radiographically, moderate opacity was observed in 57% of cases, mild opacity in 14%, and no detectable opacity in 29% (Figure 4B). The opacity and narrowing area of the growth plate defect, observed in about 71% of cases, possibly demonstrates the existence of the PRF membrane as a pad. However, the effect of releasing platelet-derived growth factor-AB (PDGF-AB) continues until the beginning of the second week to stimulate chondrocyte proliferation (He et al., 2009; Xiao et al., 2014; Kobayashi et al., 2016). Others referred to the PRF as a biological tool that delivers growth factors and cytokines to the site of injury, representing its role in the proliferation and differentiation of chondrocytes *in vitro* and its important role in cartilage repair (Brandl et al., 2010). This likely explains the high percentage of opacity in more than two-thirds of the population observed in the present study.

Both the third and fourth weeks post-surgery in the treated groups showed the existence of the growth plate defect radiographically, with no obvious higher opacity at about 57%. However, the remaining 43% exhibited higher opacity. On the other hand, the results of the area of the growth plate defect paralleled the opacity results, showing a slight narrowing in 57%, while 43% displayed a normal growth plate area (Figure 4C). It is hypothesized that the PRF membrane persisted up to the fourth week, explaining the continued opacity up to the fourth week. Previous studies have also indicated that the PRF membrane can remain functional for up to four weeks in experimental models (He et al., 2009).

By the sixth week post-surgery, the growth plate defect treated with the PRF membrane showed a normal growth appearance with no opacity in about 57% of the total population. The remaining 4343%, however, lacked clear radiographic identification of the growth plate but showed no evidence of bony bar formation (Figure 4E). Moreover, by the end of the work (the 8th-week post-surgery), approximately 57% of the cases displayed normal growth plate

appearance without increased opacity, while 43% had some opacity and a narrow growth plate defect area (Figure 4F).

It is believed that the PRF membrane did not function as a decompressed pad by the 8th-week post-surgery but acted as a regenerative material as more than half of the total number of the treated group showed the existence of the growth plate by the eighth week. To explain the above, the decompressing effect of the PRF membrane should be ended by Week Four, as it has been suggested by others that the PRF membrane exists for a maximum of four weeks within the body after implantation (He et al., 2009). Therefore, the growth plate was not replaced by the bony bar up to the end of the study, which was similarly confirmed by Wong et al. (2020). Additionally, Dirja et al. (2023), emphasized the role of growth factors in PRF membranes in stimulating chondrocyte proliferation for improving the repair of articular cartilage defects, which was explained by the role of PRF in enhancing the viability, differentiation, and migration of chondrocytes.

Figure 3. The shape of the growth plate in the medial part of the proximal tibia of an immature White New Zealand rabbit in the anteroposterior view. Demonstrating mildly increased opacity and a clear defect area after inserting the PRF membrane immediately post-surgery.



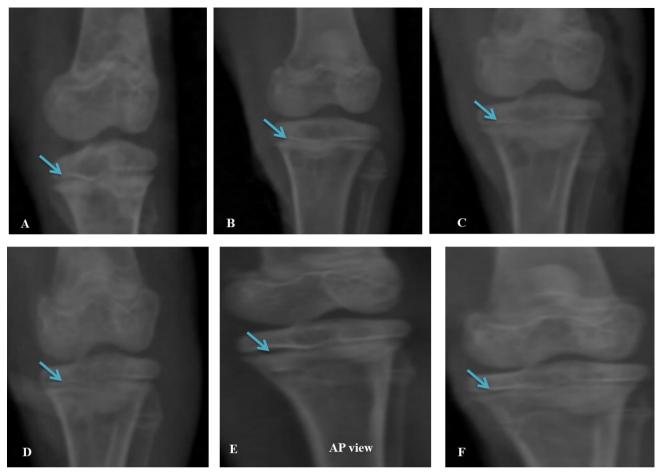


Figure 4. The growth plate defect area of an immature White New Zealand rabbit. The defect area is seen clearly (wide) in the first week post-surgery with mildly increased opacity at the medial aspect of the proximal tibial growth plate (**A**); In the second-week post-surgery, the defect area is seen slightly narrow with moderately increased opacity (**B**); the third week represents normal growth plate at the medial aspect in comparison to the lateral aspect of the proximal tibial growth plate with no obvious higher opacity (**C**); at the 4th (**D**), 6th (**E**), and 8th (**F**) weeks, the growth plate area appears normal with no obvious signs of bony bar formation from the epiphysis and metaphysis and no sign of bony bar formation at the growth plate defect.

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Figure 5. Anteroposterior view radiograph of an immature male White New Zealand rabbit representing the hind limbs eight weeks post-surgery. **L** shows the limb length, with no significant difference between the left treated limb and the contralateral limb of the same animal, and **A** shows angulation, with no significant difference between the left treated limb and the contralateral limb of the same animal, showing no sign of shortening and angulation deformities.

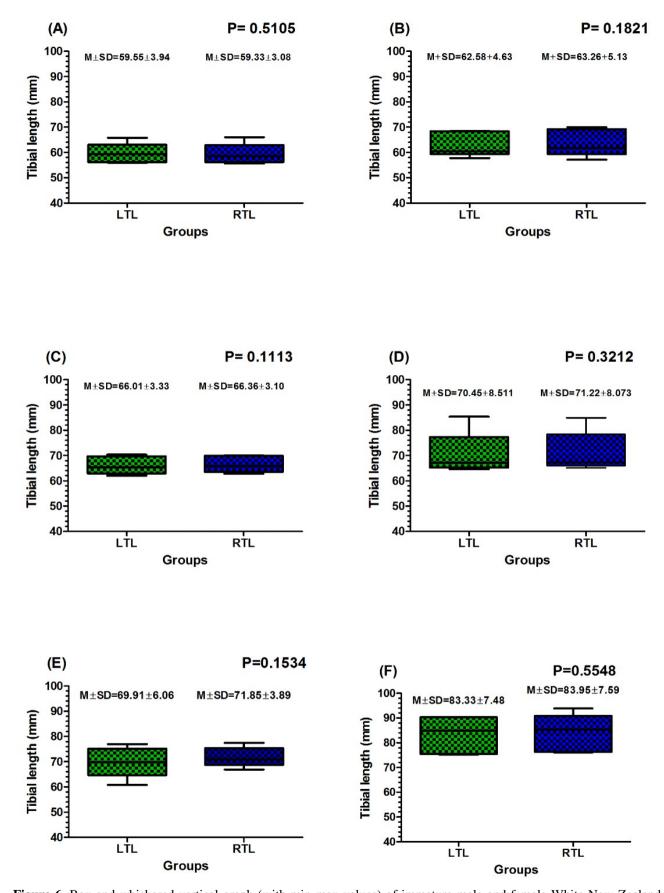


Figure 6. Box and whiskered vertical graph (with min-max values) of immature male and female White New Zealand rabbits representing the tibial length throughout the study. A: One-week post-surgery, **B**: Two weeks post-surgery, **C**: Three weeks post-surgery, **D**: Four weeks post-surgery, **E**: Six weeks post-surgery, and **F**: Eight weeks post-surgery. No significant differences (p > 0.05) were observed between the treated and contralateral limbs of the same individual White New Zealand rabbit kids. LTL: Left Tibial Length; RTL: Right Tibial Length.

P= 0.5178

P= 0.9557

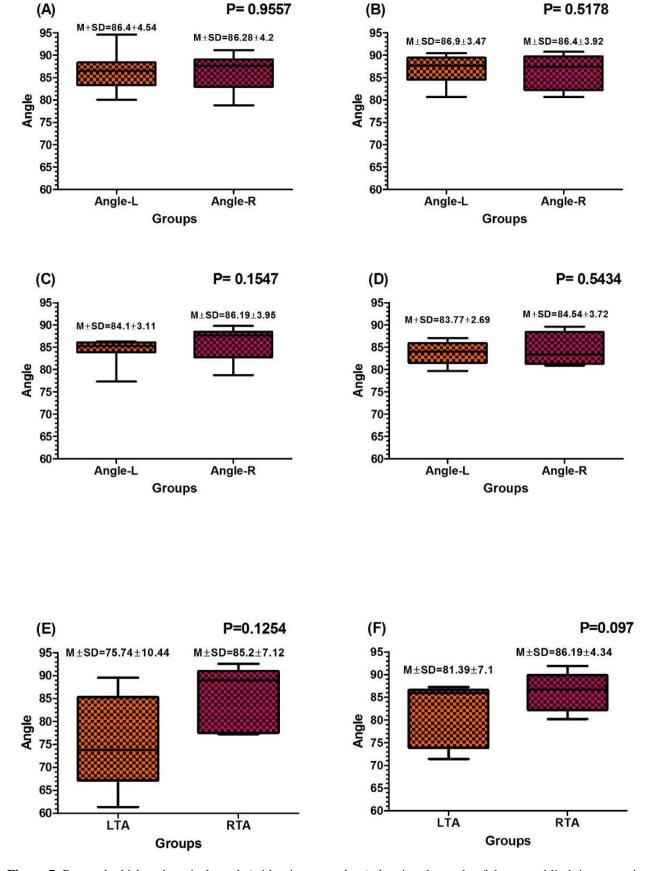


Figure 7. Box and whiskered vertical graph (with min-max values) showing the angle of the treated limb in comparison to the contralateral limb of the individual rabbit kid. There are no significant differences (p > 0.05) between the treated and contralateral limbs of the individual White New Zealand rabbit kid. A: One-week post-surgery, B: Two weeks postsurgery, C: Three weeks post-surgery, D: Four weeks post-surgery, E: Six weeks post-surgery, and F: Eight weeks post-surgery. LTA: Left Tibial Angle, RTA: Right Tibial Angle.

Radiographic evaluations of limb length and angulation throughout the study showed no significant differences between treated and contralateral limbs at eight weeks (P = 0.554 for length, P = 0.097 for angulation; Figures 5, 6, and 7). When chondrocytes of the iliac crest seeded on demineralized bone matrix as a scaffold were considered by a group of researchers, shortening and angulation were also significantly prevented when examined 16 weeks post-treatment (Jin et al., 2006). It is believed that the composition of the PRF membrane reduces angulation in the sixth- and eighth weeks post-surgery. Nonetheless, the decompressive effect of the PRF membrane during the first weeks may have a great effect in preventing the shortening of the limb and its further angulation. In contrast, other studies have used autologous bone marrow to treat growth plate defects, resulting in the osseous formation and limb shortening by the third-week postsurgery (Al-husseni, 2008). McCarty et al. (2010) used autologous bone marrow-derived mesenchymal stem cells (MSCs) for growth plate defect regeneration and showed positive results as they referred to the self-renewing and multilineage differentiation features of the mesenchymal stem cells (MSCs) for growth plate regeneration. Tobita et al. (2002) cultured autologous chondrocytes in atelocollagen gel and transplanted them into a growth plate defect, demonstrating that shortening and angulation were also significantly avoided after examination from 2 weeks up to 52 weeks. Chondrocytes implanted in atelocollagen gel may have mechanical properties to prevent a collapse with time and can proliferate and synthesize the extracellular matrix (ECM). In the present study, the shortening and angulation of the affected left limb is significantly avoided when compared to the contralateral limb in the same animals (P = 0.554). It is believed that the PRF membrane has released growth factors that might play a significant role in preventing the bony bar and reducing limb deformities. The above finding has also been supported by other studies which pointed to the effect of releasing transforming growth factor-β1 (TGF-β1) at the beginning of the third week in line with previous study (He et al., 2009) to stimulate the synthesis of ECM by chondrocytes and enhanced cartilage defect repair (van der Kraan and van den Berg, 2007). Other researchers used chitin as a scaffold for mesenchymal stem cell (MSC) transplant, which may prevent significant angulation as early as 2 weeks up to 16 weeks for shortening after excision growth plate (Li et al., 2004). It is probable that polyheterosaccharide found in chitin, similar to glycosaminoglycan (GAG), is structurally a major component of the ECM of cartilage. The angulation was also prevented. However, using other materials, such as bone wax and tissue-engineered construct (TEC), contributed to the significant prevention of angulation for up to eight weeks in rabbits (Yoshida et al., 2012), which is also explained by the significant role of bone wax as a decompressive pad from a side with extra positive results for TEC in regenerating growth plate defects. Nevertheless, using transplanted allogeneic chondrocytes for repairing growth plate defects may reduce bone angulation and shortening when examined for up to 16 weeks (Li et al., 2013). It is believed that these transplanted chondrocytes may release TGF-β1, playing a role in the chondrogenesis of bone mesenchymal stem cells (BMSCs) into chondrocytes to repair the injured growth plate and preventing collapsed growth plate defects and bony bar formation.

CONCLUSION

The present study suggests that the PRF membrane not only functions as a pad to reduce the possibility of epiphyseal and metaphyseal gap compression but also plays a role in regenerating the chondrocytes of the growth plate. This is evidenced radiographically by the prevention of bony bar formation, likely facilitated by the growth factors contained in the PRF membrane. In addition, stem cells and the above-said mechanism may play a positive role in cartilage regeneration by stimulating chondrocyte differentiation and proliferation, preventing the angulation and shortening of the limb. To further enhance the treatment outcomes, it is recommended to combine the PRF membrane with a scaffold, which could provide additional rigidity and support in the treatment of growth plate defects.

DECLARATIONS

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Author's contributions

Sura H. Abd-Alkhaleq and Aseel Kamil Hussein contributed to the planning and performed the study procedures as study design and writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The authors confirm that all data supporting the findings of this study are available upon reasonable request.

Ethical considerations

This article is not submitted anywhere else, and the findings are analyzed and written under the supervision of all authors. All authors wrote the article and checked the last draft of the manuscript for the similarity index.

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The Toxicity Assessment of Chicken Liver-Based Drug Filler Supplemented with Itraconazole

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ABSTRACT

Itraconazole is a systemic antifungal often used for fungal infection treatment in cats. This study aimed to evaluate the safety of chicken liver paste as a drug filler through an acute toxicity test when supplemented with itraconazole. A total of 25 female mice were used and divided into five groups, each consisting of five mice. The control group received chicken liver paste without any itraconazole supplementation. In contrast, the treatment groups were administered chicken liver paste supplemented with itraconazole at 5, 10, 15, and 20 g/kg body weight dosages. Observations were conducted for two weeks. The evaluated parameters included abnormal clinical signs, mortality, body temperature, weight gain, and hematology profile. During the 14 days of observation, no mortality or abnormal clinical signs were observed. Other parameters such as body temperature and weight increase showed no significant difference. Hematology profile including red blood cells (RBC), hemoglobin (HB), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), thrombocyte (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), white blood cells (WBC), lymphocytes, monocytes, and granulocytes remained within the standard ranges and showed no significant difference. The present study indicated that chicken liver paste supplemented with itraconazole falls into the practically nontoxic category with an estimated LD50 value exceeding 20 g/kg body weight.

Keywords: Chicken, Hematology, Itraconazole, LD50, Liver, Mice

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INTRODUCTION

In recent years, there has been an increase in pet ownership all over the globe. Pets, such as dogs and cats, provide companionship and passionate support, regularly considered integral members of the family. While owning pets comes with numerous benefits, it also entails responsibilities, particularly in maintaining their health. It is estimated that there are roughly around 470 million dogs and 370 million cats owned and kept as pets worldwide (Jalongo, 2021). Numerous observational studies suggest that interactions and relationships with companion animals may be beneficial for human mental and physical health, with hypothesized mechanisms involving attachment to and companionship provided by pets (Brooks et al., 2018).

Pets require regular visits to veterinarians periodically, either for preventive care or for treating diseases (Suchodolski, 2024). Cat owners frequently report challenges in administering medicine, such as cats refusing to take medicine even though it is blended with food, spitting out the medicine, or experiencing hypersalivation (Sivén et al., 2017). These challenges may lead to injuries for pet owners trying to convince unwilling cats to take medication. Additionally, failing to complete prescribed treatments can have broader health implications, including the development of antimicrobial, antifungal, and antiparasitic resistance, which is a growing concern in both veterinary and human medication (Joosten et al., 2020). One of the ways to make oral drug administration easier in cats is to ensure that the medicines are palatable (Taylor et al., 2022). The palatability of cat food is often related to flavor, smell, shape, texture, and mouthfeel sensation. If the medicine formulation suits the palatability, cats are more likely to consume it voluntarily (Petry et al., 2014). Cats are known to seldom ingest the medication in oral form such as tablets, especially if the drug is bitter or odorous (Ekweremadu et al., 2020). Cats are originally solitary hunters and obligate carnivores which often kill much smaller prey than their body size. Smaller prey is often consumed in one portion, with the flesh of larger prey ripped off and the organs such as the liver are eaten (Aldrich and Koppel, 2015). Despite being classified as carnivores, cats have specific dietary requirements, especially for protein, with a 30% protein daily requirement (Watson et al., 2023). According to the U.S. Department of Agriculture (2019), a 100-gram portion of chicken liver contains 66.8 g of water, 24.5 g of protein, 6.51 g of total lipid (fat), 1.36 g ash content, and 0.87 g of carbohydrate. Chicken liver is a

nutrient-rich food, high in protein, and is palatable to carnivorous animals (Pinto et al., 2021). Given these properties, chicken liver could serve as an ideal palatable drug filler for administering medications to cats, particularly in a semi-solid or paste form, which may be more convenient for both cats and their owners than tablets or capsules.

Skin lesions due to fungal infestation or dermatophytosis are common diseases and common health issues for cats (Thakare et al., 2019). Itraconazole, a systemic antifungal agent, is often used in the management and treatment of fungal infections. It is of the high frequently used in treating cases of dermatophytosis and is particularly effective and safer than other antifungals such as ketoconazole for treating dermatophytosis in cats (Thakare et al., 2019). This study aimed to evaluate the safety of chicken liver paste supplemented with itraconazole as a drug filler via acute toxicity tests in mice

MATERIALS AND METHODS

Ethical approval

This study was approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences (SVMBS), IPB University, Indonesia under approval number 185/KEH/SKE/III/2024. This study was conducted at the Laboratory Animal Management Unit of SVMBS, IPB University, Indonesia. The production process of hydrolyzed chicken liver was conducted at Nutricell Pacific, Indonesia, and chicken liver formulation as a drug filler was conducted at the Pharmacy Laboratory of the School of Veterinary Medicine and Biomedical Sciences (SVMBS), IPB University, Indonesia.

Formulation of chicken liver paste

The base formulation was developed using 3% xanthan gum, 5% propylene glycol, 0.1% Ethylenediaminetetraacetic acid (EDTA), and aquadest. The base formulation of the paste was prepared by dispersing gelling agent xanthan gum in distilled water containing dissolved propylene glycol and EDTA. The resulting mixture was heated at a temperature of 60°C for 5 minutes and stirred continuously until it formed a gel. Afterward, 10% chicken liver powder was added using a mortar and pestle. The base of chicken liver paste formulations was labeled as F0, while the paste supplemented with itraconazole was labeled as F1, as shown in Table 1 and Figure 1.

Acute toxicity test of chicken liver paste

The acute toxicity test followed the method of determining the lethal dose (LD50) according to the BPOM (2014) guidelines for determining the toxicity of chicken liver paste supplemented with itraconazole. To reduce stress levels, the mice were adapted to the environment for 14 days before the study. They received an oral dose of ivermectin (0.04 mg/kg body weight) as an antiparasitic agent on the first day of acclimation (Jusuf et al., 2023).

A total of 25 female mice acclimatized to the environment, were divided into five groups, each consisting of five animals. Group 1 (the control group) was given the base chicken liver paste (F0). The other four treatment groups (Groups 2, 3, 4, and 5) were administered chicken liver paste supplemented with itraconazole at doses of 5, 10, 15, and 20 g/kg body weight mice via oral gavage as shown in Figure 2. Each mouse received a single oral gavage dose on the first day of the study. During the 14 days of the study, the mice were monitored for any abnormal clinical signs, which included inappetence, skin changes such as inflammation signs, behavioral abnormalities, irregular breathing, abnormal stool consistency, abnormal signs of urinating, incoordination, and hypersalivation. Average body temperatures and mortality rates were also observed. Their body weights were measured on days 0, 7, and 14, and increases in body weight were calculated from days 0 to 7 and from days 7 to 14. At the end of the 14-day observation period, a blood sample (1 mL) was collected for hematological analysis.

Hematology analysis

One mL of blood was taken intracardiac using an EDTA tube on day 14 to analyze the hematology profile. The hematology profile was analyzed using a hematology analyzer (Vetscan HM5, PT. Mega Utama Medica, Indonesia). The parameters analyzed included red blood cells (RBC), hemoglobin (HB), white blood cells (WBC), lymphocytes, monocytes, granulocytes, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), thrombocyte (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and plateletcrit (PCT).

Data analysis

Data was analyzed using Analysis of Variance (ANOVA), with a significant level set at p < 0.05, followed by the Tukey test using SPSS 20 software.

Table 1. Formulation of chicken liver as a drug filler in paste form

Materials	Role	F0 (%)	F1 (%)
Itraconazole	Antifungal	0	0.2
Chicken liver powder	Filler	10	10
Xanthan gum	Gelling agent	3	3
Propylene glycol	Binding agent	5	5
EDTA	Chelating agent	0.1	0.1
Aquadest	Solvent	81.9	81.7

The base of chicken liver paste formulations was labeled as F0 and paste supplemented with itraconazole as F1; Source: Silva et al. (2020).

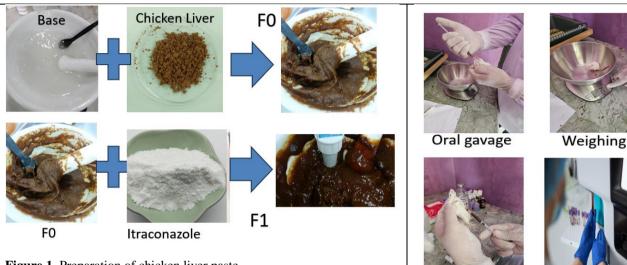


Figure 1. Preparation of chicken liver paste

Hematology **Blood sampling** Figure 2. Mice's oral gavage preparation and evaluation of body weight, blood sampling, and hematology.

RESULTS AND DISCUSSION

Acute toxicity (LD50)

Determination of toxic range is expressed by Lethal Dose 50 or LD50. If a substance with a dose of 20 g/kg of body weight does not cause mortality and toxicity clinical signs up to 14 days posttreatment, then the substance can be categorized as practically non-toxic (BPOM, 2014). In this study, none of the mice showed any mortality during the 14 days of observation, indicating that the substance falls into the practically nontoxic category, with an LD50 value greater than 20 g/kg body weight.

The results concerning the observation of abnormal clinical signs are presented in Table 2. None of the mice showed any abnormal clinical signs during the 14 days of observation. All mice showed normal behavior, normal water, and feed intake, no abnormal skin changes, regular breathing, normal stool consistency, and no abnormal signs of urinating. Furthermore, there were no signs of incoordination, convulsion, paralysis, tremors, or hypersalivation. These findings suggest that the administration of chicken liver paste supplemented with itraconazole did not cause any abnormal clinical signs.

Table 2. Body weight increase of mice per week during the observation period

Treatment group	BWI 1 (g)	BWI 2 (g)
Group 1	2.00 ± 1.10^{a}	1.67 ± 0.85^{a}
Group 2	1.50 ± 0.85^{a}	2.00 ± 0.89^{a}
Group 3	2.00 ± 0.63^{a}	$2.00.33 \pm 0.82^{a}$
Group 4	2.50 ± 0.84^{a}	1.67 ± 0.82^{a}
Group 5	2.33 ± 0.82^{a}	1.50 ± 0.55^{a}

Different superscript letters in each column indicate significant differences (p < 0.05). BWI: Body weight increase, BWI 1: Body weight increase during the first week; BWI 2: Body weight increase during the second week

The average body temperature of the mice during the observation period for Groups 1 to 5 were $36.97 \pm 0.32^{\circ}$ C, $37.15 \pm 0.55^{\circ}$ C, $36.92 \pm 0.32^{\circ}$ C, $36.83 \pm 0.44^{\circ}$ C and $37.183 \pm 0.30^{\circ}$ C, consecutively. Based on the result, no significant differences in body temperature were observed between the groups (p > 0.05). The average body temperature ranged from 36.8 to 37.1° C. It is worth noting that changes in body temperature can show signs of toxicity. Drugs and all types of substances with toxic potential can affect the body temperature of animals. This change in body temperature is mostly shown with hyperthermia (Mozafari et al., 2016). According to Ribeiro et al. (2022), the normal range of body temperature in mice falls within the range of 36.5- 38.0° C. Considering the fact that the temperatures shown by experimental animals fell within this range, it can be suggested that the administration of chicken liver paste supplemented with itraconazole did not cause any fluctuation in the body temperature of the mice.

Body weight increases per week are shown in Table 2. The purpose of observing this parameter was to find out whether the mice experienced fluctuated body weight increase after the administration of preparation. Conditions that indicate the experimental animals had, in general, experienced any kind of pain or suffering could be seen if the body weight had decreased by more than 20% in seven days or more. Animals with no experience of fluctuating changes in body weight indicate that they have not experienced pain or suffering after the administration of the substance (Nurfaat, 2016). Based on the result, the animals did not experience any body weight decrease but experienced a mean increase instead. This increase does not show any significant difference between the control group and the treatment groups. This finding indicated that the administration of chicken liver paste supplemented with itraconazole did not alter the growth of the mice under study.

According to the National Center for Biotechnology Information (NCBI, 2024), itraconazole has different LD50 values depending on the animal species. For instance, in rats and mice, the LD50 is greater than 320 mg/kg, while in dogs and guinea pigs, it is greater than 200 mg/kg and 160 mg/kg, respectively. In the present study, the chicken liver paste supplemented with itraconazole in mice showed a much higher safety range (greater than 20,000 mg/kg or 20 g/kg) compared to previous studies due to the dilution effect of the chicken liver, which acts as a drug filler. The low dose of itraconazole in this formulation likely contributed to the low toxicity observed, making it easier and more precise to administer compared to commercially available higher doses (e.g., 100 mg tablets).

Hematology profile

Hematology analysis was performed on day 14 by collecting 1 ml of blood from each mouse. The results of the hematology profile are shown in Table 3. No significant differences were observed between the groups (p > 0.05), and all parameters fell within the normal range. Hematological and clinical biochemical parameters are critical markers of the overall health status of animals and can be used to investigate the toxicity of drugs and chemicals (Niyomchan et al., 2023). Available evidence has shown that the consumption of toxic plants or agents can cause alterations in the hematological profile (Zahmati and Saljooghi, 2016). The results of this study showed no significant differences between the control group and the treatment groups, with almost all hematological parameters still falling within the normal range as compared to a study done by Haney et al. (2019).

The only hematology profile parameter that was not within the normal range was MCHC (mean corpuscular hemoglobin concentration, g/dL) in Group 4, which received the highest dose of chicken liver paste supplemented with itraconazole. However, it is not significantly different compared to other groups (p > 0.05). The MCHC in Group 4 was 32.61 g/dL, slightly above the normal range of 22.3-32 g/dL. MCHC is the hemoglobin concentration in a group of red blood cells (Cascio and DeLoughery, 2017). MCHC values above the normal range may indicate hyperchromic anemia, which can be caused by the hemolysis of red blood cells, causing the release of hemoglobin into the plasma, which ultimately leads to hemoglobinemia (Lepkov et al., 2023). This is mostly caused by vitamin B12 deficiency. Although the MCHC value in Group 4 was found to be slightly above the normal range, Hb and RBC counts were still within the normal range. This may indicate a small amount of vitamin deficiency, which will not affect overall health.

Itraconazole is an orally administered triazole antifungal agent used in the treatment of systemic and superficial fungal infections. Itraconazole therapy is associated with transient, mild-to-moderate serum elevations and can lead to clinically apparent acute drug-induced liver injury. The liver injury from itraconazole typically presents 1 to 6 months after starting therapy with symptoms of fatigue and jaundice (NCBI, 2024). Jaundice is, in fact, the yellow color of skin and mucous membranes due to the accumulation of bile pigments in blood and their deposition in body tissues. The hematology profile may provide evidence for hemolysis by demonstrating anemia (Stillman et al., 1990). According to the World Health Organization (WHO), anemia is defined as a condition in which the hemoglobin content is below normal. Based on Table 3, the hemoglobin (Hb) level for all treatments was within the normal range of 11-15.10 g/dL with no significant difference (p > 0.05). This result indicates that the chicken liver paste supplemented with itraconazole in mice would not develop anemia.

Table 3. The hematology profile of the female mice treated with Chicken Liver-Based Drug Filler Supplemented with Itraconazole

Treatment groups Hematological parameters	Group 1 (negative control)	Group 2 (5 g/kg body weight)	Group 3 (10 g/kg body weight)	Group 4 (15 g/kg body weight)	Group 5 (20 g/kg body weight)
RBC $(10^6/\mu L)$	7.57 ± 0.55^{a}	8.44 ± 0.56^{a}	8.79 ± 0.15^{a}	7.99 ± 0.85^{a}	8.21 ± 0.54^{a}
Hb (g/dL)	12.03 ± 0.31^{a}	13.03 ± 1.21^a	11.86 ± 0.53^{a}	11.44 ± 0.76^{a}	12.81 ± 0.82^{a}
Hct (%)	37.53 ± 2.11^{a}	39.48 ± 2.35^{a}	40.64 ± 3.22^{a}	39.39 ± 1.23^{a}	36.43 ± 2.88^{a}
MCV (fL)	50.54 ± 2.82^{a}	49.87 ± 0.95^{a}	50.40 ± 0.33^{a}	49.13 ± 1.15^{a}	50.30 ± 0.87^{a}
MCH (pg)	17.34 ± 0.42^{a}	16.72 ± 0.74^a	15.97 ± 1.45^{a}	17.10 ± 0.35^{a}	16.80 ± 0.95^a
MCHC (g/dL)	31.9 ± 1.07^{a}	30.73 ± 1.53^{a}	31.40 ± 0.45^{a}	31.09 ± 1.65^{a}	32.61 ± 0.49^{a}
RDW (%)	15.32 ± 0.48^{a}	16.00 ± 0.85^{a}	15.10 ± 0.78^{a}	16.50 ± 1.39^{a}	16.00 ± 0.72^{a}
PLT $(10^3/\mu L)$	1063.13 ± 21.35^{a}	1065.33 ± 18.35^{a}	1079.67±16.45 ^a	1070.70 ± 19.88^{a}	1071.67 ± 16.56^{a}
MPV (fL)	4.81 ± 1.30^{a}	5.03 ± 0.93^{a}	4.79 ± 0.65^{a}	4.93 ± 1.05^{a}	4.90 ± 1.55^{a}
PDW (%)	17.02 ± 0.22^{a}	16.70 ± 0.85^{a}	16.63 ± 0.88^{a}	17.13 ± 0.22^{a}	16.60 ± 0.85^{a}
PCT (%)	0.27 ± 0.12^{a}	0.21 ± 0.15^{a}	0.24 ± 0.05^{a}	0.32 ± 0.25^{a}	0.24 ± 0.11^{a}
WBC $(10^3/\mu L)$	5.35 ± 1.02^{a}	5.23 ± 0.55^{a}	6.13 ± 1.02^{a}	5.73 ± 0.66^{a}	5.70 ± 0.78^{a}
Lymphocyte (10 ³ /μL)	4.23 ± 0.34^{a}	4.08 ± 0.50^{a}	4.67 ± 0.51^{a}	3.93 ± 0.65^{a}	4.1 ± 0.31^{a}
Monocyte $(10^3/\mu L)$	0.25 ± 0.01^{a}	0.24 ± 0.05^a	0.23 ± 0.04^{a}	0.27 ± 0.08^a	0.23 ± 0.02^{a}
Granulocyte (10 ³ /μL)	1.50 ± 0.03^{a}	1.57 ± 0.07^{a}	1.54 ± 0.15^{a}	1.6 ± 0.23^{a}	1.56 ± 0.76^{a}
Lymphocyte (%)	71.42 ± 3.22^{a}	72.33 ± 2.24^{a}	71.30 ± 3.02^{a}	72.53 ± 3.25^{a}	73.40 ± 3.12^{a}
Monocyte (%)	2.09 ± 1.16^{a}	2.23 ± 0.98^a	2.10 ± 1.03^{a}	2.06 ± 0.22^{a}	2.31 ± 0.05^{a}
Granulocyte (%)	43.30 ± 2.16^{a}	40.43 ± 4.45^a	43.38 ± 3.12^{a}	42.87 ± 2.34^a	41.77 ± 1.78^{a}

Note: Different superscript letters in same row indicate significant differences (p < 0.05). Group 1: Control negative; Group 2 (5 g/kg body weight); Group 3 (10 g/kg body weight); Group 5 (20 g/kg body weight); RBC: Red blood cell; Hb: Hemoglobin; Hct: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width; PLT: Platelet; MPV: Mean platelet volume; PDW: Platelet distribution width; PCT: Plateletcrit; WBC: White blood cell

CONCLUSION

An acute toxicity test on chicken liver paste supplemented with itraconazole did not show any mortality or abnormal clinical signs during the 14 days of observation. Parameters of body temperature, body weight increase, and hematology profile also showed no significant differences. These findings indicated that chicken liver paste supplemented with itraconazole falls into the category of practically nontoxic medications, with an LD50 value greater than 20 g/kg body weight. It is recommended that a long-term toxicity evaluation be conducted to further assess any potential effects and side effects.

DECLARATIONS

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Authors' contributions

Prapatantio Teteg Pringgodigdoyo and Lina Noviyanti Sutardi performed the experiments and data analysis for the chicken liver-based drug formulation. Wasmen Manalu designed and drafted the experimental model and the manuscript. Andriyanto and Aulia Andi Mustika performed the experiments and data analysis for acute toxicity (LD50). The manuscript was read and approved by all authors.

Competing interests

The authors declare that there are no conflicts of interest.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Incidence and Risk Factors of *Toxocara vitulorum* Infection in Beef Cattle of Yogyakarta, Indonesia

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ABSTRACT

Toxocara vitulorum (T. vitulorum), an Ascarid nematode, infects the small intestine of cattle and buffalo, particularly in newborn calves. The present study aimed to identify the occurrence of T. vitulorum collected from cattle in Yogyakarta, Indonesia, and to examine the surface structure of its eggs by scanning electron microscopy (SEM). The present study did not observe asymptomatic clinical signs of toxocariasis, including diarrhoea and weight loss. Fecal samples were collected from 247 cattle of various breeds, consisting of 65 males and 182 females across three regions including Bantul (78 cattle), Sleman (63 cattle), and Kulon Progo (106 cattle). Qualitative and quantitative methods, including flotation and modified McMaster methods, were respectively employed to analyze nematode egg counts. SEM was utilized to characterize the surface morphology of T. vitulorum nematodes. A total of 9 cattle were found to excrete T. vitulorum eggs in their feces (3.64%). The average fecal egg count was 2.861 eggs per gram (EPG), with positive cases observed exclusively in female cattle. The risk factors influencing toxocariasis in this study were breeds and frequency of cleaning the stall. A higher odd ratio of T. vitulorum infection was found in mixed Ongole breeds than in Limousin or Simmental breeds. Moreover, cattle housed in rarely cleaned stalls showed a higher odd ratio than those in regularly cleaned ones. Factors such as age, fecal consistency, and population density factor showed no significant association with toxocariasis. The SEM analysis of T. vitulorum eggs revealed an oval shape with distinct surface ornamentations, including interlocking ridges and depressions. The cage cleanliness and cattle breed were the most common risk factors associated with infected

Keywords: Ascarid, Cattle, Prevalence, Risk factor, Scanning Electron Microscopy, Toxocariasis

INTRODUCTION

Helminth infections in livestock cause suboptimal growth, weight loss, reduction in feed conversion rate, decreased endurance, decreased reproductive capacity, and decreased carcass quality (Hamid et al., 2023). Such substantial economic and health impacts have placed helminthiasis among the strategic diseases in Indonesia (Winarso et al., 2015). Toxocara vitulorum is a gastrointestinal helminth from the neoascaris group, and the adult stage of T. vitulorum is frequently observed in calves. It has also been found to infect buffaloes, cattle, and zebu, and is particularly prevalent in tropical and subtropical regions (Dewair and Bessat, 2020). Toxocara vitulorum causes economic losses, especially in cattle and buffaloes, due to its high mortality rate of up to 37.3%, as reported by Rast et al. (2014). Clinical manifestations include anemia, diarrhea, weight loss, anorexia, and small intestine obstruction (El Shanawany et al., 2019). Toxocara vitulorum has direct life cycles without an obligate intermediate host, but its transmission is more complex than simply ingesting infectious eggs (Bowman, 2020). While ingestion of eggs containing two-stage larvae is a common route of infection, the parasite can be transmitted through ingestion of larvae in the mother's milk (Urhan et al., 2023). A female worm can lay up to 200,000 eggs per day, with eggs exhibiting thick walls that allow them to withstand extreme environmental conditions, such as heat and drought for a prolonged period (Roberts, 1990; Delling et al., 2020). Toxocara vitulorum worm eggs can live in the environment for up to two years (Ziegler and Macpherson, 2019). The eggs will hatch into their first, second, and third larval stages in moist and warm environments. The larvae require a developmental period of 7-12 days at a temperature of 28-29°C (Sihombing and Mulyowati, 2018; Aboamer et al.,

Toxocariasis is widespread across all regions of Indonesia (Purwandani et al., 2021). Nevertheless, its prevalence in the Yogyakarta region and the associated risk factors have not been reported to date. Yogyakarta is a province situated

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on the island of Java with the fourth largest cattle population (Statistics ICBo, 2022). Toxocariasis predominantly affects cattle or buffalo calves, with a reported frequency of 45% in young animals (Biswas et al., 2022). Calves aged 1-2 months exhibit higher mortality rates up to 50% if the disease is left untreated (Biswas et al., 2021). In contrast, older animals (over six months) demonstrate increased resistance to infection (Ziegler and Macpherson, 2019). The rainy season has the highest transmission rate because calves become infected with parasitic worms after ingesting worm eggs while grazing on expansive and contaminated pastures (Davila et al., 2010).

Toxocara vitulorum egg structure is protected by a thick wall, making it resistant to dry environments (Aboamer et al., 2019). In Indonesia, a tropical country with a humid climate and consistent year-round precipitation, toxocariasis remains particularly challenging to control and eradicate. This parasitic disease is commonly found in bovine and bubaline calves (Winarso et al., 2015). Third-stage larvae (L3) present in colostrum can potentially infect newborn calves. Infective eggs swallowed in the digestive tract will grow into L3 in animals older than 6 months of age (Ziegler and Macpherson, 2019).

The respiratory organ can be affected by the migration of L3, potentially leading to pneumonia. Other symptoms that may occur include difficulty passing stool, fluid loss, reduction in body mass, and swelling beneath the jaw (Davila et al., 2010). Visceral larvae migrants in adult cattle induced by *T. vitulorum* are often asymptomatic (Davila et al., 2010). *Toxocara vitulorum* larval migration in calves can induce liver and lung damage (Ziegler and Macpherson, 2019). Furthermore, the presence of adult worms in the small intestine can cause diarrhea, weight loss, and, in severe cases, mortality, which predominantly affects young animals. A significant proportion of calves up to 5 months of age are susceptible to toxocariasis when poor maternal hygiene facilitates the transmission of *T. vitulorum* through colostrum. Several drugs, including piperazine, pyrantel, febantel, and oxfendazole, are effective against *T. vitulorum* in its adult stage (Ziegler and Macpherson, 2019). Pyrantel and levamisole are both efficacious anthelmintic agents for eliminating *T. vitulorum* third-stage intestinal larvae (Ziegler and Macpherson, 2019; Afshar et al., 2023).

Several factors, including geography, season, age, gender, bodily condition, fecal consistency, nutritional status, husbandry techniques, etc., might impact the prevalence of toxocariasis. Numerous factors have been recognized as potential contributors to *T. vitulorum* infection across diverse geographical regions worldwide, including age, gender, body condition, season, and fecal consistency (Woodbury et al., 2012; Biswas et al., 2021). *Toxocara vitulorum* infection is associated with these factors; hence, studying the correlation between its prevalence and the associated factors is essential for developing strategies to mitigate the economic losses caused by this parasitic infection. Despite its significance, detailed morphological studies of *T. vitulorum* eggs using scanning electron microscopy (SEM) remain limited. SEM can reveal intricate details of the eggshell surface, including any ridges, pores, and curved structures. It has much higher magnifications than light microscopy, enabling the visualization of unique structures. This study aimed to provide valuable information on *T. vitulorum* prevalence, factors influencing infection, and detailed morphological characteristics of the parasite eggs.

MATERIALS AND METHODS

Ethical approval

This research was conceptualized and executed in compliance with animal welfare regulations promulgated by the Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia.

Materials

The tools used in this study included a scanning electron microscope (JSM-6510LA, Japan), microtubes, a vacuum critical drier, a gold coater (JEOL JEC-3000FC, Japan) object glasses, cover glasses, double object glasses, a magnetic stirrer, test tube racks, centrifuge tubes, a centrifuge, a microscope, pipettes, and syringes. Stool samples were processed using saturated sugar solution (prepared by mixing sugar from Gulaku, Indonesia, with aquadest until a specific gravity of 1.3 was reached), saturated NaCl solution (prepared by mixing salt from Refina, Indonesia, with aquadest, and aquadest. A questionnaire was used to collect research variables such as cattle breed, age, sex, stool consistency, management, and environmental variables. The authors designed the questionnaire consisting of a Likert scale and multiple-choice questions. Moreover, personal interviews with farmers were conducted to fill out the questionnaires while visiting the farm to collect the feces. To ensure validity, the questionnaire underwent expert review and content validation by authors from the Department of Parasitology.

Sampling method and sample size

In this investigation, one sample per animal was collected with a total of 247 stool samples (65 males and 182 females) from different breeds of cattle (Table 1). Three different regencies (Sleman, Bantul, and Kulon Progo) in

Yogyakarta were selected randomly from a total of 5 regencies in Yogyakarta. Two villages were chosen randomly from each regency and one cattle stall was randomly chosen. Fecal samples were collected from all cattle in the selected stalls. The feces were collected by palpation of the rectum, stored in plastic bags, and kept in a refrigerator (4°C) before the examination of samples maximally within a week. The samples were evaluated at the Laboratory of Parasitology of the Faculty of Veterinary Medicine, Universitas Gadjah Mada. Individual risk variables such as age, calf gender, cattle breed, and stool consistency were investigated. Cage management variables include cage cleaning frequency and cage density. The features of feed and water supplies were the next consideration. Sampling was distributed across six villages within the three selected districts, using a random sampling method at the sub-district, village, and breeder levels.

Table 1. The qualitative examination with the floating method and quantitative with the McMaster method on 247 samples from different breed cattle in Yogyakarta, Indonesia during May 2022 until July 2022

District		Results			
	Number of samples	Flotation method	EPG range		
Sleman	78	2.56 % (2/78)	50-9800		
Bantul	63	1.58 % (1/63)	50		
Kulon Progo	106	5.66 % (6/106)	50-9150		
Total	247	3.64 % (9/247)	50-9800		

EPG: Eggs per grams

Sample examination technique

The floating technique was used to evaluate stool samples, as described by Deplazes et al. (2016). The reference of this study is based on Zajac et al. (2021). For this technique, 3 grams of feces were placed in a mortar, mixed with sufficient water, and stirred until homogenous. The homogenous solution was then placed into a centrifuge tube (OneMed, Indonesia), filling it halfway. The centrifuge tube was then spun for 5 minutes at 3000 rpm. After discarding the clear liquid above the precipitate, saturated NaCl solution was added until ¾ parts of the tubes were mixed well. The centrifuge tube was then spun for 5 minutes at 3000 rpm. Furthermore, the centrifuge tube was subsequently placed in a rack, and saturated NaCl was added dropwise until the liquid surface formed a convex meniscus. The setup was left undisturbed for 3 minutes. After 3 minutes, an object glass slide was gently placed onto the convex surface, then promptly inverted for examination under a light microscope (Olympus, Japan) at 10×10 magnification.

For quantitative analysis, the modified McMaster technique was used. In this method, 3 grams of feces were mixed with water in a 1:14 ml of water ratio and homogenized using a magnetic stirrer. A 0.3 ml of the fecal solution was collected and deposited in the McMaster chamber, pre-filled with 1.2 g/cm3 saturated sugar. The mixture was allowed to settle for 3 minutes after homogenization with a needle. Observations were performed by counting all of the eggs in the chamber and multiplying the total number of eggs by 50 to get the total number of eggs per gram of feces.

Scanning electron microscopy

The eggs were purified and fixed in glutaraldehyde for a minimum of 35 minutes and up to three days, followed by three 5-minute double distilled water rinses. The samples were then dehydrated by suspension for 10 minutes in 30%, 50%, 70%, 80%, 90%, and three 100% ethanol treatments before being dried in a CO2 critical point drier, coated with gold, and analyzed by scanning electron microscopy (SEM; JEOL JSM-6510LA, Japan). The SEM analysis focused on comparing the morphological features of *T. vitulorum* eggs with those of other *Toxocara* species and measuring their surface characteristics.

Statistical analysis

The prevalence of infection was determined using data from the flotation technique. The collected prevalence rate statistics were examined using the following methods including Prevalence (%) = (Number of positive samples/Total number of samples) \times 100. Descriptive measurements were applied for quantitative data analysis. Univariate analysis was used to assess the effect of individual risk factors on *Toxocara* infection based on a single variable, and the strength of the connection was evaluated using the Odds Ratio (OR). Multivariate logistic regression analysis was used to identify potential risk factors associated with *Toxocara* infection based on multiple independent variables. A p < 0.05 was considered statistically significant. Descriptive analysis methods were used to examine the SEM results. All statistical analyses were performed using IBM SPSS version 26 (IBM Corporation, USA).

RESULTS AND DISCUSSION

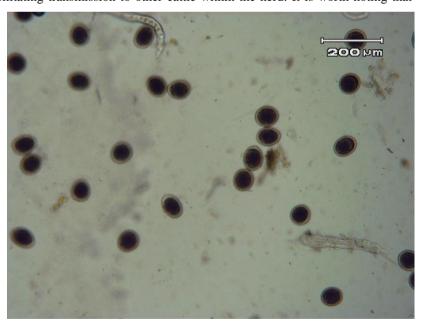
Prevalence and risk factor analysis of parasites are fundamental to understanding the epidemiology of parasitic diseases and developing effective control strategies. *Toxocara vitulorum* is difficult to manage since adult female ascarids reproduce rapidly, producing a significant number of eggs per day. Adult female ascarids are highly prolific, capable of laying a substantial quantity of approximately 110,000 worm eggs daily (Roberts, 1990; Delling et al., 2020). The eggs can remain viable for many years with protective eggshells which provide resistance against adverse climatic conditions (Venjakob et al., 2017). As a result, grazing lands can act as a permanent source of infection. Additionally, larvae migrate in the tissues, remaining dormant or hypoSE-biotic and continuing their maturation at times of stress or decreased immunity (Biswas et al., 2021).

In this study, the prevalence and risk factors of *T. vitulorum* infection in beef cattle from three districts in Yogyakarta were assessed. The overall prevalence of *T. vitulorum* infection was relatively low in the studied areas. In Sleman, only 2.56% of the samples tested positive for the infection, while in Kulon Progo, it was slightly higher at 5.66%. Out of 87 samples from Bantul, only one sample (1.58%) was positive for ascarid infection. Previous reports on *T. vitulorum* infection in Yogyakarta demonstrated a significant difference between lower-lying areas and higher altitudes. The incidence rates were found to be 12% and 19.3%, respectively (Suwito and Santoso, 2021). These findings suggest that altitude may play a role in the variation of infection rates: lower temperatures at higher altitudes may hinder parasite development (Ndamukong-Nyanga et al., 2015) Differences in soil composition, humidity, and ultra-violet (UV) radiation levels at various altitudes may impact parasite survival and infectivity in the environment (Alum et al., 2014). Notably, the McMaster method, which measures the number of eggs per gram of feces (EPG), revealed that Sleman had the highest load of *Toxocara* infection among the tested calves, with an average of 4,925 EPG, Figure 1 presents the results of fecal examination from cattle in Sleman. Kulon Progo followed with an average of 2,961 EPG. The mean EPG for *T. vitulorum* infection among calves was 2,861 EPG. Based on the results of the present study, the overall prevalence of *T. vitulorum* infection among calves was determined to be 3.64% (Table 1). According to Biswas et al. (2021), the severity of infection is categorized as light (50-500 EPG), moderate (500-1,000 EPG), and heavy (> 1,000 EPG).

In this study, cattle aged two to four years exhibited a higher prevalence of *T. vitulorum* infection compared to younger calves. This poses a greater risk of transmission to calves since cattle of that age are beginning to produce offspring, while *T. vitulorum* is mostly transmitted through larvae from lactating mothers. Furthermore, eggs can survive in the environment for up to two years, facilitating transmission to other cattle within the herd. It is worth noting that

infection in adult cattle is typically selflimiting, as frequent exposure to *T. vitulorum* induces self-cure infection (Biswas et al., 2021). Other studies have indicated that adult worms may be present in mature cattle due to immunosuppression resulting from pregnancy, infection with other pathogens, or stress-related factors (Dorny et al., 2015; Urhan et al., 2023).

Figure 1. The qualitative examination of the feces revealed the presence of a large number of *T. vitulorum* eggs from a 22-month-old cattle in Sleman, Yogyakarta. Magnification 100x



Analysis of risk factors included variables such as sex (male and female), age (< 1y, 1-2y, 2-4y, > 4y), fecal condition (normal, watery, diarrhea), breed (Ongole cross, Simental, Limousine, Simental x Ongole, Limousine x Ongole), frequency of cage cleaning (daily and weekly), and population in the cage (< 5 heads and > 5 heads, Table 2). Results showed that cleaning frequency groups across all geographic locations had a significant impact on *Toxocara* infection (Table 2). This study showed that the risk of toxocariasis infection was 10.67 times higher in calves housed in stalls that were cleaned rarely compared to those that were cleaned daily. Farmers need to maintain clean pens to reduce the risk of parasitic diseases in their cattle (Aboamer et al., 2019).

The breed of cattle was discovered to be an important risk factor, with the most at-risk races (p = 0.04) being the Simmental and Ongole crosses (OR=3.69), as well as the Ongole-cross breed (OR=2.62) compared with a Limousine-Ongole cross. Bahbahani et al.'s (2018) research has demonstrated that *Bos indicus* breeds, such as Ongole, frequently exhibit enhanced resistance to certain parasites in comparison to *Bos taurus* breeds such as Simmental and Limousine. This heightened resistance is attributed to specific immune-related genes that have undergone positive selection in tropical environments where these breeds are predominantly raised (Wang et al., 2016). Although differences in infection prevalence were observed across age groups, the association between age and infection (p = 0.57, Table 2) was not statistically significant. Similarly, no significant relationship was found between infection and fecal consistency (p = 0.87) or cage population density (p = 0.57). Comparisons with other studies provide additional context. For instance, in Central Ethiopia, *T. vitulorum* was found sporadically, with a prevalence of 2.2% (Terfa et al., 2023). The study of Bărburaş et al. (2022) demonstrated that *T. vitulorum* eggs were found in buffalo calves of various age groups, with the prevalence of infection ranging from 11% to 23% and eggs per gram values varying with age. Moreover, the presence of adult buffaloes in the same barn was identified as a risk factor for *T. vitulorum* infection in the buffalo calves. In a fatal case of toxocariasis in a yak calf aged 28-56 days in Tyrol, Austria, morphology and sequence analysis confirmed the worms as *T. vitulorum* (Schoener et al., 2020).

Table 2. The risk factors associated with 247 samples collected from different cattle breeds in the study location during May to July 2022

Variables	Number of sample	Number of positive (Percentage)	OR	p-value	
Gender					
Male	65	0 (0%)	Ref	3.34 (0.07)	
Female	182	9 (4.94%)	a		
Age					
<1 years	31	1 (3.22%)	1.70	2.003 (0.57)	
1-2 years	63	2 (3.17%)	2.98		
2-4 years	119	6 (5.04%)	1.71		
>4 years	34	0 (0%)	Ref		
Stool condition					
Normal	204	8 (3.92%)	7.93	0.27 (0.87)	
Watery	42	1 (2.38%)	2.09		
Diarrhea	1	0 (0%)	Ref		
Breed*					
Ongole cross	53	5 (9.43%)	2.62	9.77 (0.05)	
Simental	87	0 (0%)	1.28		
Limousine	11	0 (0%)	1.00		
Simental x Ongole	22	2 (9.09%)	3.68		
Limousine x Ongole	69	2 (2.89%)	Ref		
Population install					
< five cattle	134	4 (26.86%)	Ref	0.36 (0.55)	
> five cattle	113	5 (21.23%)	1.54		
Cleaning frequency*					
Everyday	137	1 (21.89%)	Ref	7.44 (0.006)	
Not everyday	110	8 (27.27%)	10.67		

Ref: Reference category. a: The response outcome is 0. OR: Odds ratio

Despite the availability of effective treatment options, *T. vitulorum* infection in calves remains a persistent challenge. A single dose of pyrantel administered at 14-21 days of age has proven effective in controlling the parasite (Rast et al., 2013; Delling et al., 2020). Rast et al. (2014) revealed poor reproduction, high calf morbidity, and mortality, coupled with limited farmer knowledge and effective control of endemic toxocariasis, hinder optimal large ruminant production in mixed smallholder farming systems in Southeast Asia. The substantial net benefit per calf attainable through a single pyrantel treatment should encourage smallholder farmers to adopt this intervention, particularly as the demand for livestock products escalates in the region, necessitating a shift towards more production-oriented farming practices.

Toxocara vitulorum lays eggs that range in form from oblong to spherical. Ripples decorate the egg's exterior and may have smaller circular or polygonal depressions that are left behind as these ridges interdigitate (Figures 2 and 3).

The present study uncovered that *T. vitulorum* eggs had characteristic ridges on the surface, similar to those found on the eggshells of the ascaridid nematodes *Ascaris lumbricoides*, *A. suum*, and *T. canis* (Ubelaker and Allison, 1975; Taira and Fujita, 1991). High-resolution imaging obtained via SEM in this study provided detailed three-dimensional visualizations of *T. vitulorum* egg surface structures. Notably, the ridge of *T. vitulorum* was found to exhibit relatively sharp profiles, neither smooth nor flat. In comparison, the ridge of *T. canis* eggs demonstrated a more uniformly connected structure (Bojanich et al., 2018). The eggs of *T. vitulorum* are rounded to oval in shape, each measuring 68-95 μm in diameter. The dimensions of *Toxocara* species eggs exhibit remarkable variations. *T. canis* ova possesses major and minor axes ranging from 71.6 to 91.2 μm and 63.4 to 79.0 μm, respectively. In contrast, *T. cati* eggs display measurements of 63.7-88.1 μm for the major axis and 53.3-73.3 μm for the minor axis. *Toxocara malaysiensis* ova, however, demonstrates dimensions of 60-68 μm by 68-76 μm for their respective axes (Kim et al., 2020). The *T. vitulorum surface* of the egg is ornamented with prominent ridges that are distinguished from other ascarid eggs. The presence of specific morphological differences between the eggshells of *T. canis* and *T. vitulorum* was reported basically in nature (Ashour et al., 1996).

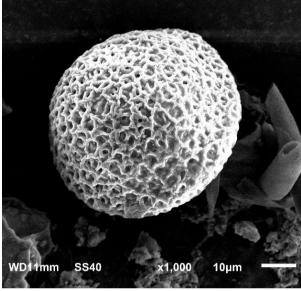


Figure 2. The surface of *T. vitulorum*'s egg isolated from Simental mix Ongole Cattle breed in Sleman, Indonesia. Magnification 1,000x

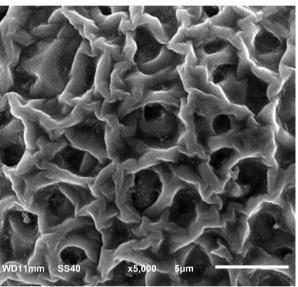


Figure 3. *T. vitulorum* isolated from Simental mix Ongole Cattle breed in Sleman, showing detailed characteristic ridges and depressions. Magnification 5,000x

CONCLUSION

The present study provided valuable insights into the prevalence and intensity of *Toxocara vitulorum* infection in cattle across three districts, revealing an overall prevalence rate of 3.64%. Among the evaluated risk factors, cage cleanliness, and cattle breed were found to be statistically associated with infection prevalence. Scanning electron microscopy revealed distinct morphological features of *T. vitulorum* eggs, including their oval shape and characteristic surface ornamentations with interlocking ridges and depressions. These findings contribute to enhancing diagnostic accuracy and facilitate the development of effective treatment or egg-elimination strategies in the environment. Future studies should focus on validating these results across diverse geographical regions and environmental conditions.

DECLARATIONS

Availability of the data and materials

All data supporting the findings of this study are available within the manuscript.

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Authors' contributions

Vika Ichsania Ninditya and Fitrine Ekawasti collected the data and drafted the manuscript, Wisnu Nurcahyo designed the study and finalized the manuscript, Joko Prastowo analyzed the data, and Irkham Widiyono reviewed and finalized the manuscript. All authors reviewed and confirmed the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

All authors have verified the ethical considerations, including plagiarism, misconduct, fabricated or false data, consent to publish double publication and/or submission, and redundancy.

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Physiological and Productive Responses to Dietary Supplementation of *Chlorella vulgaris* in Growing Rabbits

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ABSTRACT

Chlorella vulgaris (CV) acts as an immuno-modulator and growth enhancer, however, studies were concerned about its impact on growing rabbits. The present study was undertaken to evaluate CV addition on physiological responses and productive performance (feed intake, feed conversion ratio, body weight, mortality rate, and other parameters) of APRI rabbits. A total of 45 growing rabbits at their weaning age with an initial body weight of 574.8 ±11.79g were investigated. The rabbits were divided into three equal groups; the first group (G1) received a basal diet without any additions. While, the other two groups, G2 and G3 received basal diets that contained 0.5g and 1.0g CV/kg diets. The results revealed the positive impact of CV on immunity (IgA, IgM, and IgG) status, especially IgG, which was significantly higher in G3 than in G1. No negative effects of CV on kidney and liver functions, since the lowest levels of creatinine, blood urea, aspartate aminotransferase, and alanine aminotransferase were recorded in G3 compared with G1. Throughout the experimental period (8 weeks), G3 was the best group in feed intake with the lowest feed conversion ratio reflected on achieving the highest body weight compared to other experimental groups. No mortality cases were recorded in G3, while, G1 and G2 almost showed the same mortality rate (%). The histopathological examination of rabbits' intestines indicated that a less inflammation presence of rabbit intestinal cells has been noticed in G3 compared to G1 and G2. Therefore, it could be concluded that using CV at a level of 1.0 g/ kg in diet is the best level that can be used as a natural feed additive. This contributes to the health of growing rabbits by protecting their intestines against inflammation, lowering the mortality rate, and ultimately improving their overall productivity.

Keywords: APRI rabbit, *Chlorella vulgaris*, Intestinal histopathology, Physiological responses, Productive performance

INTRODUCTION

In developing countries, rabbit production is considered very important due to their unique features, such as the average of borne kits which is 40 kits/year versus one calf/year in the ruminant animals (Abdel-Rahman and Ashour, 2023). To maintain rabbit production sustainability, a critical period, that is the weaning period, must be given great attention. Rabbits at this period are facing multiple stressors, such as starting their gradual feeding on pellets that may cause digestive disorders. Additionally, they are vulnerable to immune dysfunction besides environmental stressors, including rising ambient temperature and increasing intensity of heat waves (Abdelnour et al., 2018; 2019). To overcome this critical period, natural feed additives, such as probiotics and phytogenic additives can promote rabbit growth and reduce the mortality rate around the weaning age (Abdelnour et al., 2019).

Recently, microalgae supplementation, including *Chlorella vulgaris* (CV, green algae), an unconventional source of animal feed additives has been used (Abu-Hafsa et al., 2021). It proved its incredible ability to boost animal performance by enhancing their growth rate, meat quality, immunity, and antioxidant status (Abdelnour et al., 2019). The CV contains many biological compounds, such as protein, carbohydrates, polyunsaturated fatty acids, polysaccharides, and phenolic compounds (Madeira et al., 2017; Abdelnour et al., 2019). Kang et al. (2013) and Tsiplakou et al. (2018) described CV in poultry and goats as an immuno-modulator and antimicrobial agent. Besides, CV is considered a rich protein source compared to soybean protein. It can yield 2.5-7.5 tons/hectare/year compared to 0.6-1.2 tons/ hectare/year in soybean (Abu-Hafsa et al., 2021).

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Some studies have focused on the effects of CV on rabbits' productive performance, and studies concerning its impact on immunity are rare. In Egypt, Abdelnour et al. (2019) showed that adding CV to growing rabbits' diets resulted in improving their growth rate and health. Therefore, the current study evaluated the physiological responses and productive performance to dietary supplementation of CV in APRI-growing rabbits. In addition, the economic efficiency of this supplementation was determined.

MATERIALS AND METHODS

Ethical approval

All experimental procedures followed the guidelines of the Scientific Committee of Animal Production Research Institute, coded 010303429. The current study has been conducted in the Rabbit Research Unit in Shaka Research Station, Kafr El-Sheik governorate, which belongs to the Animal Production Research Institute (APRI), Agricultural Research Center, Dokki, Giza, Egypt.

Experimental design and animal management

A total of 45 APRI rabbit bucks at their weaning age (5 weeks) with a mean initial body weight (BW) of 574.8 ± 11.79 g have been used. The rabbits were housed in galvanized wire batteries, well-ventilated and clean indoor cages. The feeding strategy was started gradually to prevent disorder in the digestive tract. The rabbits were divided into three equal groups, including 15 APRI rabbits. According to the study of De Blas and Mateos (2010), the first group (G1) received the basal balanced diet without supplementations (Table 1). The other two groups, G2 and G3 were supplied with the basal diet containing 0.5 and 1.0 g CV/kg, respectively based on Abdelnour et al. (2019) (Figure 1). Fresh and clean water (*ad libitum*) was provided freely all day time. The CV powder was obtained from the National Research Center, located in Dokki, Giza, Egypt. The feeding strategy started when the rabbits were 5 weeks old and lasted for 8 weeks. The experimental period was executed during October and November 2023.

Table 1. Feed ingredients and chemical analysis of the basal diet (Percentage of dry matter basis, as DM) provided to the APRI-growing rabbit during the experimental period

Ingredients	Percentage	Percentage Chemical analysis (1	
Barseem hay	35.00		
Barley grain	25.60	Dry matter (DM)	87.57
Soybean meal (44%)	14.50	Crude protein (CP)	17.04
Wheat bran	20.50	Organic matter (OM)	81.55
Molasses	3.00	Crude fiber (CF)	13.38
Ginger powder	0.00	Ether extract (EE)	2.275
Limestone	0.40	Digestible energy (DE, kcal/kg) ⁽²⁾	2402
Di-Calcium phosphate	0.20	Calcium ⁽²⁾	0.795
DL-Methionine	0.20	Total phosphorus ⁽²⁾	0.534
Salt	0.30	Methionine ⁽²⁾	0.445
Mineral-vitamin premix ⁽¹⁾	0.30	Lysine ⁽²⁾	0.805
Total	100		

(1): PESTMIX (Pestar company, China). Each 3 kg mixture contains: Vitamin D3 2200000 IU, Vitamin A 12000000 IU, Vitamin E 1000 mg, Vitamin B1 1000 mg, Vitamin K 2000 mg, Vitamin B2 4000 mg, Vitamin B1 210 mg, Vitamin B6 1500 mg, Pantothenic Acid 10000 mg, Niacin 20000 mg, Folic acid 1000 mg, Biotin 50 mg, Choline chloride 500 gm, Manganese 55000 mg, Selenium 100 mg, Iodine 1000 mg, Zinc 50000 mg, and carrier CaCO3, 3000 gm. (2): calculated by following De Blas and Mateos (1998). DM: Dry matter

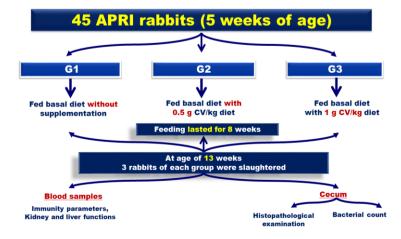


Figure 1. Experimental design of *Chlorella vulgaris* supplementation in growing rabbits' diet.

Productive performance

During the experiment, all weaned rabbits were weighed to record their initial body weight (IBW) and the final body weight (FBW). In addition, the rabbits' average daily body weight gain (ADG, g/day) feed intake (FI, g/d), and feed conversion ratio (FCR, g feed/g BW gain) were weekly recorded at 5-9, 9-13, and 5-13 weeks of their age. Besides, relative growth rate (RGR, %), performance index (PI, %), and mortality rate (MR, %) were calculated according to the following equations.

 $RGR(\%) = FBW - IBW / \frac{1}{2} (IBW + FBW) \times 100$

FCR = (g) feed/period / (g) ADG / during the same period

 $PI(\%) = FBW / FCR \times 100$

MR (%) = Number of dead rabbits / Total number of rabbits at start x 100.

Feed analytical methods

Proximate analysis of the diets was carried out through the methods of AOAC (2007). Calcium was determined by an atomic absorption spectrophotometer. Phosphorous was determined calorimetrically using a spectrophotometer (ICP Optical Emission Spectrometer, Avio 220 Max, PerkinElmer company, USA).

Carcass traits

At the end of the experiment, 3 rabbits aged 13 weeks from each group were chosen randomly for slaughtering after 12 hours of fasting. Each rabbit was weighed before slaughtering. After slaughtering, the genital organs, urinary bladder, tail, and skin were removed. Then the data of carcass and its constituents as edible parts were considered for the following formula. The average carcass weight percentage for all three groups was 48.8%. The hot parts of the carcass, main body, giblets, heart, kidney, liver, and spleen were weighed as percent of slaughter weight. Furthermore, the percentages of the abdominal fat, and gastrointestinal tract (stomach, cecum, appendix, and large intestine) were also calculated. The pH of the stomach, small intestine, and cecum was measured by a pH meter (Model 20, Digital pH meter for Orion Research).

Carcass (%) = carcass weight \times 100/live BW

Cecum bacterial count

count. Cecum bacterial count (total bacterial coliform bacteria, and lactobacilli × 10⁶) were determined using viable plate count using serial dilutions of the samples containing viable microorganisms that were plated onto a suitable growth media. A one-gram cecum sample was incubated in Tryptone Soy Agar at 37°C for 24 hours. After that, 9 ml of normal saline was labeled in test tubes 10⁻¹, 10⁻², and 10⁻⁴. Then, the nutrient agars, McConkey Agar, and MRS Agar plates from 10⁻³ to 10⁻⁴ were labeled. The samples were vortexed well to ensure bacterial distribution. After that, 1 ml of the sample was removed by a sterile pipette to transfer to a 10⁻³ dilution tube. The 10⁻¹ was vortex well and transferred 0.1 ml to a 10⁻⁴ tube. These procedures were repeated until the transfer of 0.1 ml of 10⁻⁶ to tube 10⁻⁸ and vortexed again. The agar plates must be dried well and let it incubate for 2 days. After incubation, the incubated plates were transferred to light, and the colonies were checked by using a marking pen (APHA, 1960; Difco Manual, 1977).

Blood analyses

A total amount of 3 ml of blood samples were collected on the slaughtering day in sterile and heparinized tubes. The samples were centrifuged for 15 minutes at 3500 RPM, to get blood plasma and stored at -20°C until the evaluation of the following parameters, including immunological parameters (IgA, [ng/ml], IgM [pg/ml], and IgG [ug/ml]), kidney functions (creatinine [CR, mg/dl] and urea [mg/dl]), and liver enzymes (alanine aminotransferase [ALT, U/L] and aspartate aminotransferase [AST, U/L]).

All blood analyses were conducted utilizing kits provided by Bio-Diagnostic Company, located in Dokki, Giza, Egypt. All analyzing procedures were executed according to the manufacturer's pamphlets.

Histological features

When all contents from the cecum were evacuated completely, the cecum was washed with saline solution (0.9 % NaCl) and then dried well. Thereafter, specimens from the middle region of the cecum were taken to fix in 10% neutral formalin for 24 hours for histological examination. The histological procedures were carried out in the Animal Production Department, Faculty of Agriculture, Mansoura University, Egypt. According to Bancroft et al. (1990), the specimens were washed with running tape water, dehydrated in ascending grades of alcohol, cleared, impregnated, embedded in paraffin wax blocks, cut by microtone into thin sections (7-10 µm), and stained by hematoxylin and eosin.

After that, the stained sections were examined for histopathological features in the Animal Health Research Institute, Agricultural Research Center, Egypt (H & E, X400), to test which of the used doses of CV (as an anti-inflammatory agent) can reduce the inflammation that may occur in rabbit's intestine.

Economic efficiency

Based on the Egyptian market price in 2023, the economic efficiency of using CV in a rabbit's diet has been calculated. The price of each kilogram of live BW was 80 L.E.

Net revenue = Selling price – total feed cost

Statistical model

The differences among experimental groups were statistically analyzed using the general linear model procedures of SAS (2002), applying one-way analysis of variance (ANOVA). The following statistical model was used:

$$Y_{ij} = \mu + T_i + e_{ij}$$

 Y_{ij} = The individual observation, μ = The overall mean, T_i = The fixed effect of the i^{th} treatments (i = 1, 2, 3), and e_{ij} = Random error associated with the individual. The mortality rate was analyzed by chi-square test. The differences among treatment means were separated according to Duncan's Multiple Range Test (Duncan, 1955). The significance level was set at 5%.

RESULTS AND DISCUSSION

Physiological responses

Immunological parameters

The effect of CV on growing rabbits' immunity parameters is illustrated in Table 2. The dose of 1.0 g CV/kg diet was more effective as an immune modulator compared to G2. The G3 (1.0 g CV/kg diet) recorded a significant (p < 0.05) elevation in the immunological parameters (IgM and IgG) than those recorded in G1 and G2. However, there were no significant (p > 0.05) differences among the experimental groups in the IgA values. G3 surpassed G1 in concentrations of IgA, IgM, and IgG by 4.2%, 13.3%, and 8.3%, respectively. Whereas, G1 and G2 showed almost similar and comparable values in all immunological parameters that were not statistically differed in the respective immunological parameters (Table 2, p > 0.05). According to the abovementioned results, we concluded that the greatest dose of 1.0 g CV/kg diet was more efficient in improving the immune status of growing rabbits than that of the lowest dose (G2) and the un-supplemented group (G1).

The obtained data disagreed with that of Abdelnour et al. (2019) who found that using CV at a level of 0.5 g/kg diet was better in enhancing immunity status than 1.0 g CV/kg in rabbits' diet. This difference could be attributed to the rabbit breed and the physiological and managerial conditions. The improvement in immunological parameters could be attributed to CV activities, such as anti-inflammatory anti-microbial, containing antioxidants, β -carotene, and vitamin B12 that can modulate immune functions (Abdelnour et al., 2019). A previous theory was performed by Safi et al. (2014), who proved that, carotenoid astaxanthin is present in CV, which enables CV to promote immunoglobulins production, through producing B cells in the gut-associated lymphoid tissue. Coelho et al. (2022) proposed that CV contains omega-3 fatty acids among the other biological compounds that confer to CV to act as an immune modulator and enhancer.

Immunoglobulins are released from B cells with a highly specific ability to bind to pathogens (antigens) and remove them from the body (Balan et al., 2019). The most significant immunoglobulin is IgG which represents 80% of the total immunoglobulins in the bloodstream. The IgG function neutralizes pathogens and promotes phagocytosis to remove them from the body (Borghesi et al., 2014). Immunoglobulins promote health through indirect ways, such as working on increasing growth factors and gut-weight. Additionally, they increase the beneficial bacteria and reduce the pathogenic bacteria in the gut (Balan et al., 2019). Therefore, the improved immunoglobulins in G3, specifically IgG mean increasing rabbits' ability to get rid of pathogens and have more resistance to diseases, which will reflect on their health and productive performance.

Kidney function

Verga (2002) confirmed that creatinine (CR) and blood urea (BU) are good indicators of kidney health and functions in rabbits. As can be seen in Table 2, there were no significant differences in both CR and BU concentrations between the control and treated groups (p > 0.05). However, the values of both indicators were numerically the lowest in G3 and the highest in G1. This emphasized that CV is a beneficial feed additive for rabbits with no negative effects on kidney functions. In addition, all values of both markers fell within the normal ranges as reported by Verga (2002), who

illustrated that, CR normally ranged between 0.5-2.5 mg/dl. Meanwhile, the normal range of BU is 36.84-50.28 mg/dl in rabbits. The obtained results revealed that a rabbit diet containing 1.0g CV/kg succeeded in reducing values of both CR and BU more than the other doses. While, both G1 and G2 showed similar values of CR and BU.

The findings of the present study are in line with those of Hassanein et al. (2014), which compared adding of CV with spirulina in rabbits' diet. It was found that CV surpassed spirulina in reducing CR concentrations (0.99 mg/dl) in the supplemented group with CV versus 1.33 mg/dl in another comparative group.

Table 2. Effects of *Chlorella vulgaris* on immunological parameters, kidney functions, and liver enzymes of APRI-growing rabbits during the whole experimental period

Items		G1	G2	G3	SEM	P-value
	IgA (ng / ml)	30.1	30.8	32.1	0.56	0.0936
Immunological	IgM (pg / ml)	38.0^{b}	38.3 ^b	43.4 ^a	0.63	0.0024
parameters	IgG (ug / ml)	16.0^{b}	16.9 ^{ab}	18.3 ^a	0.49	0.0339
Kidney functions	Creatinine	0.71	0.70	0.68	0.04	0.9347
(mg/dl)	Urea	43.7	43.4	42.6	2.42	0.9417
Liver enzymes	AST	40.3	39.3	38.3	2.17	0.8730
(U/L)	ALT	46.8	45.0	44.3	2.85	0.7940

a, b Means in the same row with different superscript letters are significantly different (p < 0.05). G1: Control; G2: Group fed basal diet included 0.5 g CV/kg diet; G3: Group fed basal diet included 1.0 g CV/kg diet; SEM: Standard error of means

Liver function

In the present study, both AST and ALT enzymes were determined as important biological markers of liver health and functions. The effect of adding CV at two levels on AST and ALT is presented in Table 2. The activity of AST ascertained that the dose of 1.0 g CV/kg diet was more effective in reducing AST activity. The lowest value was recorded by in G3 (38.3 ± 2.17 U/L) that followed by G2 and both G2 and G3 were the lower groups in AST values compared with G1 with no significant differences (p > 0.05). However, it should be noted all the obtained values of AST are within the normal physiological range as reported by Verga (2002) which indicated AST ranges normally between 10 to 98 U/L in rabbit blood. The ALT showed the same results as AST, which was at the lowest level in G3 in comparison with the other experimental groups. The highest value ($46.8 \pm 2.85 \text{ U/L}$) of ALT was observed in G1 which proved the safety of CV, especially at the level 1.0 g/kg diet on liver enzymes. Verga (2002) recorded that its overall mean (in the three groups) was within the normal range and the normal level of ALT is 25 to 65 U/L with no significant differences among the experimental groups. Generally, the activity of ALT in rabbits is lower than the other species as noted by Verga (2002). The current results are compatible with the findings of Abdelnour et al. (2019). That previously confirmed the ability of CV (at a level of 1.0 g/kg diet to reduce live enzymes (AST and ALT), which reflects the possibility of supplementing rabbits' diet with CV with no harmful effects on rabbits' performance and health. A recent study used marine algae in rabbit diets and did not record any abnormalities or pathological responses to AST and ALT activities (Abu-Hafsa et al., 2021).

The importance of testing AST and ALT is clarifying the liver condition since AST is an enzyme that helps the body break down amino acids. Similar to ALT, AST is usually in the blood at low levels. An increase in AST levels may cause liver damage, liver disease, or muscle damage. The ALT is an enzyme found in the liver that helps convert proteins into energy for the liver cells. When the liver is damaged, ALT is released into the bloodstream and its levels increase Verga (2002).

Productive performance Feed intake

Table 3 shows the effect of CV addition on the productive performance of APRI rabbits. It was noticed that FI was greater in G3 followed by G2 and both groups recorded the highest FI than that in G1. The findings confirmed that CV did not affect the feed palatability and encouraged rabbits to eat more. This is not consistent with the results previously reported by Sikiru et al. (2019) that used CV (at levels of 200 and 300 mg CV) in growing rabbit's diet and recorded a reduction in rabbits FI supplied with CV compared to the control group. Moreover, G3 was statistically (p < 0.05) different from G1 in FI during the experimental period. Additionally, FI was gradually increased during the 5-9 weeks of the study period in rabbits aged 9-13 weeks. However, the average FI during the experimental period (5-13 weeks) was lower than that recorded during 9-13 weeks.

Table 3. Effect of *Chlorella vulgaris* on productive performance of APRI-line rabbits

Parameters	G1	G2	G3	MES	P value
Feed intake (g/d)					
5-9 weeks	57.6 ^b	58.5 ^a	59.1 ^a	0.337	0.0080
9-13 weeks	93.9 ^b	95.0 ^{ab}	95.6 ^a	0.553	0.0485
5-13 weeks	75.7 ^b	76.8^{a}	$77.4^{\rm a}$	0.318	0.0014
Feed conversion ratio					
5-9 weeks	2.4	2.4	2.3	0.048	0.2209
9-13 weeks	4.2^{a}	3.9^{b}	3.9^{ab}	0.085	0.0434
5-13 weeks	3.2^{a}	3.1 ^{ab}	3.0^{b}	0.050	0.0469
Initial body weight (g)	572.3	576.9	575.1	11.79	0.9702
Final body weight (g)	1884.7 ^b	1957.3 ^a	1989.7 ^a	19.52	0.0020
Average daily gain (g)					
5-9 weeks	24.1 ^b	24.6^{ab}	26.0^{a}	0.522	0.0515
9-13 weeks	22.8 ^b	24.7 ^a	24.6 ^a	0.441	0.0131
5-13 weeks	23.4 ^b	24.7 ^a	25.3 ^a	0.366	0.0027
Relative growth rate (%)					
5-9 weeks	74.3	75.0	77.5	1.529	0.3874
9-13 weeks	40.7	42.9	41.9	0.902	0.1747
5-13 weeks	106.9	109.1	110.4	1.350	0.2687
Performance index (%)	58.5 ^b	63.0^{a}	65.1 ^a	1.536	0.0110
Mortality rate (%) ⁽¹⁾	6.67	6.67	0	-	_

a.b Means in the same row with different superscript letters are significantly different (p < 0.05). G1: Control; G2: Group fed basal diet included 0.5 g CV/kg diet; G3: Group fed basal diet included 1.0 g CV/kg diet; SEM: Standard error of means, (1): Chi-square test

Feed conversion ratio

It was noted that G3 and G2 showed the best FCR during periods 5-9 and 9-13 weeks, and both groups (G2 and G3) recorded approximately the same values of FCR in the same periods. The detected reduction in FCR may be related to the amended digestive enzymes, in particular protease and lipase, leading to the promotion of intestinal efficacy (El-Basuini et al., 2023). Moreover, G3 was statistically different from G1 in FCR during the whole period (5-13 weeks, p < 0.05). Whereas, G3 was not significantly different than G1 during the other two experimental periods (5-9 and 9-13 weeks; p > 0.05). The enhancement of FCR emphasized the economic benefits of adding CV to growing rabbit diets. The obtained results of FCR are compatible with Sikiru et al. (2019) who, used different levels of CV, including 200, 300, 400, and 500 mg and found that the FCR was less in the supplemented group with 500 mg CV than the other doses. This confirmed the ability of CV to enhance rabbits' productivity.

Body weight and average daily gain

The enhancement in both FI and FCR has positively impacted APRI rabbit's BW, specifically FBW. As shown in Table 3, FBW was significantly elevated in the supplemented groups with CV in comparison with the control group (p < 0.05). The amount of increased FBW through the experimental period was 3.5, 3.4, and 3.2 times in G3, G2, and G1, respectively. This is considered evidence of the improved efficiency of feed utilization. In addition, G3 was the superior in ADG during the weeks 5-9 and 5-13. However, G3 was closer to G2 in ADG during the age of 9-13 weeks.

Relative growth rate, performance index, and mortality rate

To check the relative growth rate (RGR, %), adding CV in rabbits' diet insignificantly increased RGR (%) in the supplemented groups (G2 and G3) compared to G1 (p > 0.05). The G3 was the highest group in RGR at the age of 5-9 and 5-13 weeks. However, at weeks 9-13, G2 had the highest level of RGR. Furthermore, the treated groups significantly surpassed G1 in performance index (PI, %), and G3 was the higher one than the other two experimental groups p < 0.05). Interestingly, no mortality rate was recorded in G3, as all rabbits were in good health. While, the other two groups almost had equal (6.67) MR (%, Table, 3), which is considered an acceptable percentage for rabbits at this age as cleared by Rashwan and Marai (2000), who stated that MR (%) in the post-weaning period is less than 20% and the survival rate was ranged between 81-88%. The abovementioned results showed the improvement in rabbit immunity (particularly IgG) in G3, caused by CV addition, and made them more resistant to pathogens, leading to lower MR (%) as presented in Table 3. Additionally, it could lead to suitable hygienic and managerial conditions for these rabbits. Besides the ability of

this rabbit line (synthetic line, produced from crossbreeding between Red Baladi bucks with a dose of V-line) it is more adapted to the Egyptian environmental conditions.

The noticeable improvement of the productive performance (FBW, FI, FCR RGR, and PI) of the supplemented rabbits with CV, with special reference to the dose of 1.0 g CV/kg diet, could be attributed to the ability of CV to promote feed efficiency, nutrient digestibility, and absorption (El-Banna et al., 2005). The findings are in line with those of Abdelnour et al. (2019), Abu-Hafsa et al. (2021), El-Basuini et al. (2023), and Abdurakhmanova et al. (2024) which proved the enhancement in rabbits' productive performance in response to CV inclusion in their diets.

Carcass traits

Table 4 presents the rabbits that were fed with CV inclusion produced more carcass weight compared with the control group, particularly G3 which significantly differed from G1 (p < 0.05). The relative weight of abdominal fat, liver, kidney, heart, giblets parts, spleen, stomach, and pH of the stomach, small intestine, and cecum were similar in G2 and G3 and considered close to the obtained values in G1. Meanwhile, the three experimental groups (G1, G2, and G3) had the same value of spleen percentage. It is well known that the spleen is the largest secondary lymphoid organ that has a positive role in enhancing immunological and hematological parameters. In addition, it is the main filter for bloodborne pathogens and antigens, as well as, a key organ for iron metabolism and erythrocyte homeostasis (Bronte and Pittet, 2013; Kumari et al. 2019). Moller and Erritzoe (2000) reported that the spleen mass size is closely related to the level of humoral immunity, and a large spleen size will result in high humoral immunity. Meanwhile, the percentages of small intestine, cecum, and large intestine were lower in the treated groups, specifically in the rabbits fed with a 1.0 g CV/kg, with no significant differences (p > 0.05). These results agree with the findings of Abu-Hafsa et al. (2021), who used marine algae at a level of 4% per kg rabbit diet in New Zealand White and observed that percentages of liver, spleen, and kidney were lower in the rabbits who received CV in their diets than in the control group. They attributed their results to the lower fat content in rabbit carcasses.

Table 4. Effect of Chlorella vulgaris addition on carcass traits (%) and pH values of APRI-line rabbits

Carcass traits (%)	G1	G2	G3	SEM	P-Value
Carcass weight	47.5 ^b	49.2 ^{ab}	49.8 ^a	0.501	0.0474
Liver	3.4	3.6	3.7	0.161	0.5211
Heart	0.4	0.4	0.6	0.031	0.9759
Kidney	0.98	0.97	0.97	0.049	0.9819
Giblets Part	4.7	4.9	5	0.170	0.5680
Spleen	0.1	0.1	0.1	0.006	0.9785
Abdominal fat	0.7	0.7	0.6	0.045	0.8247
Gastrointestinal tract	22.5 ^a	20.3 ^b	19.8 ^b	0.493	0.0157
Stomach	4.9	4.3	4.3	0.220	0.1879
Small intestine	4.1	3.9	3.8	0.176	0.7420
Caecum	7.2	6.4	6.2	0.434	0.2627
Appendix	1.7	1.5	1.5	0.133	0.4045
Large intestine	2.0	1.7	1.7	0.158	0.3975
pH values					
Stomach	3.13	3.27	3.37	0.067	0.1199
Small intestine	7.40	7.43	7.57	0.058	0.5787
Caecum	6.23	6.27	6.50	0.116	0.3407

a, b Means in the same row with different superscript letters are significantly different (p < 0.05). G1: Control; G2: Group fed basal diet included 0.5 g CV/kg diet; G3: Group fed basal diet included 1.0 g CV/kg diet; SEM: Standard error of means

Cecum bacterial count

The effect of CV supplementation on cecal microbiota is illustrated in Table 5. Adding CV in growing rabbits' diet improved the cecum content, via increasing total bacterial count and *lactobacilli* bacteria and reducing the count of *coliform* bacteria, especially in G3 followed by G2. Both treated groups significantly differed from G1 in the measured microbiota as shown in Table 5 (p < 0.05). The elevation of the count of *lactobacilli* bacteria is a sign of enhancing feed digestibility and nutrient efficiency utilization (Phuoc and Jamikorn, 2017). This result is considered another explanation for the increase in FI and FCR, which was reflected in obtaining a higher FBW at the end of the experiment in treated

groups compared to G1, as presented in Table 3. Whereas, the reduction in cecal *coliform* bacteria (the pathogenic bacteria) in the intestinal tract, leads to promote rabbits' health status and nutrient digestibility, thus improving their productive performance (Phuoc and Jamikorn, 2017). The findings are supported by Velankanni et al (2023), who found the positive effects of CV on animal immunity that are reflected in the gut microflora leading to the enhancement in animal health and nutrient unitization.

Histopathological features

The histopathological examination of growing rabbits' intestinal cells is shown in Figure 2. In the control group (Figures 2A, B, and C), a severe presence of sloughing tips of the intestinal villi and infusion of mononuclear inflammatory cells have been noticed with severe necrotic changes in the tips of some villi as well. Meanwhile, Figures 2D, E, and F (the photos of G2, the supplemented group with 0.5g CV/kg diet) reveal the moderate presence of sloughing and inflammation in the intestinal villi. A healthier presence of rabbit intestinal cells has been noticed in the photos of G3 (Figures 2O, H, and I). These photos illustrate a slight presence of inflammatory cells. In contrast, in Figure 2O, almost no inflammation or necrosis in the intestinal villi is observed. This means that supplemented rabbits with CV, especially with a 1.0 g / kg diet, succeeded in reducing the level of inflammation and necrosis from severe to slight level and preventing the inflammation as can be seen in Figure 2O. This could be an explanation for the elevated FBW in G3 compared to G1 and G2. As well as, the improvement in FCR, ADG, and RGR during the completely experimental period (5-13 weeks of rabbits' age). In addition, PI with zero MR % in G3 in comparison with G1 and G2 as discussed previously (Table 3).

These incredible results of CV, especially the higher dose of G3 (1.0 g/ kg diet) cloud be attributed to the anti-inflammatory activity of CV since it can reduce cytokines secretion that is associated with inflammatory activity. Furthermore, reducing the matrix called metalloproteinase, which causes tissue damage. In addition, in some species, CV might have a role in the growth and development of fibroblasts that are responsible for tissue repair (Coelho et al., 2022).

A lack of studies was concerned about the effect of CV on rabbit intestinal health except El-Basuini et al. (2023). The current data are supported by those of El-Basuini et al. (2023), who included CV in growing rabbit's diet at levels 3 and 5 g/kg diet for 8 weeks. The histological analysis of the rabbit's intestine revealed no pathological changes, such as necrosis or inflammation, when compared to the control group. This finding confirmed the health benefits of adding CV in growing rabbits' diets.

Table 5. Effect of Chlorella vulgaris on cecum microbiota of APRI-line rabbits

Parameters	G1	G2	G3	SEM	P-value
Total bacterial count (x10 ⁶)	4.66 ^b	5.60 ^a	5.87 ^a	0.252	0.0317
Coliform bacteria (x10 ⁶)	2.57 ^a	1.93 ^b	1.83 ^b	0.176	0.0411
Lactobacilli (x10 ⁶)	1.33 ^b	2.57 ^a	2.63 ^a	0.176	0.0046

a, b Means in the same row with different superscript letters are significantly different (p < 0.05). G1: Control; G2: Group fed basal diet included 0.5 g CV/kg diet; G3: Group fed basal diet included 1.0 g CV/kg diet; SEM: Standard error of means

Table 6. Effects of experimental diets on economic traits of APRI-line rabbits at 13 weeks of age

Items	G1	G2	G3
Average feed intake (kg /head)	4.242	2.298	2.332
Price /kg diet (L.E.)	12.7	13.1	13.5
Total feed cost (L.E.)	53.9	56.3	58.5
Average weight gain (kg/head)	1.312	1.380	1.415
Selling price (L.E.)	105.0	110.4	113.2
Net revenue (L.E.)	51.1	54.1	54.7
Relative revenue (%)	100	105.9	107.1

G1: Control; G2: Group fed basal diet included 0.5 g CV/kg diet; G3: Group fed basal diet included 1.0 g CV/kg diet - Other conditions like management are fixed. - Ingredients price (Egyptian pound, EGP, per ton) at 2023 were: 14000 barley; 8000 barseem hay; 8000 wheat bran; 28000 soybean meal (44%); 500 limestones; 30000 premix; 80000 methionine; 10000 di-calcium phosphate; 8000 molasses; 500 salt; 800000 *Chlorella vulgaris*. 1 USD = 49.5 EGP

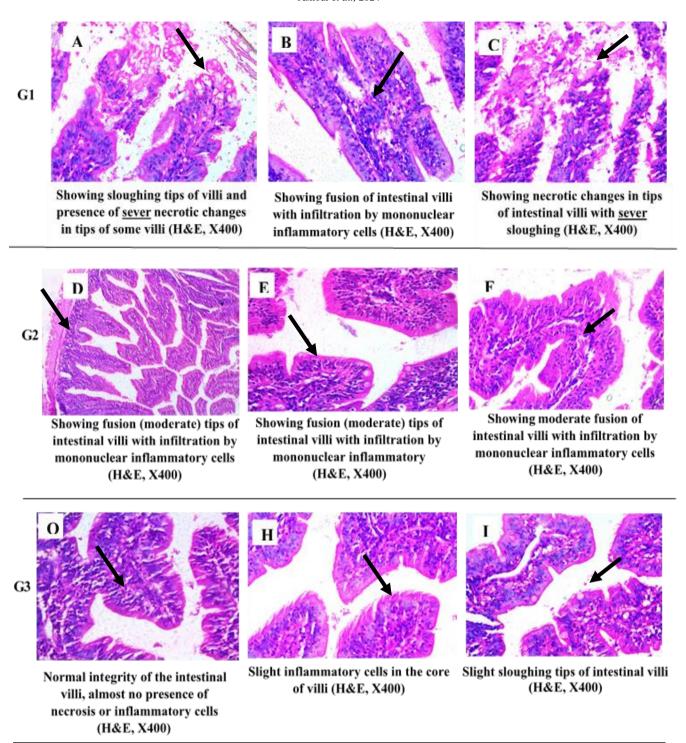


Figure 2. The examination of growing APRI rabbit's intestine at the age of 13 weeks. **G1:** Control; **G2:** Group fed basal diet included 0.5 g CV/kg diet; **G3:** Group fed basal diet included 1.0 g CV/kg diet.

Economic efficiency

The mentioned prices in Table 6 were reported based on the Egyptian market during 2023. Table 6 reveals that both treated groups recorded higher total feed costs (p > 0.05) in comparison with G1 due to CV addition. On the other side, CV supplementations, especially the level of 1.0 g CV/kg diet, succeeded in elevating the average weight gain and selling price, which led to increasing the net revenue and the relative revenue (Table 6). Referring to the positive benefits of CV on immunity and rabbits' productive performance with no hazards to their health, it is highly recommended to supplement rabbits' diets with CV and get economic benefits. Besides its health benefits through reducing the inflammation in intestinal villi confirmed by histopathological examination. The economic benefits of CV supplementation in rabbits' diet have been previously confirmed by Hassanein et al. (2014), who recorded higher net revenue and total weight gain in rabbits, despite increased total feed cost in treated rabbits compared with untreated ones.

CONCLUSION

The results of the present study confirmed the effectiveness of supplying growing rabbits' diet with CV, especially at a level of 1.0 g CV/kg diet. The dietary supplementation of CV could be severed as a natural and functional strategy that helps growing rabbits attenuate the shock of the post-weaning period. In the present study, adding CV at a level of 1.0 g/kg to the rabbits' diet improved immunity. In addition, it manifested health benefits through lowering levels of AST, ALT, CR, and urea in G3 as well as recording the highest final BW, with better FI and FCR in comparison to G1 and G2. The most significant result that has been discovered through the histopathological examination, proved the ability of CV (at a level of 1.0 g/kg diet) to attenuate the level of the sloughing and infusion of inflammatory cells in the intestinal villi from severe to slight level. This ability of CV is related to its property as an anti-inflammatory agent. Therefore, supplying growing rabbits with a 1g CV/kg diet is highly recommended, since CV is considered a natural protective agent for growing rabbits that can boost their health and productive performance, thus reducing the economic losses. Moreover, it is recommended to test higher doses of CV than 1.0 g/kg diets on intestinal cells especially in growing rabbits. Furthermore, evaluate these doses on the reproductive performance of both male and female rabbits.

DECLARATIONS

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Authors' contributions

Prof. Dr. Gamal Ashour designed the study and drafted the manuscript, Prof. Dr. Safaa Atay Barakat and Dr. Noha Mahmoud Abdel-Azeem performed the statistical analysis and tabulation of the experimental data, Dr. Esraa Mohamed performed the blood chemical analysis, Dr. Shama hosy was responsible for the applying the experimental design, Prof. Dr. George Ezzat was responsible for the histopathological examination for rabbits intestine, Dr. Samah Mohamed Abdel-Rahman participated the blood chemical analysis and participated in writing the manuscript. Dr. Hazem Gaafar applied the bacterial count. All authors approved the final version of the manuscript before publishing in the present journal.

Competing interests

The authors have not declared any conflict of interest.

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Availability of data and materials

The collected data during the current study are available from the corresponding author upon reasonable request.

Ethical considerations

The authors confirmed that all authors have reviewed and submitted the manuscript and its original data to this journal for the first time.

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Effects of Ethanolic Extracts of *Tithonia diversifolia* and *Azadirachta indica* on *Haemonchus contortus* in Goats



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ABSTRACT

Infestation of gastrointestinal worms (helminths) contributes significantly to neonatal mortality and reduced growth performance in livestock animals. The present study was conducted to determine the phytochemical composition and the *in vitro* potency of ethanolic extracts of *Tithonia diversifolia* and *Azadirachta indica* on motility inhibition on *Haemonchus contortus*. *Tithonia diversifolia* and *Azadirachta indica* were obtained from farmers in Bulambuli district of Uganda and were treated with 70% ethanol as an extraction solvent. A standard phytochemical procedure was used for qualitative analysis. The *in-vitro* experiment was conducted using 2.5 mg/ml, 5 mg/ml, 10 mg/ml, and 20 mg/ml for each plant ethanolic extract. Moreover, phosphate buffer saline (PBS) was utilized as the control. Phytochemical analysis revealed the presence of tannins, alkaloid salts, saponins, flavonoids, steroid glycosides, anthracenosides, coumarins, and anthocyanosides in ethanolic extracts. A dosage of 20 mg/l of *Tithonia diversifolia* and *Azadirachta indica* indicated motility inhibition of adult *Haemonchus contortus* after 2.55 hours and 2.1 hours, respectively, compared to the PBS control group. In conclusion, both plant extracts showed anthelmintic activity leading to the mortality of the worms. The ethanolic extracts of *Azadirachta indica* were faster in causing mortality of *Haemonchus contortus* than those of *Tithonia diversifolia* at the same dose rate of 20 mg/ml.

Keywords: Azadirachta indica, Haemonchus contortus, Plant extract, Tithonia diversifolia, Wormicide

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INTRODUCTION

Helminthiasis caused by worm infestation is among the most important diseases affecting livestock productivity, leading to reduced growth rates, decreased milk production, and in severe cases, animal mortality (Karshima et al., 2018; Reman and Abidi, 2022). Yuguda et al. (2018) reported that gastrointestinal helminths are the major source of economic losses in livestock. In Europe, the annual cost of treating helminth infections is estimated at 1.8 € billion (Charlier et al., 2020). In Indonesia, Zalizar et al. (2023) reported a 20% prevalence of worm infestation in beef cattle during the dry season, with a higher prevalence observed in bulls compared to females. Similarly, in Nigeria, Ola-Fadunsin (2020) identified helminth infections as a significant concern among cattle, noting substantial species diversity. In southern Ethiopia, Dembelo et al. (2023) documented cases of two or more gastrointestinal infections affecting cattle and concluded that deworming and effective management practices were necessary to eradicate gastrointestinal parasites. In eastern Uganda, parasitic worm burdens were reported as significantly high in cattle, with the situation worsening in small ruminants such as goats and sheep, where kid mortality rates have reached 40% (Namutosi et al., 2019). In central Uganda, approximately 56% of animals infected with helminths were found to be shedding *Haemonchus contortus* eggs (Nsereko et al., 2015). A study conducted by Kalule et al. (2023) reported a 63% prevalence of Haemonchus contortus adult worms in goats from selected districts in Uganda. This parasite, located in the gastrointestinal tracts of farm animals, causes anorexia, diarrhea, emaciation, and anemia, which can ultimately result in the death of the animal (Wright et al., 2018; Tiele et al., 2023).

The government of Uganda, through the Ministry of Agriculture, Animal Industry, and Fisheries (MAAIF), has recommended several interventions such as the use of chemotherapeutic drugs and grazing management practices to reduce helminth burdens in livestock, thereby improving productivity. The most recommended intervention is the use of

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imported wide-spectrum veterinary drugs such as Albendazole, Mebendazole, and Ivermectin. However, these veterinary drugs are costly for most farmers and are frequently unavailable in remote areas. Moreover, Aremu et al. (2012) noted that helminthiasis remains a persistent issue, particularly for poor livestock farmers.

As an alternative, some farmers rely on medical plants such as *Azadirachta indica*, *Tithonia diversifolia*, *Phytolacca dodecandra*, and *Vernonia amygdalina*, using indigenous knowledge to control helminths. These plants have been reported as effective (Bizimenyera, 2007; Nalule et al., 2011; Matovu et al., 2020). Therefore, in this study, the potency of two plant extracts of *Azadirachta indica* (*Azaradichta indica*; Neem) and *Tithonia diversifolia* (*T. diversifolia*; Mexican sunflower) on *Haemonchus contortus* were investigated. The main objective was to determine the phytochemical composition and *in-vitro* anthelminthic activity of the ethanolic extracts of *Azadirachta indica* and *Tithonia diversifolia* on *Haemonchus contortus*.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Student Research Review Committee of the College of Veterinary Medicine, Animal Resources, and Biosecurity at Makerere University, Uganda. The *Haemonchus contortus* worms used in this experiment were collected from the abomasa of slaughtered goats. Permission to collect the samples was obtained from the management of Kalerwe abattoir through the Makerere University College of Veterinary and Biosafety Sciences Laboratory.

Plant sample collection

Leaves of *Tithonia diversifolia* and *Azadirachta indica* were collected in April 2022 from Kamu sub-county, Bulambuli district in Eastern Uganda. The area lies along 1° 17' 49" N and 34° 19' 5" E, approximately 1300 meters above sea level, and receives a bi-modal rainfall pattern, with wet months from April to October and dry months from July to August and December to February. The plant parts were identified by the Department of Botany Herbarium at Makerere University as S01-*Azadirachta indica* and S02-*Tithonia diversifolia*.

Extract preparation

Leaf samples of *Tithonia diversifolia* and *Azadirachta indica* (2.5 kg each) were air-dried for three weeks at an average room temperature of 22 °C. The air-dried plant samples were pulverized using a laboratory Brook Crompton electric grinder (UK) to pass through a 1mm screen. A total of 200 g of each ground sample was then added to 1000 ml of 70 % ethanol at room temperature. The mixtures were left to stand for three days with periodic shaking, following the study of Olukotun et al. (2018). At the end of the 3-day extraction period, the mixtures were filtered twice, first through cotton wool in a Burkard funnel and then using Whatman No. 1 filter paper. The filtrates were concentrated using a rotary evaporator (CH-9230 Flawl / Schweiz, Germany) at 55°C until a constant volume was achieved. The plant extracts were dried in an oven set at a temperature of 55°C to a constant weight and then refrigerated at 4°C for the subsequent phytochemical analyses and anti-helminthic profiling.

Phytochemical screening tests

The ethanolic extracts of *Tithonia diversifolia* and *Azadirachta indica* were screened for various phytochemicals, including saponins, tannins, reducing sugars, starch, alkaloid salts, anthracenosides, anthocyanosides, coumarins, flavonosides, and steroid glycosides, as described by Das et al. (2018). Tests for the presence of saponins, tannins, reducing sugars, alkaloid salts, and anthracenocides were conducted using Frothing, Ferric chloride, Fehling's, Mayer's reagent, and Ammonia solution tests, respectively. The presence of starch was tested using an iodine solution while coumarin derivatives were detected using an ammonia solution and ultraviolet (UV) light test. The presence of flavonoids and steroid glycosides was confirmed utilizing Shibata's reaction and Liebermann-Burchard's test, respectively. The level of presence of the phytochemicals was indicated by the intensity of color change: (++) for high abundance, (+) for low abundance, and (-) for absence.

Collection of Haemonchus contortus

Adult *Haemonchus contortus* worms were harvested by washing the abomasa of goats slaughtered in Kalwere abattoir using phosphate buffer solution (PBS) following a standard procedure described by Hade et al. (2022). The washed worms were immediately put in a flask containing PBS, maintained at a temperature of 37°C, and delivered to the parasitology laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Uganda, for further management and *in-vitro* evaluation.

In-vitro anthelminthic activity assessment

The mortality of adult *Haemonchus contortus* was assessed using plant extracts at various concentrations. A stock solution of 20 mg/ml was prepared for daily treatment by dissolving 2 g or 2000 mg (dry matter) of each extract in 100 ml of PBS. Four treatments (concentrations) of 2.5 mg/ml, 5 mg/ml, 10 mg/ml, and 20 mg/ml of extracts from *Tithonia* diversifolia and Azadirachta indica were prepared for each plant sample using serial dilution with PBS as a solvent following the procedures described by Pandey et al. (2018). Concentrations of 2.5 mg/ml, 5 mg/ml, 10 mg/ml, and 20 mg/ml for each of the extracts were prepared by dissolving 250 mg, 500 mg, 1000 mg, and 2 g of dried powder, respectively, in 100 ml of the PBS solvent. The negative and positive controls consisted of 10 ml of PBS and Albendazole 2.5%, respectively. Albendazole 2.5% was used because it is the lowest commercially available concentration of the drug recommended by the National Drug Authority (NDA, 2023). Ten adult worms were put in Petri dishes containing each of the plant extract concentrations/treatments and control solutions, with three replicates for each treatment. The petri dishes were placed in an incubator set at a constant temperature of 37°C for 8 hours. Inhibition of movement of the helminths was a sign of worm death or paralysis. Movement of helminthic parasites was observed, and live and dead worms were tallied at 30-minute different time intervals. The time intervals were 30 minutes, 1 hour, 2 hours, 3 hours, and 8 hours. Worms that did not show any movement were picked from the plant extract concentrations and put in lukewarm PBS for 15 minutes. Thereafter, worms were observed and counted as live in case of restoration of any movement. Otherwise, they were considered dead when no motility was recorded (Coles et al., 1992; Aliyi Hassen et al., 2020).

Statistical analysis

Data were entered into the Microsoft Excel spreadsheet, transferred to SPSS software (version 21), and analyzed using descriptive statistics including means and standard deviations.

RESULTS

Phytochemical composition

Phytochemical screening tests showed that *Azadirachta indica* and *Tithonia diversifolia* contained several active compounds (Table 1). *Tithonia diversifolia* indicated higher levels of coumarins than *Azadirachta indica*. Starch, anthracenosides, and anthocyanosides were present in *Azadirachta indica* but absent in *Tithonia diversifolia*.

Effects of Azadirachta indica on Haemonchus contortus

The results indicated that the mean time to death of *Haemonchus contortus* decreased progressively as the concentration of *Azadirachta indica* increased (Table 2). Total mortality of *Haemonchus contortus* (100%) was achieved most rapidly at 2.1 hours with *Azadirachta indica* at a concentration of 20 mg/ml (Table 2).

Effect of Tithonia diversifolia on Haemonchus contortus

The mean time to death of *Haemonchus contortus* on exposure to different treatments /concentrations of *Tithonia diversifolia* also decreased progressively with increasing concentrations (Table 3). Total mortality of *Haemonchus contortus* (100%) was achieved after 2.55 hours of exposure to *Tithonia diversifolia* at a concentration of 20 mg/ml (Table 3). In comparison, the positive control, albendazole at a concentration of 2.5%, was highly effective and caused 100% mortality within 30 minutes, significantly outperforming all treatments /concentrations of the plant extracts.

Table 1. Phytochemical composition of ethanolic extract of Azadirachta indica and Tithonia diversifolia

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Test	Azadirachta indica	Tithonia diversifolia					
Saponins	+ (moderate)	+ (moderate)					
Tannins	+++	+++					
Reducing sugars	-	-					
Starch	+	-					
Alkaloid salts	+	+					
Anthracenosides	++	-					
Anthocyanosides	+	-					
Coumarins	+	++					
Flavonosides	++	++					
Steroidglycosides	++	++					

(+) presence; (-) absence

Table 2. Mean time to death of *Haemonchus contortus* exposed to *Azadirachta indica* extract

Items	Concentration					PC
Treatment	2.5 mg/ml	5.0 mg/ml	10.0 mg/ml	20.0 mg/ml		
Mean time to death (hours)	3.55	2.85	2.20	2.10	0.0	0.5
Variance in time to death	8.358	3.447	2.344	2.266	0.0	0.0

NC: Negative control, PC: Positive control

Table 3. Mean time to death of Haemonchus contortus exposed to Tithonia diversifolia extract

Items		NC	PC			
Treatment	2.5 mg/ml	5.0 mg/ml	10.0 mg/ml	20.0 mg/ml		
Mean time to death (hours)	3.85	3.05	2.80	2.55	0.0	0.5
Variance in time to death	6.447	4.344	3.914	3.525	0.0	0.0

NC: Negative control, PC: Positive control

DISCUSSION

The phytochemical screening tests of the two plant extracts confirmed the presence of saponins, tannins, flavonoids, alkaloids, anthracenosides, anthocyanosides, coumarins, and steroid glycosides. Both *Azadirachta indica* and *Tithonia diversifolia* contained flavonoids and steroid glycosides, as well as equally high levels of tannins. These biochemical compounds exhibit pharmacological effects, including anti-helminthic (Aremu et al., 2012; Zenebe et al., 2022; Ukwa et al., 2023), anti-oxidant (Gama et al., 2014; Gulcin, 2020; Nyero et al., 2023) antimicrobial, and anti-inflammatory properties (Bizimenyera, 2007; Gonfa et al., 2023). Tannin extracts have been reported to possess anthelmintic properties (Kotze et al., 2009; Greiffer et al., 2022). Similarly, Ferreira et al. (2013) noted that the presence of phenolic compounds in *Annona muricata* contributed to its anthelmintic effects. Alkaloids are also known to act on the central nervous system (CNS) of parasitic worms, causing paralysis, and also function as antioxidants (Adak and Kumar, 2022).

The time required to kill *Haemonchus contortus* worms on exposure to different treatments of plant extracts decreased with increasing concentrations, ranging from 2.5 mg/ml to 20 mg/ml. The shortest helminth elimination time for *Azadirachta indica* was 5 hours at a concentration of 20 mg/ml, while ethanolic extracts of *Tithonia diversifolia* achieved total mortality of *Haemonchus contortus* in 6 hours. These findings align with those of Sakti et al. (2018), who reported that dry ground leaf powder of *Azadirachta indica* significantly reduced the egg count of helminths after 28 days of treatment, likely due to the death of the parasite. Similarly, Duarte et al. (2020) used *Tithonia diversifolia* leaf powder on sheep inoculated with *Haemonchus contortus* and reported that the plant extract caused a great reduction in the population of albendazole-tolerant infective larvae of *Haemonchus contortus*. The results of the study revealed that *Azadirachta indica* extract was more effective than *Tithonia diversifolia*, possibly due to high levels of tannins and alkaloids and the presence of anthracenosides and anthocyanosides, which were absent in *Tithonia diversifolia*.

CONCLUSION

The present study provided evidence that ethanolic extracts of *Azadirachta indica* and *Tithonia diversifolia* at a concentration of 20 mg/ml have anthelmintic effects on *Haemonchus contortus*. This finding offers an excellent opportunity for advances toward developing low-cost alternative natural anthelmintic compositions to fight worm parasitic infestations in livestock. Based on the results, more *in vivo* experiments are recommended to determine the effective dosage and toxicity levels of these plant extracts for helminth control.

DECLARATIONS

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Authors' contributions

Brian Britex Owoyesigire designed the experiment, supervised the collection of plant samples, and drafted the manuscript; Laban Buyi performed the experiment, and collected and analyzed data; Joachine Idibu and Terence Odoch supervised data analysis and experimental designs, and revised the draft manuscript; and Lawrence Owere edited the manuscript. All authors read and corrected the manuscript for final approval.

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Competing interests

The authors have no competing interests.

Ethical considerations

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Availability of data and materials

The data to support this study is available upon request from the corresponding author

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Influence of Different Water Environments on the Differential Leukocyte Counts in Nile Tilapia

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ABSTRACT

Aquaculture production has become increasingly important for ensuring food security, supported by the expanding variety of cultivated species. This study evaluated the effects of different water environment characteristics on the differential leukocyte counts of the Nile tilapia (Oreochromis niloticus), a key farmed fish species in Africa. Fish from four different water sources were compared with healthy individuals from the natural water of the Nile River. A total of 64 Nile tilapia, each weighing 80-100 grams, were evenly divided into four groups and maintained at temperatures ranging from $25.00\pm2.5^{\circ}\text{C}$ to $30.00\pm2.5^{\circ}\text{C}$. The groups included fish from experimental tanks (ET), ponds managed by the General Administration of Fisheries Ponds (GAFP), the Fisheries Research Center Ponds (FRCP), and the White Nile River (WNR, control). Significant differences in water quality parameters, particularly NH₄, NH₃, NO₃, and NO₂ concentrations, were observed among the water sources. GAFP and ET waters showed higher concentrations of these compounds (NH₄, NH₃, NO₃, and NO₂) compared to FRCP and WNR. Differential leukocyte counts varied significantly across the groups. The ET group showed the highest eosinophil percentage $(9.68 \pm 0.44\%)$, while the GAFP group exhibited the highest percentages of lymphocytes $(46.40 \pm 0.13\%)$, monocytes (15.43 \pm 0.14%), and neutrophils (18.31 \pm 0.16%) compared to WNR. In contrast, the FRCP group recorded the highest platelet percentage (32.34 \pm 0.49%), while the ET group had the lowest (13.65 \pm 0.15%). Additionally, the ET group recorded the highest overall white blood cell count (191.46 \pm 0.61 \times 10³). A strong positive correlation was found between the blood profiles of Nile tilapia and the water environment parameters. This study highlighted significant differences in water quality among experimental groups, with FRCP and WNR showing lower parameters. In addition, examining white blood cells in fish is crucial for biological monitoring of surface water pollution.

Keywords: Differential leukocyte count, Nile tilapia, Water quality deterioration

INTRODUCTION

Marine organisms are an important food source for humans, as highlighted by the Food and Agriculture Organization of the United Nations (Tacon, 2019).

Nile tilapia (*Oreochromis niloticus*) farming has expanded rapidly in recent years, paralleling the global expansion of aquaculture. However, fish farming is often linked to the intensification of fish culture and the nutritional requirements (Lal, 2009; Moffitt and Cajas-Cano, 2014), which may have an impact on the well-being and, eventually, productivity, making it challenging to distinguish between overt disease and suboptimal development or reduced health status (Tacon, 2019).

Due to its extraordinary sensitivity to environmental changes, blood is a pathophysiological reflector of the body and a useful instrument for researching the impacts of toxicants (Ali and Ansari, 2023). Morphologically, seven types of cells have been identified in the blood of Nile Tilapia, including erythrocytes, thrombocytes, neutrophils, eosinophils, basophils, lymphocytes, and monocytes (Ueda et al., 2001; Handin et al., 2003).

The most important aspect of a fish farmer's skill set is the ability to recognize performance degradation during developmental stages and take corrective measures. Fish welfare can be negatively impacted by water quality degradation because it can introduce ineffective or toxic compounds into the fish (Naigaga and Kaiser, 2006; Srivastava and Reddy, 2020; Ibrahim and ElSaye, 2023). Successful fish farming relies on two critical factors, water source and water quality. Historically, the success of aquaculture intensification was largely determined by the engineer's ability to manage water and the nutritionist's ability to formulate cost-effective, nutritionally adequate diets (Shepherd et al., 2004).

In Sudan, aquaculture remains in its early stages, with most fish farming conducted by government or state organizations using low-tech methods. The small-scale sector dominates, with limited involvement from foreign entities

Received: September 27, 20; Revised: October 28, 2024 Accepted: November 23, 20; Published: December 30, 20; or organizations. *Tilapia spp.* is the most fascinating and widely used fish in fish farms. However, issues such as excessive reproduction and its associated challenges have hindered broader adoption. Various methods have been attempted to prevent overpopulation and stunted growth, including separating parents from offspring at hatching, monosex culture, introducing predators alongside tilapia, and selective breeding for larger fish. However, none have proven successful (Miller and Atanda, 2011). The current study addresses one of the critical issues in aquaculture, the accumulation of decomposed food and nitrogenous waste products from cultured fish. Excessive stocking levels in aquaculture systems can result in elevated levels of the formation of hazardous ammonia and a decline in water quality. As such, it is a significant factor that should be taken into account when evaluating fish culture. Hematology and other diagnostic tools such as enzyme activities can help identify stressors and diseases that impact fish performance (Fazio, 2019; Chew and Gibson- Kueh, 2023). These tools also assess fish health in response to changes in nutrition, water quality, and disease resulting from treatment, as reported by Fazio (2019). Over the last 20 years, there has been a rapid development of knowledge of the immunology of fish, particularly at the biochemical and molecular level, in terms of the sophisticated intercellular interactions that accompany any immune response (Sheldon et al., 2014).

The primary objectives of this study were to identify suitable water quality characteristics for aquaculture systems by comparing water from tanks, ponds, and the White Nile River, establish a method for assessing potential environmental impacts on cultured *Oreochromis niloticus*, and determine reference values for leukocyte differential counts in cultured Nile Tilapia.

MATERIALS AND METHODS

Ethical approval

Blood sample collection from the fish in this study was conducted in accordance with the National Animal Health Monitoring System (NAHMS) guidelines (USDA, 2022).

Experimental design

A Completely Randomized Design (CRD) was used to evaluate the effects of water quality parameters from four different water sources on the blood indices, specifically leukocyte differential counts, of Nile tilapia (*Oreochromis niloticus*). The studied fish were randomly collected from various water sources, regardless of their age or sex. The experiment was conducted in six glass tanks (100 cm x 35 cm x 50 cm), each equipped with an aerator positioned in the center. Two additional tanks, each fitted with two aerators, were provided to replace fish sacrificed for analysis during the study period. The experimental fish, with an average weight of 80–100 g and a total length of 19.0-22.7 cm, were collected using gillnets from the White Nile River in the El Shaggara area, located 10 km south of Khartoum (Sudan). A total of 16 fish were stocked in each tank and fed a commercial fish meal at 5% of their biomass per day for six months. The water sources included the General Administration Ponds (GAFP), Fisheries Research Centre Ponds (FRCP), and the White Nile River (WNR), which served as the control site.

Species

Nile tilapia (*Oreochromis niloticus*, [Cichlidae, Teleostei], Linnaeus, 1758) was selected for this study due to its widespread use in Sudanese fish farming and its consistent availability (Mahdi, 1972).

Blood collection and white blood cell differential count

The wedge smear technique is the most convenient and widely used method for preparing peripheral blood smears. For the present study, six fish were randomly selected from each water source to create peripheral blood smears for investigation. No anesthesia was used during blood collection. A small volume of whole blood (2.0 ml) was drawn from the caudal peduncle vein using a sterile 2.5 mL disposable syringe. The blood was transferred into a 0.5 mL mini-plastic tube containing EDTA (1.26 mg/0.6 mL) as an anticoagulant. Peripheral blood smears were promptly prepared and analyzed for differential cell counts using the wedge smear method (Dacie and Lewis, 2017). The differential counts of neutrophils, monocytes, lymphocytes, eosinophils, basophils, and total white blood cells are presented in Table 2.

Water quality parameters

Colorimetric analysis was performed to assess water quality parameters, including ionized ammonia (NH_4^+) , non-ionized ammonia (NH_3) , nitrate (NO_3^-) , and nitrite (NO_2^-) , utilizing the Lovibond test system (2000). This widely used quantitative water analysis technique measures the intensity of color produced by a chemical reaction to determine the concentration of specific substances and identify the chemicals present in the sample.

pH determination

The pH levels of water samples from the surveyed locations were measured using an electronic digital paper pH meter (Stick Meter, 2001).

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 20). A two-way Analysis of Variance (ANOVA), as described by Stern (1986), was utilized to assess the effects of water quality parameters (NH₄, NH₃, NO₃, and NO₂) on the white blood cell (WBC) counts of fish samples collected from various water sources. This method effectively evaluates interactions and differences between multiple independent variables, such as water quality parameters and fish from different water sources, on the dependent variable (WBC counts). Additionally, Pearson's correlation coefficient was applied to determine linear relationships between the physiochemical parameters of water quality and the WBC counts across the study groups.

RESULTS

Water quality characteristics

The water quality parameters of the WNR (control) and the water sources from FRCP, GAFP, and ET during the survey period are summarized in Table 1 and illustrated in Figures 1, 2, and 3a. Ionized ammonia (NH₄) concentrations were the highest in GAFP (8.36 \pm 0.05) and ET water (6.18 \pm 0.04), whereas the lowest levels were recorded in FRCP (0.57 \pm 0.06) and WNR (0.15 \pm 0.02). The pH of GAFP water (9.00 \pm 0.85) was significantly higher than that of FRCP (8.85 \pm 0.37), WNR (7.34 \pm 1.35), and ET water (7.21 \pm 0.17). Additionally, the WNR (control) exhibited the highest recorded temperature (30 \pm 0.00), showing a significant difference compared to the other sources (p > 0.05).

White blood cell count

The white blood cell counts for Nile tilapia in ET, GFAP, WNR, and FRCP groups are presented in Tables 2 and 3, as well as Figures 3b and 4. The water from the experimental tanks (ET) exhibited a significant positive correlation with the differential white blood cell count in the fish, compared to those from the White Nile River (p < 0.05, control). The total mean WBC count increased progressively from $113.26\pm0.24\times10^3$ in the FRCP group to $191.46\pm0.61\times10^3$ in the ET group, alongside an increase in neutrophils (from $10\pm0.11\%$ to $18.31\pm0.16\%$) and lymphocytes (from $27.43\pm0.12\%$ to $46.40\pm0.13\%$).

Table 1. Quality parameters for water samples procured from various water environments in Sudan

Treatment Water Environment	Samples size	Ionized ammonia (µg/dL)	Unionized ammonia (µg/dL)	Nitrate (μg/dL)	Nitrite (μg/dL)	hydrogen ions level	Temperature (° C)
WNR	16	$0.15^{c}\pm0.02$	$0.01^{c}\pm0.00$	$0.10^{b}\pm0.01$	$0.03^{b}\pm0.01$	$7.34^{b}\pm1.35$	$30.00^{a}\pm2.50$
FRCP	16	$0.57^{c} \pm 0.06$	$0.08^{c}\pm0.01$	$0.10^{b}\pm0.00$	$0.03^{b}\pm0.00$	$8.85^{a}\pm0.37$	$29.44^{a}\pm0.93$
GAFP	16	$8.36^{a}\pm0.05$	$0.42^a \pm 0.005$	$0.10^{b}\pm0.00$	$0.03^{b}\pm0.00$	$9.00^{a}\pm0.00$	$28.37^{a}\pm3.95$
ET	16	$6.18^{b}\pm0.04$	$0.31^{b}\pm0.02$	$0.65^a \pm 0.07$	$0.35^a \pm 0.04$	$7.21^{b}\pm0.17$	25.45 ^b ±4.32
Sig.		**	**	**	**	**	**

a-b Superscript letters in the identical column demonstrate a significant difference (p < 0.05). Data are expressed as averages \pm standard deviation. WNR: White Nile River, FRCP: Fisheries Research Centre Pond, GAFP: General Administration of Fisheries Pond, ET: Experimental Tanks, Sig: Significance.

Table 2. Blood profiles of Nile Tilapia (*Oreochromis niloticus*) as influenced by the various water environmental sources in Sudan

Deferential count Water Source	Eosinophils (%)	Basophils (%)	Platelets (%)	Neutrophils (%)	Monocyte (%)	Lymphocytes (%)	WBC (X 103)	Sig.
White Nile River (control)	1.11°±0.2	3.62°±0.02	29.63 ^a ±0.52	10.87°±0.04	6.22°±0.34	27.43°±0.12	116.96 ^b ±0.10	
Fisheries Research Centre Pond	1.17°±0.04	3.24°±0.05	32.34 ^b ±0.49	10.37 ^a ±0.11	5.75°±0.06	30.25°±0.08	113.63 ^b ±0.20	
General administration of fisheries pond	$5.68^{b}\pm0.18$	9.46 ^b ±0.017	14.27°±0.15	18.31°±0.16	15.43°±0.14	46.40°±0.13	113 ^b .26±0.24	**
Experimental tank	$9.68^{a}\pm0.44$	10.82 ^a ±0.12	13.65°±0.15	15.50°±0.19	7.69 ^b ±0.14	$45.57^{b}\pm0.30$	191ª.46±0.61	

Sample sizes are average \pm Standard Deviation (SD) ^{a, b, c} in the same column with different superscript letters show significant (p < 0.05) differences. **: Significant (p < 0.05). WBC: White blood cells

Table 3. Correlations between blood profile of Nile tilapia (*Oreochromis niloticus*) and different water environment parameters (NH4, NH3, NO3, and NO2)

White blood cells	Lymphocytes	Eosinophils	Basophils	Thrombocytes	Neutrophils	Monocytes	NH4	NH3	NO3	NO2
Lymphocytes	.519**									
White blood cells Lymphocytes Eosinophils Basophils Thrombocytes Neutrophils Monocytes NH4 NH3										
Eosinophils	.837**	.894**								
Basophils	.667**	.970**	.956**							
Thrombocytes	569**	995**	918**	980**						
Neutrophils	.280	.945**	.747**	.899**	930**					
Monocytes	183	.725**	.374	.607**	688**	.887**				
NH4	.369	.978**	.807**	.930**	965**	.977**	.835**			
NH3	.366	.976**	.801**	.911**	958**	.955**	.816**	.986**		
NO3	.923**	.670**	.882**	.776**	700**	.467*	.040	.542**	.559**	
NO2	.807**	.807**	.927**	.864**	837**	.667**	.297	.692**	.710**	.871**

^{**} Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). Where NH4: Unionized ammonia, NH3: Ionized ammonia, NO3: Nitrates, NO2: Nitrites and Number of Samples: 24. A correlation of - 0.96 and -0.95 are a strong negative correlation, whereas a correlation of -0.70 is stronger than +0.10.

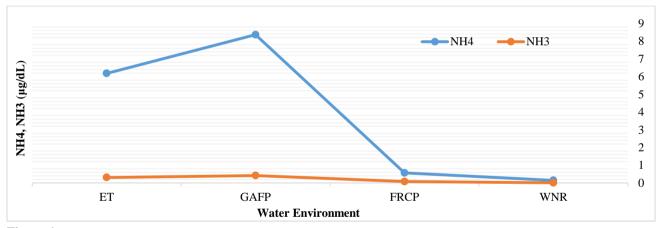


Figure 1. Ionized ammonia (NH4) and Unionized ammonia (NH3) concentrations (μ g/dL) appeared in various water environments. Where ET is the experimental Tank, GAFP is General Administration Fisheries Ponds, FRCP is Fisheries Research Centre Ponds, and WNR is White Nile River (water sources).

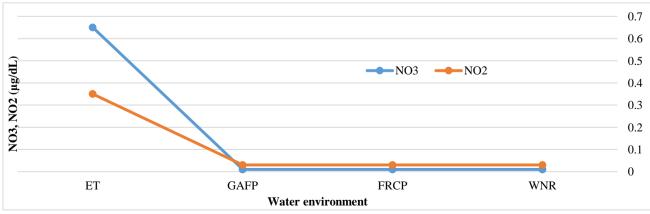


Figure 2. Nitrates (NO3) and Nerites (NO2) concentration (μ g/dL) appeared in various water environments. Where ET is the experimental Tank, GAFP is General Administration Fisheries Ponds, FRCP is Fisheries Research Centre Ponds, and WNR is White Nile River (water sources).

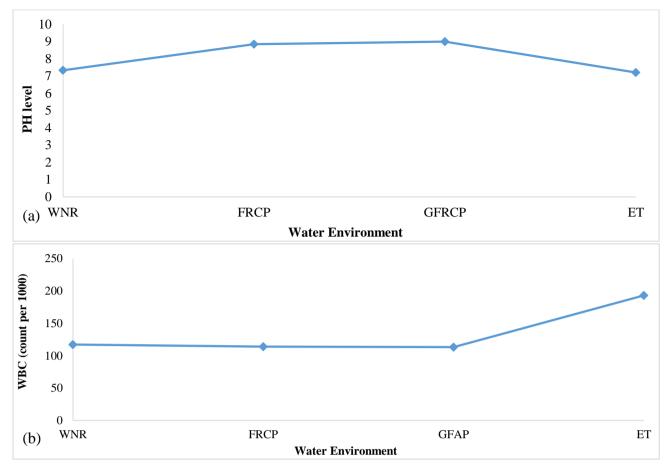


Figure 3. Hydrogen ion concentration (pH) levels as appeared at various water environment sources (a). White blood cell (WBC) count of Nile Tilapia (*Oreochromis niloticus*) per 1000 as appeared in various water environments (b). Where ET is the experimental Tank, GAFP is General Administration Fisheries Ponds, FRCP is Fisheries Research Centre Ponds, and WNR is White Nile River (water sources).

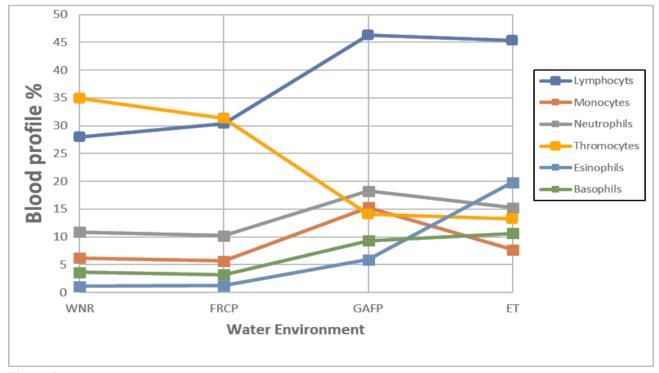


Figure 4. White blood profile (%) of Nile Tilapia (*Oreochromis niloticus*) influenced by different water environment parameters. Where ET is the experimental Tank, GAFP is General Administration Fisheries Ponds, FRCP is Fisheries Research Centre Ponds, and WNR is White Nile River (water sources).

DISCUSSION

The findings of this study revealed significant variations in the white blood cell (WBC) counts of Nile tilapia (*Oreochromis niloticus*) from different water sources. In comparison to the control, the fish from the ET had the highest eosinophils percentage and total leukocyte count. In comparison to the control fish in WNR, the GAFP fish demonstrated the highest levels of lymphocytes, monocytes, and neutrophils. Conversely, fish from the FRCP showed the highest platelet count. Significant changes in the percentage of individual leukocyte types are influenced by stress factors, particularly various forms of pollution (NH₄, NH₃, NO₃, and NO₂), including extreme pH levels (Modrá et al., 1998). This observation aligns with and supports the findings of the study by Modrá et al. (1998) and Lutnicka et al. (2016).

In general, the results of this study are confirmed by Lutnicka et al. (2016), Ueda et al. (2001), and Ibrahim and ElSayed (2023). Additionally, Ibrahim and ElSayed (2023) demonstrated that alterations in water quality variables significantly affect the blood profile of fish, as evidenced by the results of their examination. The measured water quality parameters for the White Nile River (WNR, control) and FRCP, as shown in Table 1, were within acceptable limits for Nile tilapia culture (Ibrahim and ElSayed, 2023; Aljadeff and Morlandt, 2024). Even so, the ET and GAFP water contained the highest concentration of water quality parameters. The water quality parameters measured in the WNR (control) and FRCP, specifically NH₄, NH₃, NO₃, NO₂, pH, and temperature fell within the acceptable limits for Nile tilapia culture, as reported by Ibrahim and ElSayed (2023). Research by Annas et al. (2023) indicates that ammonia can decrease dietary intake in fish at concentrations as low as 0.08 mg/L, with extended exposure to 0.2 mg/L causing serious harm.

In this study, the highest mean ammonia concentrations were observed in water samples from GAFP, ET, FRCP, and WNR, respectively. While nitrate (NO₃) is relatively non-toxic to tilapia, prolonged exposure can weaken the immune system and cause mortality (Hulata, 2001). Conversely, nitrite (NO₂) is highly toxic, impairing physiological functions and reducing hemoglobin's oxygen transport capacity, leading to growth retardation (El-Sayed, 2006).

Leukocytosis is the phenomenon characterized by an elevated white blood cell (WBC) count in a blood profile (Nussey et al., 1995; Lutnicka et al., 2016). This increase in leukocyte counts reflects the body's natural defense against foreign invaders, such as water pollutants, which can disrupt regular physiological processes in fish. The precise WBC count slightly increases in response to water environment pollutants. These data essentially demonstrate the impact of water pollution and indicate that essential physiological and immunological functions in fish may eventually be compromised after prolonged exposure to the deteriorated water quality of studied water sources.

Significant increases in percentage in neutrophils (neutrophilia), monocytes (monocytophilia), and eosinophils (eosinophilia) are indicative of changes in various water sources, as are significant increases in lymphocytes (lymphocytosis, Nwani et al., 2014; Saravanan et al., 2017). This conclusion is supported by the observation that more *Oreochromis mossambicus* leucocytes are produced at both temperatures, likely as a defense mechanism against potential pollution-related water deterioration (Nussey et al., 1995; Lutnicka et al., 2016).

The impact of contaminated water on the white blood parameters of the fish under study revealed a reduction in platelets and an increase in leukocytes, as observed in the ET water. This indicates that chemical contaminants in the water acted as antigens, triggering the proliferation of defense cells in the fish species (Svoboda et al., 2001; Venkateswara, 2006). The GAFP fish showed the highest monocyte percentage, while all fish from the WNR (control), ET, and FRCP groups remained within the normal monocyte range. Monocytosis in GAFP suggests an elevated monocyte count, which is sporadically linked to persistent water deterioration, with no clinical monocytopenia (Nussey et al., 1995; Svoboda et al., 2001; Venkateswara, 2006).

The highest percentage of neutrophils was observed in fish from GAFP. This finding aligns with studies like that of Pichhode et al. (2020), which reported increased neutrophil counts in Tilapia (*Oreochromis mossambicus*). Neutrophilia, or elevated neutrophil levels, can be triggered by factors such as stress, excitement, physical activity, and deteriorating water quality (Nussey et al., 1995; Mazon et al., 2002; Tavares- Dias and Moraes, 2007).

The lowest percentage of eosinophils and basophils were found in fish from the WNR and FRCP groups. These values resemble those reported by Henry and Kishimba (2006) and Bolade and Ndidi (2021). Basophils are extremely uncommon in all livestock species and rare in the blood of dogs and cats. According to Hrubec et al. (2000), basophils are observed only sporadically or in low numbers when present and are rarely found consistently in the blood. Eosinopenia, which is defined as a decreased eosinophil count, may be normal or result from stress or deteriorating water quality. Generally, the variation in WBC differential counts in this study may be linked to declining water quality in GAFP and ET water sources. In contrast, acute toxicity studies by Youssef et al. (2023) corroborated the findings of this study, reporting increased blood leukocyte counts linked to adverse effects observed during the study period due to deteriorating water quality.

Stress caused by high concentrations of water quality deterioration parameters, such as NH4, NH3, NO3, and NO2, leads to increased leukocyte levels, particularly neutrophils characterized by lymphocytosis and neutrophilia (Bolade and Ndidi, 2021). In this investigation, leukocyte counts considerably fluctuated to values above the control (WNR) and FRCP fish, likely due to abnormal concentrations of water quality parameters (NH₄, NH₃, NO₃, and NO₂) in GAFP and ET. Similar findings have been reported by Briggs and Bain (2017).

CONCLUSION

In conclusion, this study sheds light on the challenges affecting the growth of Tilapia (*Oreochromis niloticus*) in fish pond culture in Sudan. Poor water quality can adversely impact fish health by causing toxicosis or introducing harmful agents. Such deterioration significantly hampers fish productivity and compromises health, ultimately leading to the onset of recognizable diseases. Moreover, the study underscores the potential of blood parameter values as reliable indicators for evaluating fish health.

Further research is needed to determine whether the elevated WBC levels are caused by the deterioration of the water source or by immune stimulation from other factors, such as heavy metals.

DECLARATIONS

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Availability of data and materials

The original data supporting this study are included in the article and are available upon reasonable request to the corresponding author.

Ethical considerations

The authors of the current study declare that this study is an original study that was authored by contributions of all authors and submitted for the first time to the present journal.

Authors' contributions

Prof. Hassan Sulieman contributed to the study design and experiment scheduling, while Talaat Hassan conducted the data analysis. All authors reviewed the analyzed data and approved the final manuscript draft.

Competing of interests

The authors declare that there is no competing interest.

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Incidence of Hepatitis Hydropericardium Syndrome in Broiler Chickens Caused by a New Fowl Adenovirus Strain in Iraq

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ABSTRACT

Hepatitis Hydropericardium syndrome (HHS) is an acute infectious disease affecting broiler chickens. It is caused by a fowl adenovirus (FAdV) of Group I, serotype 4. This disease is characterized by sudden deaths in broilers as young as three weeks, with mortality rates reaching up to 65%. The current study aimed to evaluate the outbreak of HHS in three broiler farms in southern Iraq. It also sought to identify the specific serotypes of fowl adenovirus (FAdV) responsible for this outbreak, primarily focusing on its genetic characteristics and diversity. Ten liver and heart tissue samples were collected from broiler chickens (Ross 308) that had displayed clinical signs of depression, ruffled feathers, and a tendency to huddle in corners before death. Viral DNA was extracted from liver tissues for further virus detection using PCR and RT-PCR. A post-mortem examination showed a turmeric-yellow discoloration in the dividing lines between the pectoral muscles and the abdominal cavity. The livers of infected chickens were markedly enlarged, and clear, yellow-colored fluid was observed in the pericardial sac. Histopathological analysis of stained liver and heart tissues revealed small multifocal areas of necrosis and mononuclear cell infiltration, including basophilic intranuclear inclusion bodies in hepatocytes and lymphocytic infiltrates. Conventional PCR analysis of liver tissues confirmed the presence of FAdV serotype 4, identifying all samples as the Melad strain, a novel strain responsible for the ongoing epidemic in Iraq. This study confirmed the presence of FAdV serotype 4 and identified all samples as the Melad strain. This research also addresses the need to investigate FAdV with molecular techniques for a better understanding of the epidemiology of the disease.

Keywords: Fowl adenovirus, Hepatitis Hydropericardium Syndrome, Melad strain serotype 4.

INTRODUCTION

Hepatitis Hydropericardium Syndrome (HHS) has emerged as a critical challenge in the global poultry industry, leading to substantial economic losses and high mortality rates, particularly in Iraq since its recognition in the early 1990s (Abdul-Aziz and Al-Attar, 1991; Abdul-Aziz and Hassan, 1995). HHS primarily affects broiler chickens aged 3 to 6 weeks and is characterized by abrupt outbreaks that severely impact poultry production (Liu et al., 2022; Oraibi and Abdalmaged, 2022). Key clinical manifestations of HHS include hepatomegaly, hepatic discoloration, and the accumulation of gelatinous fluid within the pericardium, highlighting the severe pathophysiological effects on infected flocks (Li et al., 2016). The etiological agent responsible for HHS is a non-enveloped double-stranded DNA virus, classified within the Adenovirus family and specifically under the fowl adenovirus (FAdV) group (Meulemans et al., 2004; Fitzgerald et al., 2020). Fowl adenoviruses (FAdVs) are classified into twelve serotypes, grouped into five species including FAdV-A to FAdV-E (Mirzazadeh et al., 2020; El-Shall et al., 2022). Among these, FAdV-C, particularly serotype 4, is most frequently linked to HHS outbreaks worldwide and is recognized as the principal causative agent of the disease (Pallister et al., 1996; Liu et al., 2016; Niczyporuk, 2016; Mirzazadeh et al., 2020; El-Shall et al., 2022). The hexon protein, located on the virion capsid, plays a crucial role in classifying FAdVs into specific groups and types, while the fiber protein at the distal C-terminal head of the virion contains type-specific antigens (Hess, 2000; Mase et al., 2010; Zhao et al., 2016; Li et al., 2023). HHS is known by various names across different regions, reflecting its widespread impact and local nomenclature. In Pakistan, it is called "Angara disease," named after the region near Karachi, where it was first reported (Cheema et al., 1989; Kataria et al., 1997). In India, it is commonly called "Leachy" (Afzal et al., 1991; Gowda and Satyanarayana, 1994). To differentiate it from classical inclusion body hepatitis (IBH), some researchers call it "infectious hydropericardium" (IH) (Mirzazadeh et al., 2020). Additionally, it is recognized as "inclusion body hepatitis-hydropericardium syndrome" (IBH-HPS) and "hydropericardium hepatitis syndrome" (HHS) in various scientific literature.

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Traditionally, the disease is diagnosed by post-mortem examination, histological findings of the liver and other organs in the affected bird, and various serological tests (Zhao et al., 2016). However, more accurate and sensitive molecular techniques include polymerase chain reaction (PCR), Real-time PCR (RT-PCR), and restriction fragment length polymorphism (RFLP) (Meulemans et al., 2004; EL-Shall et al., 2022).

The current study aimed to evluate the outbreak of Hepatitis Hydropericardium Syndrome (HHS) in broiler farms located in the Shatra district of Thi-Qar Governorate, southern Iraq. It also sought to identify the specific serotypes of fowl adenovirus (FAdV) responsible for this outbreak, primarily focusing on its genetic characteristics and diversity.

MATERIALS AND METHODS

Ethical approval

The authors considered ethical concerns and farmers' consent before the surveys. This article was originally written without copying from other articles. All broiler chickens involved in this research were treated humanely, according to the guidelines outlined by the College of Veterinary Medicine, Baghdad University, adhering to the procedures outlined by international and national animal care, and using criteria of ethical standards defined in the 1964 Declaration of Helsinki.

Environmental conditions

This study began following a notification from a poultry farm owner in December 2023, who reported a significant loss of broilers at his farm in the Al-Shatra district of Thi-Qar Governorate, Iraq. Alarmed by the high mortality rate, the owner contacted the research team to investigate the sudden deaths of the broiler chickens. Initial observations were conducted during a comprehensive visit to the farm, spanning from December 2023 to May 2024. The farm owner reported that the mortality rate was about 65%.

During the visit, the research team meticulously assessed the farm environment. The farm consisted of three broiler houses (Breed Ross 308) situated in a rural area with a semi-arid climate, characterized by significant temperature fluctuations, reaching around 30°C between day and night, and a humidity of 60%. The farm operates under an openhouse system with essential ventilation and temperature control measures. The broiler chickens are raised on deep litter bedding, and the farm follows a standard feeding regimen, utilizing commercially available poultry diet plate fodder that contains 22% starter protein and has a metabolizable energy content of about 3200 kcal. Routine management practices at the farm include vaccinations against common poultry diseases. At one day old, the chicks receive the Newcastle Disease Virus (NDV) vaccine, Clone, and Ma5 IB (Inervet, Netherlands). At five days old, they are injected with the Avian Influenza virus (AIV Hg) and Newcastle disease virus (NDV) Lasota killed vaccine (Vaxxon, Italy). The Newcastle disease (ND) Lasota vaccine is administered again at eight days of age through drinking water. Finally, on day 14, the chicks receive the Infectious Bronchitis Disease (IBD) vaccine D78 (Intervet, Netherlands) via drinking water. Periodic health check-ups and regular cleaning and disinfection of the poultry houses are also conducted.

Clinical signs

The clinical signs observed in the affected broilers included depression, ruffled feathers, and a tendency to huddle in corners before death. The droppings were discolored, varying from yellowish to greenish, and the chickens exhibited lethargy and elevated body temperatures. These observations, combined with a high mortality rate, necessitated further investigation through post-mortem examinations and molecular diagnostic techniques to identify the causative agent of the outbreak (Chen et al., 2019).

Post-Mortem examination

Post-mortem examinations adhered to the protocols described by Kalai (2024) with additional precision to ensure comprehensive analysis. The external examination involved a detailed inspection for signs of trauma, external parasites, or abnormalities in the plumage and skin. A systematic approach was followed, opening the body cavity to expose the thoracic and abdominal organs. Standard dissection instruments, including scalpels, forceps, and scissors, were used. The organs were inspected in situ for any gross lesions before being removed for further examination. Particular attention was given to the liver, heart, lungs, kidneys, and gastrointestinal tract.

Sample collection

Ten tissue samples from the liver and heart of affected broiler chickens were collected and divided into two parts. Part 1 was used for molecular techniques, and Part 2 was fixed in 10% neutral buffered formalin for histopathological analysis.

Histopathology

The liver and heart samples of the affected chickens were processed with care for the histological examination. The samples were fixed in 10% neutral buffered formalin for 24 hours. After fixation, the tissues were dehydrated through a graded series of ethanol, cleared in xylene, and then embedded in paraffin wax. Paraffin blocks were sectioned to a thickness of 5 µm using a rotary microtome. The sections were stained using the hematoxylin and eosin (H and E) protocol (Hess, 2000). Hematoxylin staining was performed for 5 minutes, followed by differentiation in acid alcohol, and then rinsed in alkaline tap water. Eosin staining was carried out for 2 minutes. After staining, the sections were dehydrated, cleared, and mounted with coverslips. Any deviations from the standard H and E protocol were carefully noted to ensure consistent staining quality. The prepared slides were examined under a light microscope (Olympus, Japan) at a specified magnification to identify and evaluate the presence and extent of lesions. The detailed histopathological analysis aimed to detect cellular and tissue-level abnormalities indicative of the pathological condition (Gallina et al., 1973).

Molecular technique

A polymerase chain reaction (PCR) assay was developed following the protocol outlined by Ganesh et al. (2000) to detect fowl adenovirus (FAdV) associated with Hepatitis Hydropericardium Syndrome (HHS). According to the manufacturer's instructions, viral DNA was purified from infected liver tissues using the KYLT RNA/DNA Purification Kit (Anicon, Germany). Primers targeting the *hexon gene* of FAdV were designed based on previously published sequences (Werner et al., 2024).

PCR protocol

The PCR was conducted using a thermocycler (Applied BiosystemsTM VeritiTM 96-Well Thermal Cycler, USA) with the following cycling conditions, thirty cycles at 90°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. The target products were detected using specific *hexon* gene primers and subsequently subjected to DNA sequencing. The results of nucleotide sequencing confirmed the nature of the product (Liu et al., 2016; Werner et al., 2024).

Controls and replicates

To ensure reliability, each PCR assay included positive and negative controls. The positive control consisted of known FAdV DNA, while the negative control utilized nuclease-free water. all PCR reactions were performed in triplicate to ensure consistency and reproducibility of the results.

Detection of virus DNA

The PCR products were analyzed using agarose gel electrophoresis, which confirmed the specific *hexon* gene by comparing the band patterns with a molecular weight marker. The amplified products were then purified and subjected to DNA sequencing using the Sanger sequencing method on an ABI 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequencing results were analyzed to verify the specific nature of the amplified products, ensuring accurate identification of the fowl adenovirus associated with HHS (Liu et al., 2016).

RT-PCR for variant identification

Hexon gene-specific and variant-specific RT-PCR assays were conducted according to the protocols provided by AniCon Labor GmbH (AniCon Labor GmbH, Muehlenstr. Hoeltinghausen, Germany). Hybridization probes specific to HHS-FAdV, *Hexon* A, *Hexon* B, and *Hexon* C were utilized, following the instructions for the Kylt® HHS-FAdV assay (Liu et al., 2016; Werner et al., 2024).

Variant identification

To identify and detect variant mutations and single nucleotide polymorphisms (SNPs), DNA sequences were analyzed using bioinformatics tools and software recommended in the protocol (Yamaguchi et al., 2022; Werner et al., 2024). The sequencing data were aligned and compared with reference sequences to identify any mutations or variations. The sequences of the new strain were validated and deposited in the GenBank database, which is accessible through the National Center for Biotechnology Information (NCBI, Bethesda, MD20894, USA). This comprehensive approach ensured the accurate detection and characterization of FAdV variants, providing valuable insights into the genetic diversity and epidemiology of the virus (Liu et al., 2016).

RESULTS

Clinical signs

The research team identified several factors that may have contributed to the outbreak, including high broiler chicken density, potential breaches in biosecurity protocols, and the possible introduction of the virus through contaminated feed or equipment. The mortality rate reached about 60%; the affected broiler chickens exhibited a range of clinical signs indicative of severe illness, with symptoms progressing consistently across the observed population, though some variability in severity was noted. Elevated body temperatures were observed, suggesting a systemic inflammatory response or infection. The chicken's feathers appeared fluffy and unkempt, a common sign of poor health or systemic disease. The droppings were notably yellowish-green and phosphorescent, indicating possible liver dysfunction or biliary involvement. The comb and wattles of the affected chickens were pale, signifying anemia or circulatory compromise. Many chicks were found dead while lying on their stomachs, a posture suggesting respiratory distress or sudden cardiac failure. Difficulty breathing was noted, attributed to the fluid-filled sac around the heart (pericardial effusion), which likely compromised cardiac function and contributed to the respiratory difficulties observed (Choi et al., 2012; Chen et al., 2019).

Post-mortem

Post-mortem examinations revealed several distinctive pathological findings that provided significant insights into the disease process. Externally, a notable turmeric-yellow discoloration was observed in the dividing lines between the pectoral muscles and the abdominal cavity (Figure 1). The broiler duodenal loop showed multifocal necrosis in the pancreas. Additionally, the subcutaneous fat exhibited a dark yellow hue, indicating jaundice or extensive lipid deposition (Figure 1a). Internally, significant congestion and an increase in the thymus were observed, suggesting an immune response or inflammation. Sporadic hemorrhagic spots were present in the leg and chest areas, indicative of vascular damage or coagulopathy. The livers of the infected chickens were markedly enlarged, and clear, yellow-colored fluid was present in the pericardial sac (Figures 1b, c). A yellow, watery sac was found surrounding the heart within the endocardium, which initially appeared as a yellow liquid and later turned gelatinous in the advanced stages of infection, impairing cardiac function (Figure 1d).

Necrotic foci were evident in the pancreas, indicating localized cell death likely due to viral infection or secondary complications. The spleen was pale and enlarged, with signs of congestion in the surrounding intestines, suggesting splenomegaly and an active immune response (Figures 1-4). The kidneys were also enlarged and pale, indicative of renal compromise or infection. Blood spots were observed in the duodenal area, pointing towards gastrointestinal involvement and possible hemorrhagic enteritis. These detailed post-mortem findings provided crucial insights into the pathophysiological changes occurring in the affected chickens, highlighting the systemic nature of the infection and its impact on multiple organs.

Histopathological findings

Histopathological examination of liver samples from infected chickens revealed significant pathological changes, providing crucial insights into the disease's impact on cellular structures. Liver tissue sections were stained using the hematoxylin and eosin (H & E) technique, a standard method for highlighting cellular and tissue morphology. These sections were then examined under a light microscope at a magnification of X400 to allow detailed observation of cellular changes.

The findings showed numerous necrotic foci within the liver parenchyma, indicating areas where hepatocytes had died due to the infection (Figure 2). This necrosis disrupted normal liver function, suggesting an advanced stage of disease progression. Additionally, basophilic intranuclear inclusion bodies were prominently observed within the hepatocytes. These inclusions, stained dark blue to purple, indicate viral replication, confirming the presence of a viral pathogen actively infecting the liver cells. Furthermore, the examination revealed blue and red corpuscles within the nuclei of hepatocytes. These corpuscles represent abnormal nuclear structures and inclusions, signifying severe cellular distress and disruption of normal nuclear functions. The presence of these nuclear inclusions and necrotic areas are hallmark features of viral hepatitis, suggesting an aggressive and extensive viral infection. These histopathological findings underscore the severity of the liver damage caused by the infection, highlighting the aggressive nature of the disease and its profound impact on the affected broiler chickens. The extensive necrosis and the presence of intranuclear inclusion bodies are critical indicators of the pathological processes at play, providing essential information for understanding the disease mechanism and progression.

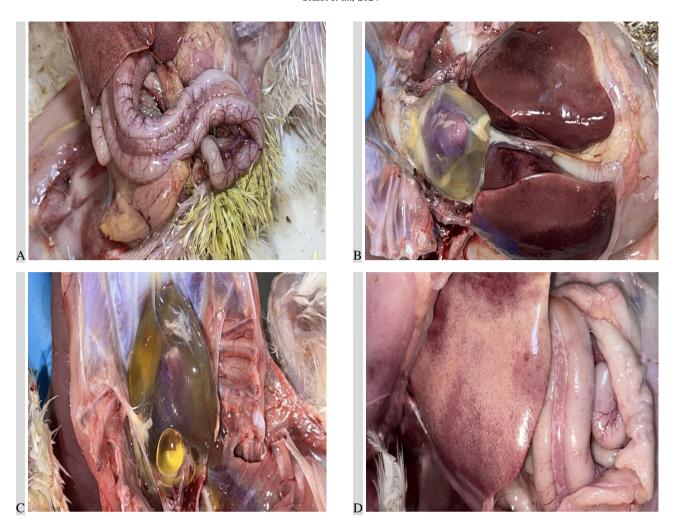


Figure 1. Gross lesions in 24-day-old broiler chickens affected by hepatitis hydropericardium syndrome. **A:** The broiler duodenal loop shows multifocal necrosis in the pancreas. **B:** Enlargement of the liver of infected chicken and the accumulation of clear, yellow-colored fluid in the pericardial sac. **C:** A yellow watery sac surrounding the heart within the endocardium in a bird affected by HSS. **D:** Enlarged spleen and congested intestines in the affected broiler chickens.

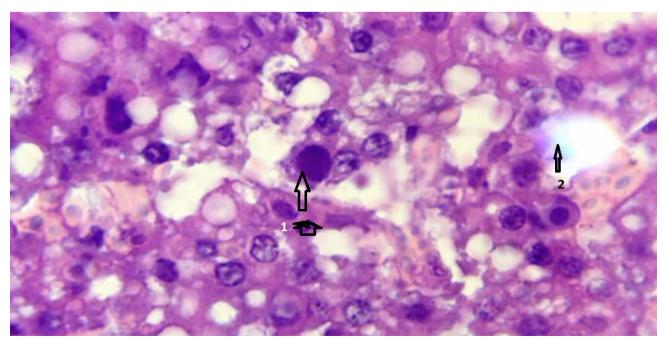


Figure 2. The liver of an affected broiler chicken with hepatitis hydropericardium syndrome at 24 days of age. The numerous basophilic intranuclear inclusion bodies in the hepatocytes. (1) Small multifocal areas of necrosis (2). Blue and red corpuscles within the nuclei of hepatocytes are also observed (H and E stain, magnification X400).

Molecular techniques

Liver samples from affected broilers collected from the farm located in the Al-Shatra district tested positive for HHS adenovirus using the polymerase chain reaction (PCR) technique.

PCR protocol

The PCR protocol followed a meticulous step-by-step process to ensure the accuracy and reliability of the results.

All ten samples taken from the affected liver were positive. Each PCR run included both positive and negative controls. While the positive control used known HHS adenovirus DNA, the negative control used nuclease-free water instead of DNA. Each sample and control were run in triplicate to ensure the reproducibility and reliability of the results. The PCR products were analyzed using agarose gel electrophoresis, which confirmed the presence of the specific *hexon* gene fragment at approximately 580 bp (Figure 3).

DNA sequences

The PCR products were then subjected to DNA sequencing to confirm the identity of the adenovirus. The sequencing was performed using the Sanger sequencing method on an ABI 3730xl DNA Analyzer (Applied Biosystems). The complete genomic sequences of FAdV-4 were analyzed and deposited in the GenBank database under accession number PP129627 (Figure 4).



Figure 3. Conventional PCR test of liver tissue from broiler chickens infected with adenovirus. The size of the fragment is approximately 580 bp.

ORIGIN

- 1 cgacagaaaa ggetcaacgg etgeaaatee gettttacee cateeaaace gacgacacgt
- 61 cgacgggcta ccgcgtgcgg tacaacatca atgtgggcga cggttgggtc ctggacatgg
- 121 ggtcgaccta tttcgacatc aagggaatcc tagaccgagg gccgtccttc aagccctact
- 181 geggeaegge ttacaaceeg etggeteeca aggagteeat gtttaacaac tggteggaga
- 241 eggeaeeegg geagaaegtg teegeeteeg gteagetgte eaaegtetat aceaaeaega
- 301 gcacctccaa agacacgacg gcggcgcagg tgacgaagat ttccggcgtc ttccccaatc
- 361 ccaaccaggg acceggaaga aateetetge gaegggtaga aaacgccaac accggegtge
- 421 teggtegett egecaagtet eagtacaatt aegettaegg tgeetaegte aagecegteg
- 481 ccgccgacgg ttcccagtcc ctcacgcaga ccccctactg gatcatggat aacacgggca
- 541 ccaattacct gggagcggtg gccgtcgagg actacaccaa cagcctctcg tacccagata
- 601 ccatagtcgt gccgcctccc gaggactacg acgattataa cataggcacc acgcgtgcgc
- 661 teaggeceaa etacateggg tteagggata aetteattaa eetgetgtat eaegaeteeg
- 721 gegtgtgete gggeacecte aacteggage gttegggeat gaaegtggtg gtegagetge

781 ccgaccggaa taccgagete agetaccagt acatgetgge

Figure 4. Gene sequences used in NCBI-based BLAST searches identify Fowl Adenovirus C from Ross 308 chickens affected by Hepatitis Hydropericardium Syndrome.

DNA sequencing

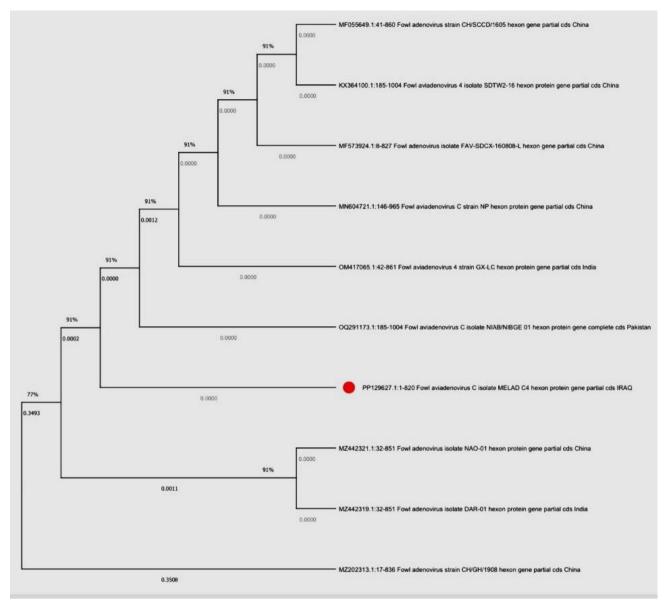


Figure 5. Sequence analysis of the new strain using NCBI-based BLAST search confirmed the identification of Fowl Adenovirus C in Ross 308 chickens as the causative agent leading to hepatitis hydropericardium syndrome.

Analysis results and validation

The nucleotide sequences of the two-flank regions, Fib F and Fib R, were analyzed, revealing two sequences with molecular weights of 6011 and 6287, respectively. The guanidine-cytosine (GC) content for both fragments was determined to be 60%. The sequencing data were cross-checked with existing sequences in the GenBank database using the NCBI BLAST tool to confirm the identity of the virus. This analysis confirmed that the causative agent of HHS in the Al-Shatra district was adenovirus serotype 4 type C, aligning with the gene sequencing data provided by the National Center for Biotechnology Information (NCBI). The samples were identified as Fowl Adenovirus C, supporting the conclusions of other studies, particularly those referencing the Fowl Aviadenovirus C isolate MELAD *hexon* protein gene and partial coding sequences (Figure. 5; Liu et al., 2016). The detailed analysis and validation steps ensured the accuracy and reliability of the molecular diagnosis, providing a comprehensive understanding of the genetic makeup and variation of the adenovirus strain involved in the outbreak.

DISCUSSION

Hydropericardium hepatitis syndrome (HHS) is a fatal disease caused by fowl adenovirus serotype 4 (FAdV-4, Figure 5). The current study unequivocally confirms that HHS is a lethal infectious disease caused by FAdV-4, affecting broilers

aged 24 days on farms located in southern Iraq. This finding is consistent with global reports of HHS outbreaks, highlighting the widespread nature of the disease with no specific geographic limitations (Cheema et al., 1989; Afzal et al., 1991; Asrani et al., 1997; Philippe et al., 2005; Choi et al., 2012; Liu et al., 2016; Niczyporuk, 2016; Cui et al., 2020; Mirzazadeh et al., 2020; El-Shall et al., 2022; Yamaguchi et al., 2022). In Iraq, the disease has been well-documented since the 1990s (Abdul-Aziz and Al-Attar, 1991; Abdul-Aziz and Hassan, 1995; Abdulrahman et al., 2022; Al-Taee and Saeed, 2022; Oraibi and Abdalmaged, 2022) and continues to pose a significant threat, with recent cases consistently attributing the causative agent to FAdV-4 (Ye et al., 2016; Li et al., 2017; Mete et al., 2021). The virulence of HHS appears to have increased over time, a trend that the current study substantiates, with reported mortality rates reaching up to 65%. Post-mortem examinations revealed classic HHS lesions, including those depicted in Figures 1, 2, and 3, which are consistent with the findings of other researchers (Naeem et al., 1995; Asrani et al., 1997). This study also emphasizes critical issues by detecting a novel strain responsible for the ongoing epidemic in Iraq. Adenoviruses (FAdVs) may have undergone significant genetic changes over the years through multiple passages, resulting in the emergence of highly virulent strains. The genome of FAdV is composed of double-stranded DNA, encapsulated by protein coats that include two specific proteins, hexon, and fiber (Zhao et al., 2016; Sharif et al., 2020). The hexon protein, which houses genes responsible for pathogenicity, is located at the end of the genome, while the central region of the genome shows conserved organization among different FAdVs. Notably, most mutations occur at the genome ends, which are crucial in distinguishing the five genera of the virus (Mittal et al., 2014; Zhao et al., 2016; Niczypouk, 2016; Sharif et al., 2020). Pallister et al. (1996) observed that variations in FAdV virulence could be attributed to a single gene. Ye et al. (2016) reported numerous deletions in serotype 4 FAdV, while Li et al. (2017) identified ORF19 deletions in the FAdV-4 HB 1510 strain as investigated by NBIC.

Furthermore, Yamaguchi et al. (2022) and Chen et al. (2019) indicated that serotype 4 FAdV isolates with truncated ORF19 displayed higher virulence than other isolates, particularly those in Japan and China. These findings hold significant implications for poultry health management, emphasizing the necessity for targeted intervention strategies by avian disease specialists to mitigate the impact of the disease.

Routine monitoring of HHS is necessary because adenovirus infections can severely compromise the efficacy of vaccinations for other poultry diseases, such as Newcastle disease (ND), influenza, infectious bursal disease, and infectious bronchitis. This interference is likely due to the destruction of lymphoid tissues and the induction of immunosuppression, either directly or indirectly (Nakamura et al., 1999; Mase et al., 2010; Yan et al., 2020). Additionally, co-infections with avian Ortho reoviruses (ARVs) have been shown to enhance FAdV-4 replication in specific pathogen-free (SPF) chickens, resulting in significant changes in cytokine levels in co-infected groups compared to those with single infections (Nakamura et al., 1999; Tian et al., 2020). Another critical consideration is the presence of adenoviruses in apparently healthy chickens. The isolation of adenovirus from such broiler chickens in China suggests that the virus can circulate asymptomatically within the host, only to become activated and mutate into more virulent forms under certain conditions (Yan et al., 2020; De Luca et al., 2022). This phenomenon supports the hypothesis that new, more virulent strains may emerge through deletion processes at the genome ends, significantly affecting the hexon genes and thus causing more severe disease outbreaks.

Meanwhile, the current study underscores the increasing virulence of FAdV-4 and its substantial impact on poultry health. This finding necessitates ongoing vigilance and advanced research into adenovirus mutations and their epidemiological trends. Such efforts are essential for developing effective strategies to control and prevent HHS, safeguarding poultry populations from this devastating disease. The current findings align with global reports, emphasizing the widespread and escalating threat posed by this adenovirus strain. Molecular genetic analysis revealed notable mutations and deletions in the ORF19 region associated with increased virulence. The finding supports the hypothesis that FAdVs undergo significant genetic modifications over time, leading to the emergence of more virulent strains. The identification of the Melad strain of FAdV-4, unique to this study, underscores the ongoing evolution of the virus and its implications for poultry health management. The study highlights the need for continuous monitoring and advanced research into FAdV mutations and their epidemiological trends. Comprehensive post-mortem, histopathological, and molecular examinations elucidated the severe pathological impact of FAdV-4 on affected broiler chickens, characterized by extensive hepatic necrosis, basophilic intranuclear inclusion bodies, and gelatinous fluid accumulation within the pericardium.

While this study provides significant insights, certain limitations should be noted. The geographic scope is confined to broiler farms in southern Iraq, which may only partially be representative of other regions. The sample size, although significant, may not capture the total genetic diversity of FAdV strains present. The study spans from December 2023 to May 2024, offering a limited temporal snapshot of the impact of the disease and the genetic evolution of the virus. While the focus on FAdV-4 is essential, the potential role of co-infections and other adenovirus serotypes warrants further exploration. However, this study underscores the need for vigilant monitoring, advanced research, and tailored

intervention strategies to combat HHS in poultry. By addressing these challenges, the poultry industry can better safeguard against the devastating impacts of this disease, ensuring healthier flocks and more stable production outcomes.

CONCLUSION

This study confirmed the incidence and outbreak of HHS in broiler farms in southern Iraq, attributing the causative agent to fowl adenovirus serotype 4 (FAdV-4). The findings highlight the significant virulence of the disease, resulting in mortality rates reaching up to 65%. Future research should focus on developing region-specific vaccines tailored to the genetic makeup of prevalent FAdV strains, implementing stricter biosecurity protocols to prevent the introduction and spread of FAdVs, conducting long-term studies to track the evolution of FAdV strains and their impact on poultry health, and integrating disease management strategies combining vaccination with improved management practices.

DECLARATIONS

Finding

This study received no financial support.

Availability of data and materials

All data from the current study are available in this article.

Authors' contributions

Melad Ibrahim Oraibi, Majid Hagi Khaleel, and Amer Abdulameer A. Al-Baldawi conceived the idea, developed the theory, and performed the computations. Majid Haqi Khaleel and Amer Abdulameer A. Al-Baldawi verified the analytical methods. All authors are involved in laboratory work. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no conflicts of interest.

Ethical considerations

The authors considered ethical concerns and farmers' consent before conducting the surveys. This article was originally written without copying from other sources and submitted to this journal for the first time.

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Sebaceous Adenitis in an Akita: Symptoms and Therapeutic Approaches

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ABSTRACT

Sebaceous adenitis is observed in different animal species, with dogs being the most commonly diagnosed. This study aimed to report a case of sebaceous adenitis in a 5-year-old male Akita dog weighing 35.8 kg, initially presented with pruritus and alopecia on the inner ear surfaces, face, and head. Over time, signs of changes in sebaceous glands appeared in other body parts. The dog was sent to a veterinary clinic in Kamyanets-Podilsky, Ukraine. During the clinical examination, the body temperature was 38.4°C, heart rate 78 bpm, and a respiratory rate 27 breaths per minute, with no change in appetite. Clinical and dermatological methods and histopathological examination were used to detect the disease. The hair on the head was curling, and the lesions appeared on the dorsal tail, the distal front and hind legs, the groin, and the abdomen. Initially, redness and itching occurred in the affected areas, and the skin lost elasticity and became dry. The hair was stuck together with gray crusts. The diagnosis was confirmed based on pathohistological studies. An inflammatory infiltrate with migration of lymphocytes, histiocytes, neutrophils, and atrophy of sebaceous glands was diagnosed, focusing on the pathology of the dermis. Treatment included topical therapy with shampoo applied for 3-5 minutes, then rinsed and followed by Conditioner for 2 minutes, over 30 days. Omega-3 was administered orally at 1000 mg twice daily. Isotretinoin (Roaccutane[®], 20 mg) was administered orally twice daily for 30 days. Moreover, Cyclosporine was dosed at 5 mg/kg once daily orally, on an empty stomach, for 30 days. During the treatment, signs of inflammation gradually disappeared, with itching and hyperemia disappearing from day 3. From day 7, desquamation and hair loss decreased, and on day 12, signs of new hair growth appeared. The areas were completely restored on day 28 of treatment. Sebaceous adenitis was found to lead to the destruction of sebaceous glands, causing scaling, hair loss, and skin inflammation, which could be a hereditary condition in Akitas. Immunomodulation and normalization of trophic processes in the skin are crucial in the treatment.

Keywords: Akita, Diagnosis, Dog, Sebaceous Adenitis, Treatment

INTRODUCTION

Sebaceous adenitis is defined as inflammation of the skin glands characterized by degenerative changes and their atrophy. Sebaceous adenitis is observed in various animal species, though it is most frequently diagnosed in dogs. Sebaceous glands play an important physiological role in dogs and provide protective mechanisms for the body's natural defense and maintaining homeostasis. These glands also play a vital role in dogs in maintaining the health of the skin and coat by secreting sebum, an oily substance that provides moisture and protection. Sebum forms a barrier that prevents moisture loss, reduces the risk of microbial infections, and enhances skin elasticity. Moreover, sebaceous glands play a crucial role in sustaining the natural equilibrium of the skin's microbiome (Abbas et al., 2021; Harris et al., 2022; Anderson et al., 2023).

Skin glands secrete sebum, an oily substance that moisturizes the skin and hair, ensuring their flexibility (Wang et al., 2022; Older et al., 2023). Located primarily near the hair follicles, their activity is regulated by hormones and other physiological factors. Neutered or spayed dogs may experience changes in sebaceous gland activity, which can affect the condition of their skin and coat (Lee et al., 2020; Older et al., 2021; Outerbridge and Jordan, 2021). Sebum forms a protective barrier on the skin's surface, helping to prevent the entry of bacteria, fungi, and other pathogens (Zhelavskyi et al., 2023a).

Since dogs spend a lot of time outdoors, with their skin exposed to various environmental factors, the protective function of sebum is particularly significant. Sebaceous glands are situated in the dermis, typically at the upper part of hair follicles (Cugmas and Olivry, 2020). Sebaceous glands are distributed throughout the dog's body, with the highest concentration found in areas with dense hair, such as the back, neck, and tail.

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Diet plays a key role in preventing and controlling sebaceous adenitis in dogs, as proper nutrition supports skin and coat health. A diet rich in omega-3 and omega-6 fatty acids reduces inflammation and improves the skin barrier (Olivry et al., 2020). Sebaceous adenitis in dogs can be triggered by foods containing allergens or inadequately balanced nutrients. Products high in artificial additives, preservatives, or low-quality protein sources can negatively impact skin health and cause inflammation of the sebaceous glands (Outerbridge and Jordan, 2021).

Previous studies have shown that sebaceous adenitis is influenced by genetic factors, breed predisposition, and autoimmune mechanisms (Hayashi et al., 2021; Patel et al., 2021; Zhelavskyi et al., 2023b). Key mechanisms in the pathophysiology of sebaceous adenitis are attributed to immune defense mechanisms. The immune system mistakenly attacks sebaceous gland cells, which leads to inflammation and their gradual destruction. Lymphocytes, histiocytes, and other inflammatory cells surround the affected sebaceous glands (Hayashi et al., 2021). This causes chronic inflammation and gradual atrophy of the glands. Excessive keratin formation in the epidermis can lead to scales and crusts on the skin (Tomotake et al., 2021). Immune mechanisms are central to the development of sebaceous adenitis in dogs, which is regarded as an autoimmune condition. The immune system mistakenly targets and destroys the sebaceous glands, resulting in their dysfunction. T lymphocytes, particularly CD8+ cytotoxic T cells, play a significant role in the destruction of sebaceous glands by triggering an immune response to specific antigens within the glands or their products. They recognize antigens on the surface of sebaceous gland cells and initiate an autoimmune response (Sartori and Peruccio, 2020). Sebaceous glands are surrounded by inflammatory cells, including lymphocytes, histiocytes, and neutrophils. Immune cells release proinflammatory cytokines (Patel et al., 2021), including interleukins (IL-1, IL-6) and tumor necrosis factor-alpha (TNF-α), which amplify the inflammatory response (Jolly et al., 2023). Some cells, such as macrophages and cytotoxic T lymphocytes, directly attack sebaceous gland cells and destroy them.

The first step in diagnosing sebaceous adenitis involves taking a detailed clinical history and observing the dog's symptoms. Sebaceous adenitis typically manifests as hair loss (alopecia), scaling, dull or brittle hair, and, in some cases, secondary bacterial infections. Akitas, for instance, may develop severe generalized scaling and thickened skin. A veterinarian will typically begin by conducting a broad clinical examination to rule out other dermatological conditions such as hypothyroidism, allergies, or fungal infections (Sharkey et al., 2020).

The age of onset and breed predisposition are important clues. Sebaceous adenitis commonly affects dogs in their young adult years, and certain breeds, such as Akitas and Standard Poodles, are genetically predisposed. A thorough clinical history, including any familial history of skin disorders, can help narrow down the diagnosis (Brown et al., 2023). A detailed dermatological examination is crucial for identifying characteristic skin changes, such as patchy hair loss, excessive scaling, and follicular casts (which are accumulations of keratin around hair shafts). Follicular cysts are one of the hallmark signs of sebaceous adenitis, particularly in Standard Poodles. This physical examination helps the veterinarian determine whether sebaceous adenitis is a likely diagnosis or if further testing is necessary (Olivry et al., 2020).

Trichography, or microscopic examination of hair samples, can be used as a non-invasive method to evaluate hair follicle health. In sebaceous adenitis, the hair may show signs of brittleness, breakage, and other abnormalities due to the lack of sebum. Follicular casts around the hair shafts can also be observed under a microscope, which is a significant indicator of sebaceous adenitis. While this method is useful for initial suspicion, it is not conclusive and usually needs to be combined with more specific tests like skin biopsies (Cugmas and Olivry, 2020).

Cytology involves taking samples of the skin surface (e.g., through skin scrapings or impression smears) and examining them under a microscope to detect signs of inflammation or secondary infections (Thompson et al., 2023). In cases of sebaceous adenitis, cytology might reveal inflammatory cells like lymphocytes and macrophages, but these findings are not unique to the disease (Diaz, 2021; Sharkey et al., 2020).

A definitive diagnostic tool for sebaceous adenitis is a 'skin biopsy' followed by histopathological examination. A biopsy involves taking small samples of the affected skin, usually from multiple sites, to ensure that representative areas are analyzed (Brown et al., 2023). In the early stages of sebaceous adenitis, histopathology will typically reveal "granulomatous or pyogranulomatous inflammation" targeting the sebaceous glands, along with the destruction or absence of these glands. As the disease progresses, sebaceous glands may completely disappear, and the skin may develop hyperkeratosis, i.e., the thickened outer layer of the skin (Sharkey et al., 2020).

Histopathology is critical for differentiating sebaceous adenitis from other skin disorders that might present with similar symptoms, such as demodicosis or certain autoimmune diseases. Importantly, this test can confirm the diagnosis with a high degree of accuracy, making it the gold standard for diagnosing sebaceous adenitis (Reichler et al., 2001). In certain cases, advanced diagnostic imaging techniques, such as "dermoscopy" or "confocal microscopy" may be used to provide further insight into the structure of the skin and hair follicles. These imaging methods allow for non-invasive or minimally invasive visualization of the skin, helping to identify abnormalities in the sebaceous glands or hair follicles

(Forbes et al., 2024). While not commonly used in routine veterinary practice, these tools can be helpful in research settings or in complicated cases where a biopsy may be inconclusive or too invasive for the patient.

Immunohistochemistry is a more specialized diagnostic method that involves staining biopsy samples with antibodies to identify specific immune cells or proteins involved in the disease process (Tomotake et al., 2023; Zhelavskyi et al., 2024). This method is particularly useful in distinguishing sebaceous adenitis from autoimmune skin disorders that may present with similar histopathological findings. While not always necessary for routine diagnosis, immunohistochemistry can provide valuable additional information in complex or atypical cases (Abbas et al., 2021).

While molecular testing is not yet widely available for routine clinical diagnosis, ongoing research is exploring the genetic basis of sebaceous adenitis in dogs. In the future, genetic testing may become an important diagnostic tool, allowing for earlier detection and better management of dogs predisposed to the condition (Sharkey et al., 2020).

Although sebaceous adenitis is not primarily an allergic condition, dogs with the disease may develop secondary allergies or sensitivities due to the impaired skin barrier. In some cases, veterinarians may perform allergy testing or conduct food elimination trials to rule out concurrent allergic dermatitis. While these tests do not directly diagnose sebaceous adenitis, they can help manage the overall skin health of the affected dogs (Lee et al., 2020).

These treatments predominantly target the underlying disease mechanisms, focusing on the enhancement of the trophic processes in the skin. In addition to antibiotics, veterinarians also prescribe immunosuppression (Palmeiro, 2013; Tomotake et al., 2021; Zhelavskyi et al., 2023a). Treatment often needs to be tailored individually for each patient.

The differential diagnosis of sebaceous adenitis in dogs includes conditions, such as hypothyroidism, demodicosis, and autoimmune skin diseases like pemphigus foliaceus (Reichler et al., 2001). Hypothyroidism can lead to similar hair loss and skin changes but is usually accompanied by systemic signs (Diaz, 2021). Demodicosis, caused by mites, can mimic sebaceous adenitis with hair loss and scaling but typically involves other diagnostic findings like mite presence. Autoimmune diseases, such as pemphigus, often present with pustules and erosions, which are less common in sebaceous adenitis (Abbas et al., 2021).

Treatment of sebaceous adenitis in dogs is often complex and requires a long-term and comprehensive approach. There are many factors to consider when treating this disease, including sebaceous adenitis. Sebaceous adenitis is often chronic, requiring long-term treatment and constant monitoring. The disease may periodically worsen, which requires adjustment of therapy. Certain medications, such as cyclosporine or corticosteroids, may not always be effective for all dogs. In addition, long-term use of these drugs can lead to side effects. Even with successful treatment, recurrences of sebaceous adenitis are frequent. Consequently, therapy must be monitored and corrected constantly (Olivry et al., 2020). The present study aimed to report a case of sebaceous adenitis in an Akita dog with a description of the methods employed for diagnosis and treatment.

CASE REPORT

Clinical signs

A 5-year-old male Akita weighing 35.8 kg was presented for pruritus and alopecia on the inner surface of the ears, face, and head and was sent to a veterinary clinic (21 April 2024) located in Kamyanets-Podilsky, Ukraine. During the clinical examination, the body temperature was recorded at 38.4 °C, the heart rate at 78 bpm, and the respiratory rate at 27 breaths per minute. The appetite did not change. Over time, changes appeared in other parts of the skin. During the clinical examination, dermatological examinations were performed including trichogram, acetate tape test, skin scraping, mycological examination (luminescent method, inoculation on indicator nutrient media), and parasitological dermatology (Diaz, 2021). The results did not reveal any change in the characteristics of infections. Verification of the diagnosis (sebaceous adenitis) was based on histopathological examination (Diaz, 2021).

The first signs were the erythema of the skin on the ears (Figure 1) and on the snout (Figure 2). The hair on the head was becoming curly (Figure 3). The alopecia and clumping of hair were also found on the dorsal part of the tail (Figure 4), the distal part of the front and hind legs, the groin, and the abdomen. At first, redness and itching appeared in the areas. The skin lost elasticity and became dry. The hair was stuck together with gray crusts. Inflammatory infiltration was accompanied by cellular migration of lymphocytes, histiocytes, and neutrophils (Figure 5).

Changes in the size and structure of hair follicles with signs of hyperkeratosis, cysts, and atrophy of sebaceous glands were diagnosed. During the treatment, the gradual disappearance of inflammation signs was well noted. Starting from the third day, itching and hyperemia disappeared. From day 7, desquamation and hair loss decreased. On day 12, signs of new hair growth appeared. The areas were completely restored on day 28 of the treatment.



Figure 1. Sebaceous adenitis in the left ear of a 5-year-old, Akita male dog. a: visualization of alopecia.

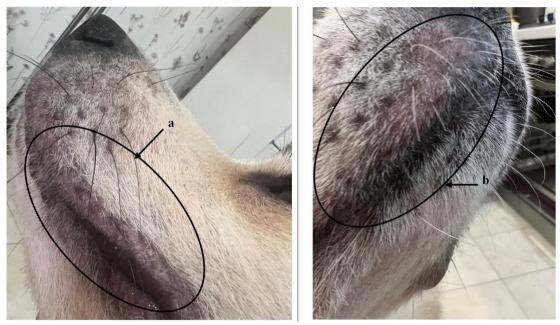


Figure 2. Clinical characteristics of sebaceous adenitis of a 5-year-old, Akita male dog (on the snout). **a:** Visualization of hair loss around the lips, **b**: Redness of the skin and gluing of hair.



Figure 3. Sebaceous adenitis of a 5-year-old, Akita male dog. Clinical signs: Curling hair is visible on the head.

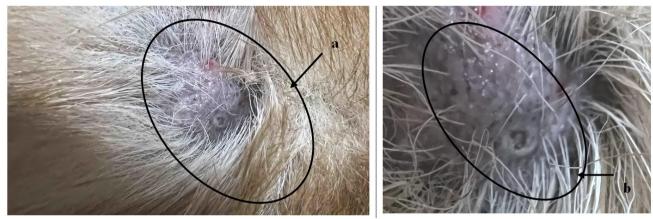


Figure 4. Sebaceous adenitis in a 5-year-old, Akita male dog (on the dorsal part of the tail). **a:** Alopecia and glued hair are visible, **b:** Accumulation of sticky exudate and damage to the epidermis (magnification x4).

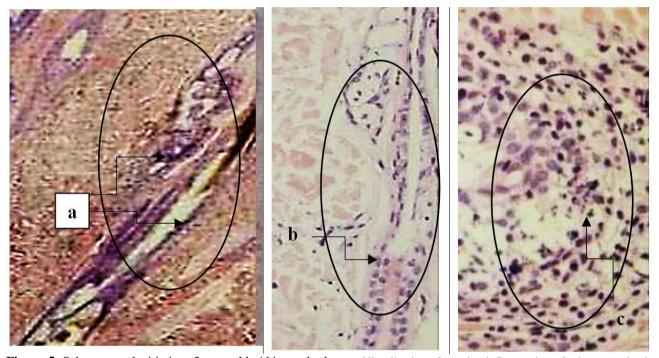


Figure 5. Sebaceous adenitis in a 5-year-old, Akita male dog. **a:** Visualization of reactive inflammation of sebaceous glands (magnification x800), **b**: Cellular infiltration phagocytes and destruction of sebaceous glands, **c**: Reactive phagocytes and lymphocytes (magnification x3000), stained with H and E.

Sample selection and drug preparation

A laboratory test was performed in the clinic and a biopsy location was determined. A marker MEANT (Mini Traditional Gentian Violet Ink Surgical Marker, Viscot Medical LLC, USA) was used to mark the place of biopsy selection. For local anesthesia, 2% lidocaine hydrochloride (lidocaine, B. Braun Medical, UK) was used. The area was cleaned with 70° ethyl alcohol (Emsure®, Sigma-Aldrich, Germany). A skin sample (epidermis, dermis, hypodermis) was taken with a biopsy needle (Diaz, 2021). The biopsies were placed in a container with a 10% aqueous solution of formaldehyde (Sigma-Aldrich, Germany), marked, and sent to the laboratory (Kamyanets-Podilsky, Ukraine). A sterile dressing was applied to the biopsy. The skin biopsy was fixed in 10% formaldehyde (Sigma-Aldrich, Germany) for at least 24-48 hours. Next, the preparations were prepared according to the protocol (Diaz, 2021) for staining with hematoxylin and eosin (H and E, Sigma-Aldrich, Germany).

Treatment

The treatment involved the use of shampoo (8 in 1 Natural Oatmeal, USA), which was applied to the wool and gently treated for 3-5 minutes on the affected skin areas. After that, the shampoo was washed off with clean water and thoroughly wiped with a towel. After treatment with shampoo, Conditioner (8 in 1 Hypoallergenic Conditioner, USA) was applied to lesions for 2 minutes. Afterward, it was thoroughly washed with clean water and dried with a towel. This treatment course lasted for 30 days. Omega-3 (Omega-3 for Dogs/Cat, Now Foods, USA) was administered orally in a

dose of 1 capsule (1000 mg, containing 180 mg of eicosatrienic acid, 120 mg of docosapentaenoic acid), twice a day with an interval of 12 hours, in the course of 30 days (Diaz, 2021). Isotretinoin (stereoisomer of trans-retinoic acid (tretinoin, Roaccutane[®], F. Hoffmann-La Roche Ltd., Switzerland) was also administered orally. The drug was prescribed in a dose of 1 capsule (20 mg) twice a day. The course of treatment was 30 days (Pye, 2021). Cyclosporine (Atopica®, Novartis, UK) was administered orally at 5.0 mg/kg once daily (over 30 days), on an empty stomach, 2 hours after a meal (Palmeiro, 2013).

DISCUSSION

Sebaceous adenitis is a rare disease among dermatological diseases of animals. The disease was first described in a poodle dog in 1987. Subsequently, sebaceous adenitis was diagnosed in cats, rabbits, horses, and humans (Reichler et al., 2001; Olivry et al., 2020; Forbes et al., 2024). Sebaceous gland disease is infrequently observed in cats, typically manifesting as chronic and progressive dermatologic issues. These include non-itchy scaling, crusting, hair loss, and localized skin depigmentation (Sartori and Peruccio, 2020; Thompson et al., 2021; Gonzalez et al., 2023). In dogs, the breeds at higher risk include Poodles, Akitas, Hungarian Sheepdogs, Samoyeds, and Springer Spaniels (Brown et al., 2023). Parasitic infections, dermatomycoses, allergies, autoimmune processes, and metabolic disorders are taken into account during differentiation (Denti et al., 2022; Jolly et al., 2023). In the clinical case, the main lesions were localized on the inner and outer surfaces of the auricle of the left and right ears. The lesions were localized on the dorsal part of the tail. The patient had itching, alopecia was formed on the affected areas, and hair was sticking together, which formed gray crusts. Similar signs have been described in previous studies (Reichler et al., 2001; Olivry et al., 2020).

In the Akita, the disease may have a genetic determination (Reichler et al., 2001; Olivry et al., 2020; Pye, 2021). Additionally, climatic factors should be considered as well. In this case, the patient's main sebadenitis lesions were on the ears, the dorsal part of the tail, and the distal parts of the forelimbs. Clinical signs were consistent with those observed in other dog breeds, including hypotrichosis, alopecia, follicular casts, comedones, scaling, and bilateral otitis externa. Pruritus ranged from moderate to severe, likely linked to skin and coat dryness (xerosis).

The diagnosis was based on dermatological signs and confirmed by biopsy and histopathology. For treatment, the drugs were used with a pathogenetic and etiotropic direction. The treatment consisted of normalizing the animal's trophic processes and modulating its immune response. Using specialized shampoos offers several key benefits in managing sebaceous adenitis in dogs. Natural ingredients, including oatmeal and aloe vera, help soothe and moisturize irritated skin, reducing inflammation and dryness. Massaging the shampoo into the dog's coat for 3-5 minutes not only ensures even application but also promotes circulation, aiding in skin healing. After thoroughly rinsing the shampoo, applying a hypoallergenic conditioner further enhances the treatment by moisturizing both skin and fur, reducing irritation, and improving overall coat texture. Recent formulations of conditioners may also include essential fatty acids, which help restore the lipid barrier of the skin, providing long-term benefits in managing dryness and irritation associated with sebaceous adenitis (Reichler et al., 2001).

Omega-3 fatty acids in a dog's diet can be a valuable additive to the overall treatment plan for sebaceous adenitis. Fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), help reduce inflammation by suppressing the production of pro-inflammatory cytokines and other inflammatory mediators.

Additionally, omega-3 fatty acids help retain moisture in the skin, reducing dryness and flakiness that often accompany sebaceous adenitis. It has been shown that omega-3 fatty acids affect immunomodulatory, helping to balance the immune response and reduce autoimmune reactions, which may cause or contribute to the development of sebaceous adenitis. Isotretinoin stimulates skin cell renewal, which aids in the regeneration of damaged skin and the restoration of its normal structure. As a result of the medication, keratinization processes are normalized, reducing excess keratin production that can block sebaceous glands and result in crusts and scales on the skin (Cugmas and Olivry, 2020). Isotretinoin dramatically decreases sebaceous gland activity, leading to a reduction in sebum production. This helps to reduce the inflammatory processes associated with blocked sebaceous glands. The applied therapy yielded positive results (Outerbridge and Jordan, 2021; Patel et al., 2021; Pye, 2021).

The effectiveness of cyclosporine has been described in several publications (Palmeiro, 2013; Outerbridge and Jordan, 2021; Jolly et al., 2023). Cyclosporine is an immunosuppressant widely used for the treatment of sebaceous adenitis in dogs. Its action involves suppressing the activity of the immune system, which helps to reduce inflammation and alleviate the symptoms of the disease. Cyclosporine suppresses the activation and proliferation of T lymphocytes, which are crucial cells in the immune response. This reduces the production of pro-inflammatory cytokines, including interleukins and interferons, reducing inflammation in the sebaceous glands (Olivry et al., 2020; Denti et al., 2022; Tomotake et al., 2021). Possible complications have been reported in recent studies (Pye, 2021; Jolly et al., 2023).

CONCLUSION

Sebaceous adenitis of Akita has a spontaneous nature, often without determining the cause. Treatment is aimed at using the means and methods necessary to restore the functioning of the skin and the sebaceous glands. Further research may focus on studying the immune mechanisms involved in the development of this pathology, which will, in turn, open new avenues for improving diagnosis and treatment. The immune system plays a key role in many diseases, including those associated with autoimmune processes, inflammatory responses, or dysregulation of immune function. Understanding how these mechanisms interact with other systems in the body will allow for the development of more precise diagnostic markers and targeted therapies, leading to more effective and personalized treatments for patients.

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Availability of data and materials

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

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Authors' contributions

Mykola Zhelavskyi developed the initial concept, verified the medical history, collected data, and conducted the experiment. Mykola Maryniuk was responsible for designing the study and conducting clinical research. Maryna Drobot carried out laboratory research. Each author played a significant role in critically reviewing and giving final approval to the manuscript, ensuring the integrity and quality of the work. All the authors thoroughly reviewed the content and contributed to refining the final version of the manuscripts. After careful evaluation, they unanimously approved the manuscripts for submission, confirming that the content accurately reflects their research findings, perspectives, and collective input. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Ethical considerations

The authors addressed all relevant ethical considerations, including but not limited to, ensuring the originality of the work, obtaining publication consent, preventing research misconduct, avoiding data fabrication or falsification, and preventing duplicate publication or redundancy.

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An Overview of Cyanide Poisoning in Humans and Animals

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ABSTRACT

Cyanide poisoning poses a significant global health risk, affecting both humans and animals due to its rapid and often fatal effects. Cyanide compounds, such as hydrogen cyanide, potassium cyanide, and cyanogenic glycosides found in certain plants, interfere with cellular respiration by blocking cytochrome oxidase, causing cytotoxic hypoxia and organ failure. Human exposure to cyanide is primarily linked to industrial activities such as mining and electroplating, as well as smoke inhalation during fires. Symptoms of poisoning include respiratory distress, dizziness, and, in severe cases, cardiac arrest and death if left untreated. In animals, particularly livestock, poisoning often results from consuming cyanogenic plants such as sorghum and specific grasses. Different species have varying susceptibility to cyanide, with cattle showing signs such as respiratory distress, seizures, and death after ingesting these plants. Detection methods for cyanide, including spectrophotometry, gas chromatography, and ion-selective electrodes, which analyze cyanide levels in blood, urine, or tissues. Treatment generally involves the administration of antidotes, such as hydroxocobalamin, sodium thiosulfate, and sodium nitrite, which neutralize cyanide in the body. The current study aimed to highlight the importance of stringent regulatory measures on cyanide usage in industrial and agricultural contexts to prevent accidental poisoning. Environmental monitoring and processing of safe food are vital to reduce the incidence of cyanide poisoning in both humans and animals.

Keywords: Animal, Antidote, Cyanide, Human, Law, Poisoning

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INTRODUCTION

Cyanide poisoning is a significant global health concern that affects both humans and animals. Cyanide, a highly toxic chemical compound, exists in various forms, including hydrogen cyanide, cyanide salts, and cyanogenic glycosides found in certain plants (Parker-Cote et al., 2018). It is widely recognized for its rapid and potentially lethal effects on living organisms by inhibiting cellular respiration, thus halting the production of adenosine triphosphate (ATP), which is essential for cellular energy (Dorooshi et al., 2020). This disruption hinders cells from utilizing oxygen, resulting in rapid organ failure (Dorooshi et al., 2020). The accumulation of cyanide toxins in the body can cause various organ dysfunctions, including dizziness, seizures, loss of consciousness, and arrhythmia (Woolf, 2022). Without timely medical intervention, this condition may lead to death (Abeyasinghe et al., 2011).

In humans, cyanide poisoning often results from accidental or intentional exposure through industrial processes such as mining, electroplating, and chemical manufacturing (Jadav et al., 2022). Additionally, the combustion of certain materials, such as plastics and tobacco, releases hydrogen cyanide gas, which can be inhaled, leading to acute poisoning (Jadav et al., 2022). In some regions, the consumption of foods containing cyanogenic compounds, such as cassava, improperly processed bitter almonds, and apricot seeds, poses a risk of chronic cyanide toxicity, which may lead to neurological disorders (Akvildiz et al., 2010).

Animals are vulnerable to cyanide poisoning, with livestock, wildlife, and pets at risk (Brasel et al., 2006; Oruc et al., 2006; Kennedy et al., 2021). Accidental ingestion of cyanogenic plants, such as sorghum, cherries, and certain grasses, is a common cause of poisoning in animals, particularly grazing livestock, including cattle (Giantin et al., 2024). Wildlife, especially birds and aquatic species may be exposed to cyanide through industrial effluents, such as those released during gold mining, where cyanide is used to extract gold from ore (Eisler and Wiemeyer, 2004; Brasel et al.,

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2006). In pets, there have been reports of dogs ingesting apricots in households, these fruits containing cyanide and dogs developing poisoning (Houlton et al., 2024). This environmental contamination can lead to mass mortality events, significantly impacting biodiversity and ecosystems (Eisler and Wiemeyer, 2004).

There are similar clinical signs of cyanide poisoning in both humans and animals which include difficulty breathing, confusion, dizziness, and cardiac arrest (Sabourin et al., 2016; Woolf, 2022). In severe cases, death may occur within minutes to hours if left untreated so, rapid diagnosis and treatment are critical (Giantin et al., 2024). Preventing cyanide poisoning requires stringent regulation of industrial activities, proper food processing, and comprehensive environmental monitoring (Saingam, 2018). The present study aimed to highlight key aspects of cyanide poisoning to enhance understanding of its impact and the necessity for effective prevention and treatment strategies.

Characteristics, properties, and sources of cyanide

Cyanide is a highly toxic substance composed of carbon and nitrogen atoms, commonly found in compounds, such as hydrogen cyanide (HCN), potassium cyanide (KCN), and sodium cyanide (NaCN) (Dorooshi et al., 2020). Cyanide compounds can exist in various physical states, depending on their chemical form and environmental conditions. HCN is a colorless, highly poisonous gas with a faint, bitter almond-like odor, while KCN and NaCN are typically found in solid form, appearing as white crystalline powders or pellets (Parker-Cote et al., 2018). Cyanides are present in a range of sources, including industrial processes, including mining, electroplating, the production of synthetic fibers, pesticides, and fumigation (Jaszczak et al., 2017). Natural sources include plants that produce cyanogenic compounds known as cyanogenic glycosides, such as cassava, stone fruits, and bamboo shoots, which release hydrogen cyanide (HCN) when plant tissue is damaged (Jaszczak et al., 2017). In 2024, reports from certain regions indicated a preference for cultivating Sorghum species due to their favorable growth, particularly in the Northwest of Italy. However, an outbreak of cyanide poisoning was observed in cattle that consumed Sorghum in these areas (Giantin et al., 2024). Eisler and Wiemeyer (2004) discussed the impact of cyanide poisoning on fish, reptiles, and amphibians, resulting from the contamination of rivers due to gold mining activities.

Mechanisms and dosages of cyanide poisoning

Cyanide poisoning exerts its lethal effects primarily by inhibiting cytochrome oxidase in the mitochondrial electron transport chain, thereby obstructing cellular respiration (Dorooshi et al., 2020). The mechanism of this inhibition involves cyanide blocking the activity of an enzyme called cytochrome c oxidase (complex IV), a key enzyme in mitochondrial respiration (Dorooshi et al., 2020). This enzyme is crucial for the production of energy in the form of ATP (adenosine triphosphate) through oxidative phosphorylation (Dorooshi et al., 2020). The inhibition of cytochrome c oxidase prevents cells from utilizing oxygen, leading to cytotoxic hypoxia despite adequate oxygen levels in the blood (Woolf, 2022). The resulting energy crisis forces cells to switch to anaerobic metabolism, which leads to lactic acid accumulation and metabolic acidosis (Yadukul et al., 2014). The depletion of cellular energy subsequently causes widespread cellular damage, organ failure, and potentially death (Dorooshi et al., 2020). The toxicity level of cyanide is influenced by its specific form and route of exposure (Jadav et al., 2022; Woolf, 2022). In humans, previous research has established that the lethal dose fifty (LD₅₀) for KCN ingestion is 140 mg, while the lethal dose hundred (LD₁₀₀) ranges from 200 to 300 mg (Wolnik et al., 1984; Woolf, 2022). In animals, cyanide poisoning in swine has been documented, with the lethal dose of HCN by inhalation reported to be 2.21 mg/kg or 5893 mg/minute/m³ (Staugler et al., 2018). The specific amounts of cyanide toxins that cause toxicity in other species are shown in Table 1.

Table 1. Dosage of cyanide poisoning in different species

Species	Types of cyanide	Dosage	Clinical signs/ Disorder	References
Human	KCN	140-300 mg/person (oral)	Cardiac arrest, seizures, cyanosis, and cherry- red skin coloration	Woolf (2022)
Swine	HCN	2.21 mg/kg or 5893 mg/minute/m³ (inhalation)	Asphyxia, cardiorespiratory arrest, and death	Staugler et al. (2018)
Mice	KCN	9.9-11.8 mg/kg (oral)	Difficulty breathing, convulsions, tremors, seizures, and death	Sabourin et al. (2016)
Fish	NaCN	5-20 μg/l (bathe)	Impact on swimming disrupted respiration, and death	Ramzy (2014)
Coyote	NaCN	2.1-8.3 mg/kg (oral)	Respiratory failure and death	Sterner (1979)
Dog	HCN	2-2.5 mg/kg (oral)	Vomiting, lethargy, ataxia, and unresponsiveness	Nagy et al. (2023); Houlton et al. (2024)
Cat	HCN	2-2.5 mg/kg (oral)	Hypersalivation, vomiting, and diarrhea	Caloni et al. (2013); Nagy et al. (2023)
Cattle	HCN	2 mg/kg (oral)	Hyperventilation, diarrhea, convulsions, shock, and death	Gensa (2019); Kennedy et al. (2021)
Avian	NaCN	4-21 mg/kg (oral)	Lethargy and death	Wiemeyer et al. (1986); Brasel et al. (2006)

KCN: Potassium cyanide, HCN: Hydrogen cyanide, NaCN: Sodium cyanide

Sample collection and methods for detecting cyanide poisoning

Due to the non-specific clinical manifestations observed in animals or humans exposed to cyanide, laboratory testing is necessary to confirm the presence of the toxin (Oruc et al., 2006). Cyanide toxicity can be detected through various analytical techniques, depending on the sample type and method employed. Samples should be collected carefully to avoid contamination with hazardous chemicals. Blood samples are most frequently utilized and should be collected in tubes containing anticoagulants such as heparin or ethylenediaminetetraacetic acid (Kennedy et al., 2021). In some cases, urine samples may also be used for analysis of cyanide (McGorum and Anderson, 2002). Internal organs, including stomach contents, liver, and kidneys, should be tested to confirm poisoning (Jadav et al., 2022). Clothing suspected of toxin contamination should also be examined (Jaday et al., 2022). Methods for cyanide detection include the picrate paper test, which is a qualitative method that utilizes picric acid to observe a color change (Oruc et al., 2006). The Prussian Blue Test is another qualitative analysis method for detecting cyanide concentration in a solution, involving the reaction of ferric compounds to form a colored complex (Oruc et al., 2006). Other techniques that have been developed and used include spectrophotometry, which assesses cyanide concentration by measuring the color change in a solution reacting with cyanide (Nnoli et al., 2013); gas chromatography (GC), which separates and quantifies cyanide using a GC instrument (McGorum and Anderson, 2002); high-performance liquid chromatography (HPLC), which employs liquidphase separation to determine cyanide levels (Tobarran et al., 2022); and ion-selective electrode (ISE) techniques, which detect cyanide ions in samples via a specialized electrode (Sankaran et al., 2020). Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is a highly accurate quantitative method for detecting cyanide, capable of identifying trace amounts and simultaneously analyzing multiple compounds (Giantin et al., 2024). Liquid Chromatography-Fluorimetry (LC-Fluorimetry) combines liquid chromatography with fluorimetric detection to separate and measure cyanide and is sensitive enough to detect even trace amounts of cyanide (Kennedy et al., 2021).

Cases of accidental exposure to or misuse of cyanide in different species

Cyanide poisoning has been documented in both humans and animals across numerous countries, with incidents in animals often resulting from accidental exposure. For example, a study by Giantin et al. (2024) linked cattle fatalities to the consumption of sorghum. The affected cattle exhibited clinical signs such as severe respiratory distress, recumbency, and seizures. Necropsy findings revealed typical indicators of cyanide poisoning, including bright red blood, an almond-like odor, and emphysema in the lungs. Further analysis of sorghum samples confirmed cyanide levels exceeding the safety threshold (Giantin et al., 2024). Similarly, in 2002, a case of cyanogenic poisoning in horses was attributed to the ingestion of white clover, with the animals displaying signs of ataxia and neuropathy (McGorum and Anderson, 2002). Another case reported in Turkey in 2006 involved canine deaths due to cyanide poisoning (Oruc et al., 2006). The affected dogs exhibited symptoms such as dyspnea, excitability, and convulsions before death. Postmortem examinations confirmed the diagnosis, with pathological findings showing severe inflammation and hemorrhage in the blood vessels, lungs, and heart (Oruc et al., 2006). Additionally, there have been reports of hawk fatalities, with carcasses found near a water source adjacent to a gold mine in Nevada (Franson, 2017). Postmortem examinations revealed cyanide accumulation in the blood, liver, and brain, which is suspected to have originated from contamination linked to gold mining activities (Franson, 2017).

Cyanide intoxication in humans is frequently associated with accidental exposure, homicide, and suicide. In 2022, an accidental death from cyanide inhalation in an industrial setting was reported in India (Jadav et al., 2022). The incident occurred in an electroplating chamber, and autopsy findings along with toxicological analyses confirmed cyanide poisoning as the cause of death (Jadav et al., 2022). Another notable case occurred in 2012, where an individual who consumed beer at a bar was later found died due to cyanide poisoning (Nnoli et al., 2013). Initially, the death was attributed to myocardial infarction, but further investigation revealed cyanide as the underlying factor. Nnoli et al. (2013) conducted a comprehensive examination, detecting cyanide in the victim's stomach contents, blood, bile fluid, and a mouth swab, thereby confirming cyanide poisoning as the cause of death. This case highlights the risk of cyanide contamination in food or beverages, whether deliberate or accidental, which can lead to fatal consequences (Nnoli et al., 2013). Additionally, in 2011, a suspected homicide involving cyanide injection was reported in Sri Lanka. The victim exhibited signs of rapid breathing before death, and subsequent analysis of blood samples and the contents of a syringe confirmed the presence of cyanide (Abeyasinghe et al., 2011). Further details are shown in Table 2.

Guidelines for the treatment of cyanide toxicity in humans and animals

In cases of cyanide exposure in humans or animals, the initial step involves determining the likely route of toxin entry into the body. Decontamination should be carried out by thoroughly washing the body and removing any clothing that may have been contaminated with cyanide (Jadav et al., 2022). Monitoring and stabilizing vital signs should be prioritized, with immediate management of life-threatening conditions (Reade et al., 2012). Supportive care is crucial in

managing clinical signs such as respiratory distress, seizures, or unconsciousness, and may include hyperbaric oxygen therapy along with comprehensive intensive care measures such as ventilation, circulatory support, and renal replacement therapy (Reade et al., 2012). Hydroxocobalamin has been reported as an effective antidote, binding cyanide ions to form cyanocobalamin, a less toxic compound that is subsequently excreted in the urine (Kiernan et al., 2020). Additionally, sodium nitrite is used to induce the formation of methemoglobin by oxidizing Fe²⁺ in hemoglobin to Fe³⁺ (Johnson-Davis, 2020). Methemoglobin cannot efficiently bind oxygen, but it has a high affinity for cyanide. It acts as a cyanide binder, preventing cyanide from inhibiting the activity of the cytochrome oxidase enzyme, which facilitates the removal of cyanide from the mitochondria (Johnson-Davis, 2020). However, caution is advised, as excessive use can lead to methemoglobinemia, impairing oxygen transport (Johnson-Davis, 2020). Sodium thiosulfate is also used to convert cyanide into thiocyanate through the action of rhodanese, an enzyme composed of various amino acids and antioxidants, in the liver (Avais et al., 2018). Thiocyanate is a less toxic compound that is excreted by the kidneys (Avais et al., 2018). Sodium thiosulfate is often administered in combination with hydroxocobalamin and sodium nitrite)Tobarran et al., 2022, Table 3).

Table 2. The reported cases of cyanide poisoning in different species

Source of cyanide	Method for detection	Samples	Species	References
Sorghum	LC-MS/MS	Sorghum	Cattle	Giantin et al. (2024)
Laurel	LC-Fluorimetry	Blood	Cattle	Kennedy et al. (2021)
Apricot (Prunus armeniaca)	The owner is the informant	Gastric lavage	Dog	Houlton et al. (2024)
No reports were identified in the article	Picrate paper and Prussian blue	Contents in the stomach and intestines	Dogs	Oruc et al. (2006)
White clover (Trifolium repens)	Gas chromatography	Blood and urine	Horses	McGorum and Anderson (2002)
The water source adjacent to the gold mine	Spectrophotometry	Heart blood, liver, and brain	Bird	Franson (2017)
Beverages or food	Spectrophotometry	Stomach contents, blood, bile fluid, and mouth swab	Human	Nnoli et al. (2013)
Syringe	Benzidine-copper acetate	Blood and content in the syringe	Human	Abeyasinghe et al. (2011)
Electroplating chambers	Toxicology laboratory (Method not indicated)	Blood, internal organs, and clothes	Human	Jadav et al. (2022)

Table 3. The effective agents in the treatment of cyanide toxicity in humans and animals

Agent	Species	Dosage	References
Dobetin® (cyanocobalamin, thiamine hydrochloride)	Cattle	60 ml (The solution contains cyanocobalamin at 1 mg/ml and thiamine hydrochloride at 100 mg/ml)	Giantin et al. (2024)
Sodium thiosulfate	Cattle	250-500 mg/kg	Gensa (2019)
Hydroxocobalamin	Dog	150 mg/kg	Houlton et al. (2024)
Hydroxocobalamin and sodium thiosulfate	Human	5 g and 12.5 g	Tobarran et al. (2022), Meillier and Heller (2015)
Sodium nitrite and sodium thiosulfate	Rabbit	20 mg/kg and 600 mg/kg	Avais et al. (2018)

Legislation related to cyanide in animals and humans

Due to the presence of cyanide in various plant species, several countries have established regulations to limit permissible levels of cyanide in animal feed. Compliance with these regulations varies by region. For instance, in Asian countries, such as Thailand, the Animal Feed Act (2015) stipulates penalties for exceeding allowable cyanide limits, including imprisonment for up to one year, a fine of up to 2,700 USD, or both (Office of the Council of State, 2015). Japan enacted the Pet Food Safety Law in 2008, which imposes penalties for contamination with harmful agents (Sugiura et al., 2009). Violations can result in imprisonment, fines, or other penalties, depending on the court's discretion (Sugiura et al., 2009). In European countries such as the United Kingdom, regulations governing cyanide levels in food include the Animal Feed Regulations 2015 and the Feed Hygiene Regulation No. 183/2005 (Cheli et al., 2013). Violations of

these regulations may result in substantial fines amounting to several thousand pounds or imprisonment, depending on the court's discretion (Cheli et al., 2013).

In industries where cyanide chemicals are used, various countries have established regulations to control the storage and use of these substances to prevent misuse. In Thailand, the Chemical Substance Control Act of 2012 stipulates penalties for violations, including imprisonment for up to 5 years or fines exceeding 13,500 USD, depending on the nature of the infraction (Saingam, 2018). In South Korea, the Chemical Substance Control Act of 1987 mandates penalties for breaches, which may include imprisonment for up to 5 years or more, and fines potentially exceeding several million dollars (Yoon et al., 2014). In the United States, the Toxic Substances Control Act (TSCA) of 1976 regulates the production and use of hazardous chemicals. Violations of the TSCA may result in imprisonment for up to 5 years or more, and fines reaching up to \$50,000 per day, depending on the court's discretion (Ozymy, and Ozymy, 2023).

CONCLUSION

Cyanide poisoning remains a significant concern in both medical and veterinary fields due to its rapid onset and high lethality. The widespread presence of cyanide in industrial processes, and natural environments, and its potential use in toxicological crimes underscores the need for stringent safety measures, timely detection, and effective treatment protocols. Understanding the mechanisms, sources, and treatment options for cyanide poisoning is essential for reducing its impact on both human and animal populations. Regulatory enforcement and public awareness are crucial for mitigating the risks associated with cyanide exposure. Future studies should focus on developing methods for the rapid and accurate detection of cyanide in biological and environmental samples, as well as enhancing novel antidotes to minimize side effects.

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Author's contributions

Narong Kulnides and Athip Lorsirigool were responsible for drafting the writing guidelines and preparing the initial manuscript. Narong Kulnides, Athip Lorsirigool, and Rachada Fongtanakit contributed to the conceptualization and design of ideas for each subtopic. Kanokporn Saenkaew, Narong Kulnides, and Athip Lorsirigool gathered data and contributed to the sections on cyanide characteristics and relevant legislation. Nontachai Santichat, Kanokwan Tawinwang, and Pornchai Thumrin were involved in data collection and writing the sections on the mechanisms of poisoning and dosage information. Pradipat Sonti and Chanon Phiphittaphan handled data collection and writing for the section on sample collection and detection methods. Arunroj Kullaya and Theeradon Sophaporn contributed by collecting data and writing the case reports and treatment sections. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors have no conflicts of interest to declare.

Ethical considerations

All authors have checked for plagiarism, fabrication and/or falsification, dual publication and/or submission, and redundancy.

Availability of data and materials

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

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Contribution of Cytochrome P450s to Development of Insecticide Resistance in *Musca domestica L.*: A Review

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ABSTRACT

Musca domestica L. (Diptera: Muscidae) is recognized as one of the most prevalent fly species globally, playing a significant role in the transmission of infections and pathogens that are important in both veterinary and medical contexts. This includes the dissemination of eggs from intestinal helminths, as well as ectoparasites, endoparasites, and protozoan cysts. Several approaches to combating insect pests include biological, physical, chemical, and agrotechnical methods. Chemical methods remain the main strategy for controlling the population of insect pests; however, excessive use, increased dosages, and frequency of treatments have led to the development of resistance. To date, numerous documented cases of resistance to insecticides have been registered in natural populations. A significant mechanism for resistance development is the detoxification of xenobiotics by enzymes of the cytochrome system. This study aimed to summarize the current knowledge on the role of P450 monooxygenase in developing insecticide resistance in houseflies. This overview focuses on the diversity of Cytochrome P450 monooxygenases in Musca domestica that contribute to resistance against the most popular classes of insecticides and their location in the genome. Throughout this work, the main P450 candidate genes associated with insecticide resistance were identified and described. The authors also summarized and systematized recent research results in this area.

Keywords: Gene expression, Housefly, Insecticide, Insecticidal resistance, Monooxygenase, Cytochromes P450

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INTRODUCTION

Currently, Musca domestica is one of the most common fly species around the world. Living in perpetual close contact with humans and animals. It is a vector for more than 100 species of bacterial, viral, and fungal pathogens. It also carries worm eggs, cysts, and trophozoites of protozoa (Adenusi et al., 2013; Al-Aredhi, 2015; Nayduch and Burrus, 2017; Issa, 2019; Geden et al., 2021). This species is most commonly found in restaurants, hospitals, landfills, and agricultural facilities (El-Sherbini and El-Sherbini, 2011). Chemical insecticides remain the first line of defense in insect pest control (Freeman et al., 2019). However, extensive use of different insecticides has led to the development of resistance. To date, Musca domestica has identified more than 460 cases of resistance to various classes of insecticides. There are four main mechanisms for the development of insecticidal resistance including changes in the permeability of the integument, target site resistance, metabolic resistance, and behavioral resistance. Changes in the permeability of the integument occur due to thickening or changes in the composition of the cuticle of insects. Consequently, these changes prevent insecticide molecules from entering the insect's body (Balabanidou et al., 2018). Target site resistance primarily arises from modifications (mutations or losses) in the protein-coding region of the target site of the insecticide, which leads to incompatibility of the target site for activation. Mutations in the same target gene often cause resistance to the same insecticide in different insect species. The four main types of resistance to various drugs include modified acetylcholine esterase-based (MACE), knockdown resistance (KDR), nicotinic acetylcholine receptor-based (nAChRs), and duplication of resistance to dieldrin (RDL, Khan et al., 2020). Metabolic resistance develops due to enhanced detoxification, which usually stems from overexpression of CYP450 monooxygenases, nonspecific esterases, glutathione S-transferases, and uridine diphosphate (UDP)-glycosyltransferases (Li et al., 2012; Lopatina and Eremina, 2018; Khan et al., 2020; Pu and Chung, 2024). Behavioral resistance refers to an aversion to insecticides caused by simple repulsion and avoidance, or by an acquired factor (Zalucki and Furlong, 2017). Therefore, the current study aimed to summarize the current knowledge on the role of P450 monooxygenase in developing insecticide resistance in the housefly.

Data collection

The data was obtained by searching publications in databases such as Google Scholar, PubMed, Web of Science, and Scopus by keywords including housefly, insecticides, monooxygenase, resistance, cytochromes *P450*, and gene expression. The criteria for selecting publications included, the study subject being *Musca Domestica* L.; Changes in the expression of *P450* monooxygenase genes; and the presence of insecticidal stress. Publications before 2000 were not included in the study. Moreover, experimental articles were mainly considered. Thus, the search for the keywords "*Musca domestica*" and "Housefly" yielded a total of 5131 publications. When adding other keywords, the final number of publications was reduced to 272. These articles were analyzed, and only those publications in which the level of *P450* gene expression changed under the influence of insecticides were selected, resulting in no more than 52 suitable publications.

General characteristics of cytochromes p450

The metabolism of xenobiotics, including pesticides, depends heavily on cytochromes *P450* (Scott, 2001). Cytochromes are typically localized in the smooth and rough endoplasmic reticulum or the mitochondria (Cribb et al., 2005; Rewitz et al., 2006). Most *P450* enzymes associated with xenobiotic metabolism are membrane-bound and are located in the endoplasmic reticulum. They possess an N-terminal zone which includes a transmembrane helix responsible for lodging (Feyereisen, 2012). Cytochrome *P450*-dependent monooxygenases play a significant role in the metabolic system, regulating the titers of endogenous compounds, such as hormones, fatty acids, and steroids, as well as in the catabolism and anabolism of xenobiotics, such as pharmaceutical products, pesticides, and plant toxins (Parolini, 2020). These enzymes are found in the organs and tissues of a diverse range of organisms including mammals, fish, plants, arthropods, fungi, and bacteria (Feyereisen, 2005). Monooxygenases in insects were first detected in 1965 (Brooks, 2008). There are 146 *CYP* genes in the genome of the housefly (Scott et al., 2014), and a significant amount of them have not yet been functionally defined.

Insecticidal resistance to pyrethroids

Pyrethroids are classified as neurotoxins; they disrupt the metabolism of sodium and calcium ions, and depolarize the membrane, which leaves the insect paralyzed (Chrustek et al., 2018). Pyrethroids are the most commonly used insecticides against houseflies, and the development of resistance to pyrethroids presents a significant challenge today (Scott, 2016; Khan et al., 2017; Wang et al., 2019). For example, the metabolic resistance of the housefly to insecticides has been most thoroughly described for the Learn Pyretroid Resistant (LPR) strain, which is resistant to unsubstituted phenoxybenzyl pyrethroids. This strain was established through laboratory sampling of houseflies captured in 1980 from a dairy factory in New York City, following four years of localized indoor spraying with permethrin (Kasai and Scott, 2000). Biochemical studies on this strain revealed the P450 isoform, responsible for pyrethroid detoxification as CYP6D1. Overexpression of this gene is correlated with resistance to pyrethroids. It has been demonstrated that, at the time of xenobiotic detoxification, CYP6D1 expression is ninefold higher in LPR specimens than in sensitive strain specimens (Karunker et al., 2008). Overexpression of CYP6D1 is due to increased gene transcription (Kasai and Scott, 2000; Markussen and Kristensen, 2010). CYP6D1 is also one of the most well-studied P450 genes involved in the development of insecticide resistance in houseflies (Freeman, 2020; You et al., 2022). A study on the deltamethrinresistant (BJD) and the sensitive (TJS) strains revealed that enhanced expression of the CYP6A40, CYP6D8, and CYP6G4 genes is associated with pyrethroid resistance. Altogether, CYP6G4 is not transcribed in eggs and larvae, and CYP6A40 and CYP6D8 are expressed at all life stages of the houseflies (Gao et al., 2012).

Insecticidal resistance to neonicotinoids

For centuries, humans have utilized the insecticidal properties of nicotine to control insect populations (Steppuhn et al., 2004). Nowadays, nicotine derivatives, such as neonicotinoids, are widely used. They affect postsynaptic nicotinic acetylcholine receptors, which block the transmission of nerve impulses, and the insect dies from overexcitation (Markussen and Kristensen, 2010). Resistance to insecticides from the neonicotinoid class (imidacloprid and thiamethoxam) has been detected in natural populations of *Musca domestica*. Strain 766b, collected in Denmark in 2005, showed 130-fold and 140-fold resistance to imidacloprid, as well as 17-fold and 28-fold resistance to thiamethoxam in males and females, respectively (Markussen and Kristensen, 2010). The 791a strain was obtained from a natural population that was collected in 1997 from a farm located in Store Karleby, Denmark. This strain was highly resistant to pyrethroids, dimethoate, propetamphos, methomyl, and azamethiphos, and had some resistance to cyromazine and fipronil (Kristensen et al., 2001; Kristensen and Jespersen, 2003; Kristensen et al., 2004). Selection with imidacloprid 791a increased resistance to imidacloprid by 75-fold in males and 150-fold in females, whereas selection with

thiamethoxam showed minimal effect. Three cytochrome *P450* genes—*CYP6A1*, *CYP6D1*, and *CYP6D3*—were overexpressed in resistant strains compared to the laboratory-sensitive strain (Markussen and Kristensen, 2010).

Insecticidal resistance to spinosad

Spinosad is an insecticide whose active toxins (spinosin A and D) are produced by the soil actinomycete Saccaropolyspora Spinoza (Hertlein et al., 2011). The mechanism of action of spinosad is unique; it first targets the nicotinic acetylcholine receptor and subsequently affects GABA receptors (Elsayed et al., 2022). A comparison of P450 gene expression levels among three Musca domestica strains allowed for the identification of genes associated with spinosad resistance (Højland et al., 2014; Højland and Kristensen, 2017). Two resistant strains were analyzed including strain 766b, resistant to imidacloprid and thiamethoxam, and strain 791spin, derived from multidrug-resistant strain 791a through selection with spinosad. It should be noted that the selection with spinosad not only led to the development of resistance to this insecticide but also caused a reduced resistance to fipronil, imidacloprid, and thiamethaxam, which was characteristic of the parental strain 791a. The sensitive WHO-SRS strain obtained in 1988 at the University of Pavia, Italy, was used for comparison as a reference strain. The findings of the study revealed that 19 out of 100 P450 genes associated with insecticide resistance, including CYP4D9, CYP4D63, CYP4D13, CYP4G13, CYP4G98, CYP6A1, CYP6A24, CYP6A36, CYP6A37, CYP6D1, CYP6D3, CYP6G4, CYP6G7, CYP6G1, CYP9F12, CYP12A1, CYP12A2, CYP12G2 and CYP313D1. The highest expression among the P450 genes was characteristic of the CYP4D63 and CYP6A24 genes in both resistant strains. Additionally, the CYP4G98 and CYP6G7 genes were overexpressed in strain 766b compared to WHO-SRS and 791spin, which indicates their significant contribution to resistance to neonicotinoids. Furthermore, CYP4D9 demonstrated increased expression in the 791spin strain compared to spinosage-sensitive strains, indicating its important role in the formation of spinosage resistance (Højland et al., 2014; Højland and Kristensen, 2017; Mahmood et al., 2016).

Insecticidal resistance to various classes of insecticides

The Alabama house fly strain (ALHF), which is resistant to several insecticides (permethrin, deltamethrin, fipronil, imidacloprid, chlorpyrifos, etc.), was collected in 1998 from a poultry farm in Alabama (Liu and Yue, 2000). In this strain, 86 of the currently known 146 *P450* genes were expressed. Researchers from Auburn University, Alabama, performed a comparative analysis of *P450* gene expression in a resistant strain of ALHF and sensitive strains, abyss and CS. As a result, they identified 11 genes with expression levels more than 2-fold higher in the multidrug-resistant line compared to the sensitive strains. The identified genes were primarily related to *CYP4* and *CYP6* (*CYP4G13*, *CYP4G99*, *CYP4S24*, *CYP4E10*, *CYP4E11*, *CYP6A36*, *CYP6A40*, *CYP6A52*, *CYP6A58*, *CYP6D3*, and *CYP6D10*; Li et al., 2023). In studies by Zhu et al. in 2008, conducted on the same strain, three additional gene expressions—*CYP4D4*, *CYP4G2*, and *CYP6A38*—were found to be upregulated in response to permethrin exposure (Zhu et al., 2008).

Expression systems are often used to assess the involvement of a gene in the detoxification of specific insecticides, or recombinant proteins are introduced into other insect species. For instance, in several studies conducted in 2022 and 2023, Chinese scientists demonstrated that the *CYP6G4* gene from the multidrug-resistant line of *Musca domestica* can metabolize insecticides of the carbamate class (Zhu et al., 2022, 2023; You et al., 2023). Experiments were conducted in the *E.coli* expression system and *in vivo* on *Drosophila melanogaster*. The results of the studies demonstrated that *CYP6G4* is capable of metabolizing propoxur through O-depropylation, N-demethylation, and hydroxylation (Zhu et al., 2022). Earlier studies noted that *CYP6A1* and *CYP12A1* are highly expressed in the dianin-resistant Rutgers strain, indicating their ability to participate in the metabolism of organophosphorus insecticides (Li et al., 2007; Højland et al., 2013).

Table 1 depicts the genes responsible for resistance to a particular class of insecticides. To understand the mechanisms underlying the development of insecticide resistance and to design effective methods for its elimination and prevention, it is essential to learn not only which *P450* genes are responsible for resistance development but also their location and regulatory mechanisms.

Table 2 provides information about several *P450* genes and their locations on autosomes (Meisel and Scott, 2018; Li et al., 2023). For some of the genes listed in the table, the location of trans elements that affect gene expression has been studied. Specifically, trans elements located on autosome 1 regulate the *CYP4G99* gene, while those on autosome 2 regulate *CYP4E11*, *CYP6A1*, *CYP6A40*, and *CYP6D3*. The *CYP4G13*, *CYP4S24*, *CYP4E10*, *CYP6A3*, *CYP6A58*, *CYP6D1* genes are regulated by aggregated trans elements located on autosomes 1 and 2. Co-regulation is also typical for elements located on autosomes 2 and 5, which regulate the *CYP6D10* gene (Gao and Scott, 2006; Pu et al., 2016; Li et al., 2023; Freeman and Scott, 2024).

Table 1. CYP450 enzymes involved in insecticide detoxification in Musca domestica

Insecticide class	CYP family	CYP genes	References	
Multidrug resistance (based	CYP4	CYP4G2, CYP4G13, CYP4G99, CYP4D4, CYP4S24, CYP4E10, CYP4E11	71 - 4 1 2000 I.: 4 1 2002	
on the study of the ALHF strain)	СҮР6	CYP6A5, CYP6A36, CYP6A38, CYP6A40, CYP6A52, CYP6A58, CYP6D3, CYP6D10	- Zhu et al. 2008; Li et al., 2023	
Pyrethroids	CYP6	CYP6A40, CYP6D1, CYP6D8, CYP6G4	Karunker et al., 2008; Gao et al., 2012	
	CYP4	CYP4D9, CYP4D63, CYP4D2, CYP4G98	Højland et al., 2014, Højland and Kristensen, 2017	
N	СҮР6	CYP6A1, CYP6A24, CYP6D1, CYP6D3, CYP6G4, CYP6G7, CYP6G1	Markussen and Kristensen, 2010; Højland et al., 2014; Højland and Kristensen, 2017	
Neonicotinoids	СҮР9	CYP9F12		
	CYP12	CYP12A1, CYP12A2, CYP12G2	_	
	CYP313	CYP313D1	_	
	CYP4	CYP4D9, CYP4D63	_	
	СҮР6	CYP6A1, CYP6A24, CYP6A36, CYP6A37, CYP6D1, CYP6D3, CYP6G4, CYP6G1	 Højland et al., 2014; Højland and Kristensen, 2017 	
Spinosyns	СҮР9	CYP9F12	_	
	CYP12	CYP12A1, CYP12A2, CYP12G2	_	
	CYP313	CYP313D1	_	
Carbamates	СҮР6	CYP6G4	Zhu et al., 2022	
Organophosphor	CYP6	CYP6A1	Li et al., 2007	
us compounds	CYP12	CYP12A1	Højland et al., 2013	

Table 2. Location of some cytochrome *P450* genes in the genome

Autosome	<i>P450</i> genes	References
1	CYP6D1, CYP6D3, CYP6D10	Meisel and Scott, 2018; Li et al., 2023
2	CYP6D8, CYP9F12, CYP12A1, CYP12A2, CYP313D1	Meisel and Scott, 2018
3	CYP4D9, CYP4G13, CYP4G2, CYP4G98, CYP4G99, CYP4S24	Meisel and Scott, 2018; Li et al., 2023
4	CYP12G2	Meisel and Scott, 2018
5	CYP4E10, CYP4E11, CYP6A1, CYP6A36, CYP6A40, CYP6A52,	Meisel and Scott, 2018; Li et al., 2023
	CYP6A58, CYP6G4	Meiser and Scott, 2018, Li et al., 2023

CONCLUSION

At present, the role of some cytochrome P450 genes of *Musca domestica* in the detoxification of several insecticides is well described, particularly the *CYP6D*1 gene. While an increase in expression levels has been noted in response to insecticidal exposure for many *CYP* genes, the specific mechanisms of insecticide detoxification for these genes are still being elucidated. Based on the literature reviewed, it can be concluded that *CYPs* are collectively capable of metabolizing numerous insecticides, but for most insects, there are gaps in terms of which *CYPs* are responsible for the metabolism of a particular insecticide. Additionally, the locations in the genome, linkage groups, and mechanisms of transcription regulation for many *P450* genes remain unknown. More recent publications are interested in this area and more studies are emerging aimed at unraveling the workings of regulatory networks in the context of the development of insecticide resistance. For the most part, the resistance due to the gene activity of the cytochrome monooxygenase family

is polygenic in nature, indicating that resistance to one class of insecticide can be conditioned by several genes. Furthermore, the same genes may be active in conferring resistance to various classes of insecticides, which may contribute to the development of cross-resistance. Thus, molecular genetic studies are essential for each case of insecticide resistance to identify patterns and learn how to predict the development of resistance to other active ingredients. The accumulation of knowledge regarding the insect genome and the mechanisms of resistance to insecticides would facilitate the development of rational strategies for the long-term use of insecticides in pest control programs.

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Availability of data and materials

The data of the current study are available by reasonable requests.

Authors' contributions

Ksenia Krestonoshina conducted project administration, conceptualization, information search and analysis, writing development, and revising. Anastasia Melnichuk did strategic contemplation, information search and analysis, review writing, and editing. All authors reviewed and confirmed the final draft of the manuscript.

Competing interests

The authors have declared no conflicts of interest.

Ethical considerations

All authors have verified all ethical issues including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been addressed.

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Pathological and Economic Effects of Bovine Skin Tumors on Cattle Production in Ethiopia: A Review

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ABSTRACT

A tumor is an abnormal mass of tissue that exceeds normal boundaries, resulting from uncoordinated and uncontrolled cell proliferation. Tumors can affect various parts of cattle animals, including the skin, bones, glands, and visceral organs. The present study aimed to explore the pathology of bovine skin tumors and their health and economic impacts on cattle. Skin tumors are the most frequently diagnosed neoplastic disorders in bovine species. The most common skin tumors in bovine include bovine papilloma, squamous cell carcinoma, and bovine lymphosarcoma. These tumors pose significant health challenges and have a negative economic impact on cattle production and its byproducts. Clinical features of skin tumors often include hyperkeratosis, acanthosis, elongated rete pegs, large nodular structures, exophytic and cauliflower-like lesions, and friable lesions. Melanomas, another type of proliferative skin tumor, are characterized by spindle to round cell shapes containing abundant black pigment. More than 90% of skin tumors are linked to prolonged exposure to ultraviolet radiation. Diagnosing a skin tumor in cattle typically involves skin biopsy and fine needle aspiration cytology. Histologically, skin tumor cells exhibit an increased nuclear-to-cytoplasmic ratio, cellular and nuclear pleomorphism, and a discohesive arrangement of cells. In addition to their health implications, skin tumors in cattle result in significant economic losses due to reduced productivity, decreased reproduction rates, carcass condemnation, and the downgrading of skins and hides. Common treatment options for skin tumors include chemotherapy, radiation, and surgical removal. Given that skin tumors are an economically significant disease in Ethiopia, they require increased attention from researchers and the centers for control and prevention. Early diagnosis and effective management of these tumors are crucial issues that must be addressed.

Keywords: Bovine, Cattle, Diagnosis, Skin tumor, Tumor

INTRODUCTION

A tumor is an abnormal growth of tissue resulting from the uncontrolled proliferation of cells (Vasconcelos et al., 2023). Based on its behavior, a tumor may be classified as benign or malignant (Constable et al., 2017). A benign tumor refers to a cluster of cells that do not spread throughout the body; instead, it invades only the surrounding tissue of its origin (Mithila Bisht et al., 2020). In contrast, a malignant tumor is more serious, with the ability to spread to other healthy tissues or organs (Khalid et al., 2020). Notably, both forms of tumors increasingly pose a significant threat to the health and well-being of cattle (Flores-Balcázar et al., 2020).

Tumors can affect various parts of the animal body, including both hard and soft tissues (Khalid et al., 2020). Commonly affected areas in cattle include the skin, bones, glands, and various visceral organs (Flores-Balcázar et al., 2020). Skin, as the largest organ of the body, is composed of three distinct anatomical layers, including the epidermis, dermis, and hypodermis (Khalid et al., 2020). It plays crucial roles in physiological regulation, protection from the external environment, and serving as a boundary for internal structures (Achalkar, 2019; Mathewos et al., 2021). Given that the skin is the outermost layer, it is frequently exposed to various pathogens and physical injuries (Khalid et al., 2020).

Tumors represent a significant challenge for the skin at multiple levels, reducing its function for cattle and its value for human consumption (Mathewos et al., 2020). Research indicates that skin tumors are among the most frequently observed tumors in cattle (Dabbagh Moghaddam et al., 2021), primarily due to the skin's continuous exposure to the external environment (Vasconcelos et al., 2023). Prolonged exposure to sunlight can lead to DNA damage in skin cells, preventing the body from recovering and resulting in uncontrolled cell proliferation (Mathewos et al., 2021). The development of tumors often occurs when DNA is damaged and the body cannot repair it (Flores-Balcázar et al., 2020). Currently, the incidence of bovine skin cancer is increasing, negatively impacting animal productivity and leading to mortality (Mathewos et al., 2020).

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Constable et al. (2017) noted that the incidence of skin tumors is rising over time in cattle, causing significant damage to the skin and hide industry. This trend contributes to economic losses, particularly in Ethiopia, where the skin and hide industry faces challenges related to quality. The objective of this review was to summarize the issue of bovine skin tumors and their economic impact.

Common skin tumors occur in cattle

Currently, many researchers report that cattle rank second after dogs for the incidence of all types of tumors occurring in domestic animals (Mathewos et al., 2020). The most common type of tumor affecting bovine species is skin tumor (Khalid et al., 2020). Many cattle are more likely to develop skin tumors, although the rate of occurrence may vary among different breeds, coat colors, agro-climates, and management practices (Moharram et al., 2019).

As indicated in Table 1, the different types of cutaneous tumors affecting cattle include bovine papilloma, squamous cell carcinoma (SCC), bovine lymphosarcoma, and melanoma. Bovine cutaneous papilloma and SCC are particularly prevalent forms of tumors in cattle (Flores-Balcázar et al., 2020). Skin tumors have been identified as serious diseases that significantly impact the health and welfare of cattle (Khan et al., 2022).

Different types of skin tumors exhibit varying prevalence across the skin of cattle. Not all parts of the skin are equally susceptible to different types of tumor diseases (Jamieson and Mohamed, 2020) as illustrated in Table 2.

Table 1. Spontaneous skin tumors prevalence in farm animals

Organ	Tumor type	Animal	Number/Percent	Sex (M/F)	Age
Skin	Fibropapilloma	Cattle	110 (54.4%)	40M/70F	M 2m-3y F 4m-6y
Skin	Equine Sarcoid	Equine	65 (32.1%)	39M/26F	M 2m-6y F 2m-7y
cutis	Fibroma	Cattle Equine	14 3(8.4%)	4M/10F 1M/2F	M 2-4y F 2-5y M 5y and F 3y
Skin and subcutis	SCC	Cattle Sheep	31(1.9%)	1M/2F 1M	M 3y F 2-3y F 3m
sub-cutis	Lymphosarcoma	Cattle Buffalo	11(0.9%)	1F 1F	2y 8m
Liver	Haemagiosarcoma	Cattle	2 (0.9%)	2M	3y
subcutis	Liposarcoma	Goat	1 (0.4%)	1F	3y
Ovary	Malignant Teratoma	Cattle	1 (0.4%)	1F	8m

M: Male, F: Female, m: Months, y: Year, Source: Moharram et al. (2019).

Table 2. Tumor description on the skin of cattle and buffaloes

T	Number of		Landin		ex	A
Tumors	Animals	tumors	Location	F	M	Age
Cutaneous Papillomatosis	Cattle	15	Head, around the eyes, neck, back, shoulder, axilla, and all over the body	13	2	5m to 3y
Cutaneous Fibropapilloma	Cattle	5	The skin of the head, around the eyes,		1	8m to 1y
	Cattle	25	Eye	10	15	2-10y
Scc	Cattle	1	Skin of muzzle	1	-	9y
	Cattle	8	Perineum	8	-	7-9y
Scc	Buffalo	1	Skin of face	1	-	3y
	Bullato	1	Buccal cavity	1	-	8y
Epulis	Cattle	2	Gum	1	1	8m and 2y
Leiomyoma	Cattle	2	2 Vagina		-	4y
Fibroma	Cattle	1	Submandibular space		-	6y
Liposarcoma	Cattle	1	Neck		-	1y
Total		61		43	19	

SCC: Squamous cell carcinoma, m: Month, y: Year, Source: Moharram et al. (2019)

Bovine papilloma

Bovine papilloma is the most commonly identified type of skin tumor in cattle. It is an exophytic growth of squamous cell epidermis caused by bovine papillomavirus (BPV, Constable et al., 2017). This tumor rarely regresses without causing serious clinical problems for the infected animal (Vasconcelos et al., 2023). However, it can occasionally persist for an extended period, serving as a focus for malignant transformation into squamous cell carcinoma (SCC, Moharram et al., 2019). Bovine papilloma (BP) can affect cattle of all ages, but it is most commonly observed in young animals (Feyisa, 2018). The virus spreads between animals through direct or indirect contact, such as grooming materials and other fomites (Ugochukwu et al., 2019).

Gross lesion

Bovine papilloma can be found in various locations throughout the body, including the head, neck, udder, around the eyes, shoulders, limbs, and ears (Vasconcelos et al., 2023). It is characterized by elevated and diffuse multi-nodular proliferations (Constable et al., 2017), as well as a lichenified appearance, thickened epidermis, and pedunculated, firm, dense masses with rough, scaly, and dry surfaces (Feyisa, 2018). Cauliflower-like lesions are also common (Mathewos et al., 2020). The color of the affected areas can range from grayish-white to black (Moharram et al., 2019). The gross appearance of Bovine papilloma on the neck, shoulder, and face of the cattle is shown in Figure 1, and also on the teats and thoracic region of cattle is shown in Figure 2.



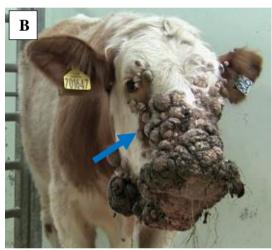


Figure 1. Gross appearance of Bovine papilloma. **A**: Bovine papilloma located on the neck and shoulder of cattle (Yellow arrows, Kumar et al., 2015). **B**: Bovine papilloma presented on the face of the cattle (blue arrow). Source: (Flores-Balcázar et al., 2020).



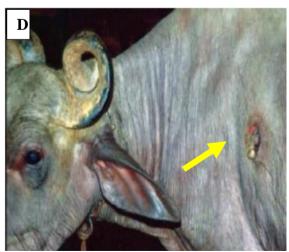


Figure 2. Gross appearance of Bovine papilloma. C: Bovine papilloma affected the teats of cattle (Yellow arrow, Kumar et al., 2015). **D**: Papilloma wart in a buffalo on the thoracic region (yellow arrow). Source: Flores-Balcázar et al. (2020).

Histopathological features

Bovine papilloma exhibits prominent hyperkeratosis, acanthosis, and elongated rete pegs surrounded by fibrovascular stroma when observed under a light microscope (Xiao et al., 2020). Additionally, hydropic degeneration of epidermal cells (Meuten, 2020) and hypergranulosis of the stratum granulosum are also evident, as shown in Figure 3. Fibropapillomas, a subtype, are characterized by fibromas that arise from the stroma, exhibiting hypercellularity of fibroblasts forming whorls around blood vessels (Hunt, 2017). As indicated in Figure 4, cutaneous bovine papilloma has the features of proliferation of epithelial cells at the stratum spinosum with varying degrees of ballooning degeneration, several epithelial cells have enlarged, condensed nuclei, and some are apoptotic (Crespo et al., 2019).

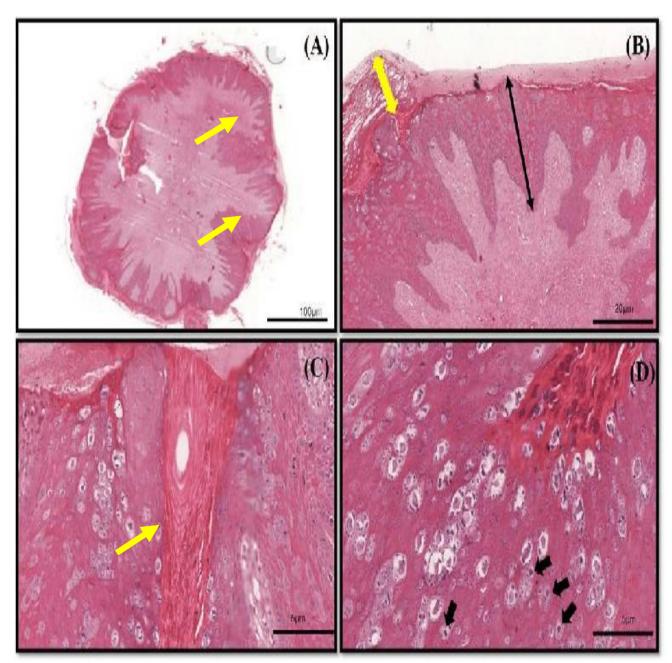


Figure 3. The histopathology findings of bovine papilloma tissues. **A**: Low magnification showing a well-developed finger-like projecting papillae rising from the surface of the epidermis to the subcutaneous layer (yellow arrows). **B**: Hyperkeratosis of the epithelial layer (double yellow arrow) with acanthosis formation (double black arrow). **C**: Epidermal proliferation with elongated rete pegs and neoplastic fibroblast (yellow arrow). **D**: Multiple koilocytosis formation on the dermis layer (black arrows). H&E staining 10X and 40X. Source: Khalid et al. (2020).

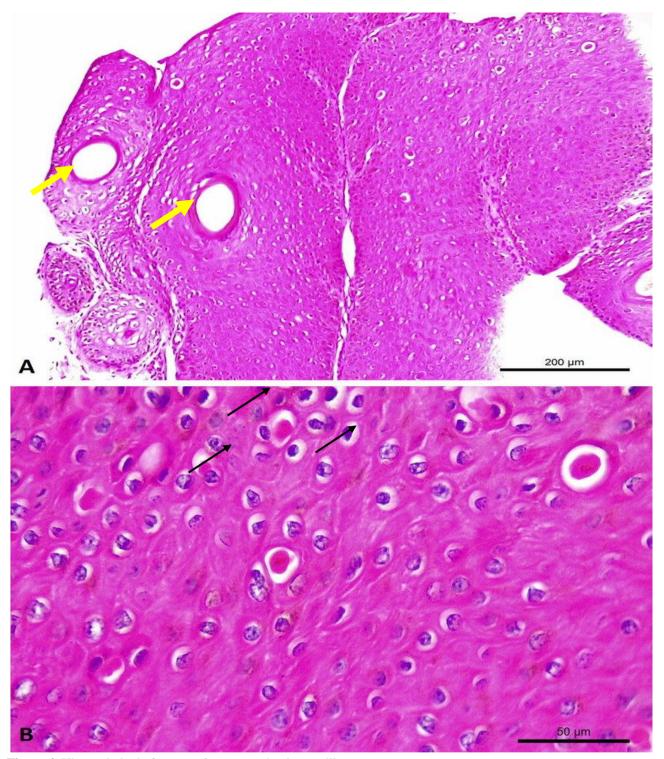


Figure 4. Histopathologic features of cutaneous bovine papilloma. **A**: There is the proliferation of epithelial cells at the stratum spinosum with varying degrees of ballooning degeneration (yellow arrows). **B**: Several epithelial cells have enlarged, condensed nuclei, and some are apoptotic (black arrows). Hematoxylin and eosin stain; Bar, A. 200 μm, B. 50 μm. Source: Crespo et al. (2019).

Squamous cell carcinoma

Bovine squamous cell carcinoma (SCC) is a cancer that develops in the squamous cells of the skin, specifically keratinocytes (Moharram et al., 2019). It is the second most common type of tumor in cattle, following bovine papilloma (Mithila et al., 2020). Bovine SCC tends to grow rapidly on various body parts, with a particular prevalence on the eyelids, especially following prolonged exposure to sunlight (da Cruz Campos and Pimentel, 2023). Additionally, mechanical irritations, injuries, and burns can also contribute to the development of SCC (Moharram et al., 2019).

This type of cancer generally occurs in cattle over the age of seven and is rare in those younger than three years old (da Cruz Campos and Pimentel, 2023). Bovine SCC is more common in cattle with white hair and light-colored skin, particularly in breeds such as Holsteins and Ayrshires (Moharram et al., 2019). Besides ultraviolet (UV) light, bovine

ocular SCC may also be caused by viral agents, although the specific causative agent remains unknown (Vasconcelos et al., 2023).

Macroscopic appearance

Squamous cell carcinoma (SCC) appears as large nodular and cauliflower-like lesions, characterized by exophytic, ulcerated, and friable features (da Cruz Campos and Pimentel, 2023). As indicated in Figure 5, bovine SCC can present as nodular and hemorrhagic, accompanied by purulent discharge, and may metastasize to the retro-pharyngeal lymph nodes (Mathewos et al., 2020). Figure 6 shows Gross pictures of cattle affected with ocular squamous cell carcinoma (Timurkan and Alcigir, 2017). Similarly, Figure 7, indicates gross pictures of cattle affected with ocular Squamous cell carcinoma (Timurkan and Alcigir, 2017).

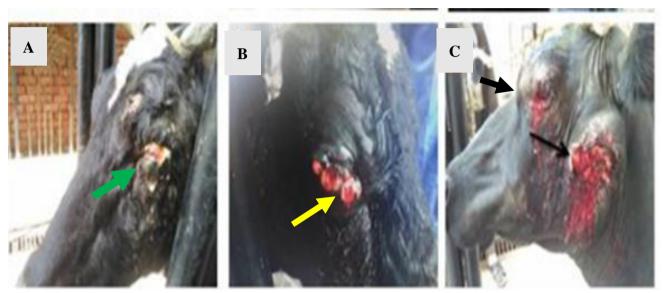


Figure 5. Gross pictures of cattle affected with squamous cell carcinoma. **A:** Bovine SCC on the eyelid (green arrow), **B:** SCC developed with hemorrhagic on the eyelid (yellow arrow), and **C:** The eyelid and the proximal part of the mandible region are affected and an ulcer is obvious (black arrows). Source: Moharram et al. (2019).



Figure 6. Gross pictures of cattle affected with ocular squamous cell carcinoma (yellow arrow). Source: Timurkan and Alcigir (2017).



Squamous cell carcinoma on the conjunctiva

Figure 7. Gross pictures of cattle affected with ocular Squamous cell carcinoma (black arrow); Source: Timurkan and Alcigir (2017).

Microscopic appearance

The tumor reveals the proliferation of anastomosing nests, sheets, and strands of atypical keratinocytes originating in the epidermis and infiltrating into the dermis (Hunt, 2017). The neoplastic cells differentiate to form distinct keratin pearls, which are characteristic of squamous cell carcinoma (Flores-Balcázar et al., 2020). At higher magnification, keratin tonofilaments are visible as intracytoplasmic eosinophilic fibrillar material (Moharram et al., 2019). Additionally, mitotic figures, pleomorphism, hyperchromatism of the neoplastic cells, enlarged and prominent nucleoli, and vacuolation of neoplastic cells are evident (Timurkan and Alcigir, 2017). As indicated in Figure 8, Microscopic appearance of SCC in cattle, tumor masses show round masses of keratin pearls in SCC (Habte, 2022). Similarly, Figure 9, indicates the Microscopic appearance of SCC in cattle, Tumor masses show round masses of keratin pearls in SCC (Habte, 2022).

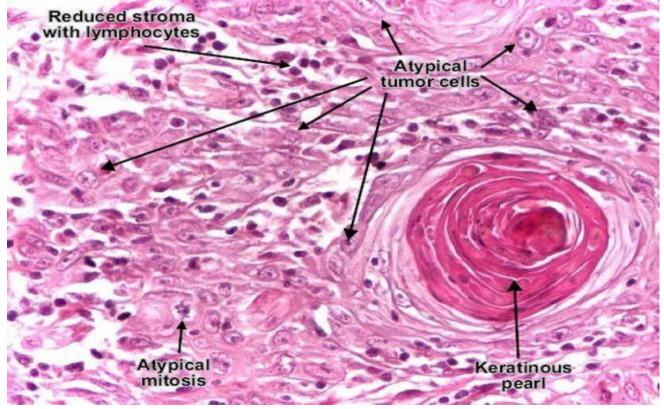


Figure 8. Microscopic appearance of Squamous cell carcinoma in cattle. Tumor masses show round masses of keratin pearls in Squamous cell carcinoma (black arrow). Haematoxylin & Eosin, 40x, Source: Habte (2022).

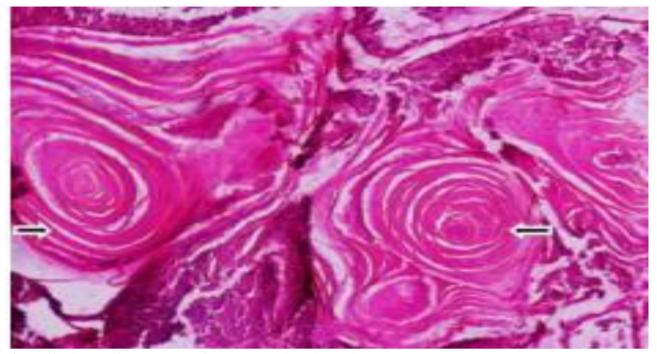


Figure 9. Microscopic appearance of Squamous cell carcinoma in cattle. Tumor masses show round masses of keratin pearls in SCC (arrows), Haematoxylin & Eosin, 40x, Source: Habte (2022).

Bovine lymphosarcoma

Cutaneous lymphosarcoma is another common type of tumor in cattle (Mathewos et al., 2020). Lymphoma is a cancer of the lymphatic system that develops from lymphocytes (Vasconcelos et al., 2023). It is classified as a diffuse malignant lymphoma that can arise in the skin, lymph nodes, or other lymphoid tissues (da Cruz Campos and Pimentel, 2023). Lymphoma is considered primarily cutaneous when the lymphatic proliferation is confined to the skin, with no involvement of the bone marrow, lymph nodes, or viscera at the time of diagnosis (Xiao et al., 2020).

Lymphosarcoma in cattle may be sporadic, occurring in calves and young herds without a known cause, or it may result from infection with the bovine leukemia virus (BLV), commonly referred to as enzootic bovine leucosis, which primarily affects adults (Vasconcelos et al., 2023). Bovine lymphosarcoma is transmitted through contaminated blood containing infected lymphocytes (Habte, 2022). Cutaneous lymphosarcoma is most common in cattle aged 1–3 years (Xiao et al., 2020) and is extremely rare in sheep, goats, and swine (Xiao et al., 2020). While lymphosarcoma can occur in all breeds, it is most frequently observed in Holsteins (Moharram et al., 2019).

Gross lesion appearance

Cutaneous lymphosarcoma presents as cutaneous plaques measuring 1–5 cm in diameter, appearing as yellow-tan discrete nodular masses or diffuse tissue infiltrates on the neck, back, rump, and thighs (Mathewos et al., 2020). This form of lymphosarcoma may undergo spontaneous remission; however, relapses can occur (Xiao et al., 2020). Additionally, local lymph nodes may become enlarged, exhibiting colors that range from white to tan (Neerja et al., 2018). Lymphosarcoma tumors are often multifocal and commonly involve the neck and trunk surfaces, presenting as variable-sized firm nodules and lesions that resemble an urticarial reaction (Constable et al., 2017). The overlying skin may appear normal, or it may show variable alopecia, hyperkeratosis, or ulceration, as indicated in Figure 10 (Sokołowska-Wojdyło et al., 2015).

Microscopic appearance

Histological and cytological preparations reveal sheets of a relatively homogeneous population of neoplastic lymphocytes (Moharram et al., 2019). The tumor masses are composed of closely packed, monomorphic lymphocytic cells (Mathewos et al., 2020), along with extensive dermal invasion by lymphoblasts (Crespo et al., 2019). As indicated in Figure 11, Microscopic appearances of lymphosarcoma have shown tumor mass showing pleomorphic small to medium and round to oval neoplastic lymphocytes separated by a delicate collagenous fibrous tissue in lymphoma (Habte, 2022). The tumor mass shows pleomorphic small to medium and round to oval neoplastic lymphocytes separated by a delicate collagenous fibrous tissue in lymphoma (Habte, 2022), as indicated in Figure 12.

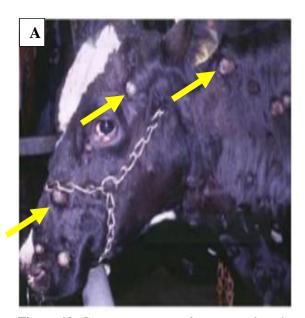




Figure 10. Gross appearance of cutaneous lymphosarcoma. **A:** Scattered lymphosarcoma on the skin (yellow arrows) and **B:** Over congested lymphosarcoma on the skin (star). Source: Mathewos et al. (2020).

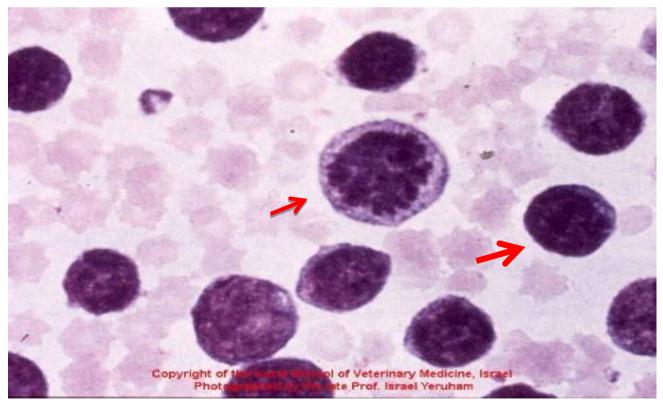


Figure 11. Microscopic appearances of lymphosarcoma. Cytological features of Lymphosarcoma (bovine leukemia) from Fine Needle Aspiration Cytology; and tumor mass showing pleomorphic small to medium and round to oval neoplastic lymphocytes separated by a delicate collagenous fibrous tissue in lymphoma (red arrows), Haematoxylin & Eosin, 40x, Source: Habte (2022).

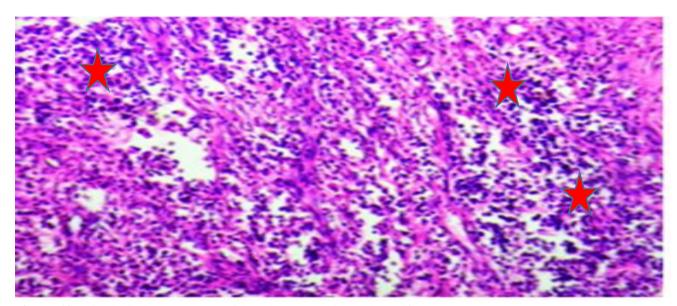


Figure 12. Histological appearances of lymphosarcoma in cattle (heifers). Tumor mass showing pleomorphic small to medium and round to oval neoplastic lymphocytes separated by a delicate collagenous fibrous tissue in lymphoma (red stars), Haematoxylin & Eosin, x40, Source: Habte (2022).

Melanoma

Melanoma is a benign tumor that arises from melanocytes, or pigment-producing cells (da Cruz Campos and Pimentel, 2023). The majority of melanoma cases occur on the skin surface of the head, neck, trunk, or legs; however, they can very rarely develop in the mouth, intestines, or eye (uveal melanoma) (Hunt, 2017). Despite being predominantly benign, there are rare reports of locally invasive tumors as well as metastasis to distant sites (Habte, 2022). The main cause of melanoma is exposure to UV light, particularly in individuals with low levels of the skin pigment melanin (da Cruz Campos and Pimentel, 2023).

Gross lesion appearance

The size of melanoma tumors in cattle varies widely, ranging from less than 5 cm to up to 25 cm (Constable et al., 2017). These tumors can appear as single or multiple, raised, firm masses that are typically black (Mathewos et al., 2020). The mass is composed of a proliferation of spindle to round-shaped cells that frequently contain abundant black pigment (Flores-Balcázar et al., 2020). On cross-section, melanoma appears as pigmented brown or black and oily (Figure 13).

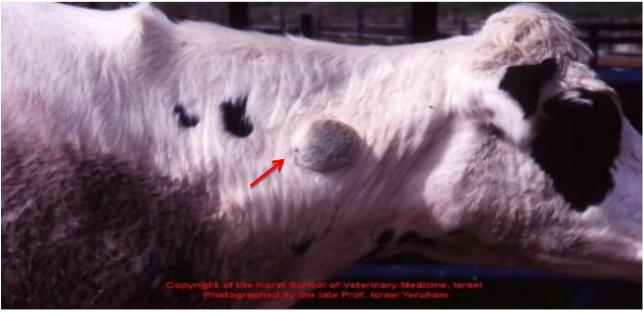


Figure 13. Gross lesion of skin melanoma on the neck of cattle (red arrow), Source: Javanbakht (2014).

Histopathological appearance

Neoplastic melanocytes are arranged in sheets that resemble a band-like pattern (Mithila et al., 2020). Melanocytic tumors exhibit characteristic melanin-containing neoplastic cells, often mixed with heavily pigmented melanophages and arranged in nests and clusters, Cellular pleomorphism and mitotic activity are noted as variable features (da Cruz Campos and Pimentel, 2023).

In dendritic cell-type melanoma, dermal and/or hypodermal melanoma consists of cells with a dense, disordered, or band-like arrangement and an extremely high melanin content (Khalid et al., 2020). Cellular details can typically be observed only after discoloration (Mathewos et al., 2021). The cells are polyhedral or round, with numerous small dendritic cells present. Although mitoses are not abundant, necrotic foci are frequently observed (Mithila et al., 2020). As indicated in Figure 14. Microscopically melanoma has shown a proliferation of spindle to round-shaped cells containing abundant black glandular pigment (Khalid et al., 2020). As shown in Figure 15, Histological appearances of dermal melanoma in cattle, dermal melanocytoma, and round cell type occurred (Baba and Câtoi, 2007). Also, it shows, a proliferation of spindle to round-shaped cells (dendritic cell type) containing abundant brown glandular pigment (Figure 16) (Šitum et al., 2014). Moreover, Malignant melanoma shows, pleomorphic, and giant cell type proliferation (Figure 17) (Goldschmidt, 2016). Histological appearances of malignant melanoma, epithelioid cell type (Baba and Câtoi, 2007), as indicated in Figure 18.

In Ethiopia, various types of tumors from different origins are a common problem in domestic animals, including those of epithelial and mesenchymal origins (Feyisa, 2018; Mathewos et al., 2021). The most prevalent tumor in cattle among those of epithelial origin is squamous cell papilloma (Table 3). Similarly, among tumors of mesenchymal origin, cutaneous fibrosarcoma is observed on the dewlap of bulls (Habte, 2022). Cutaneous fibrosarcoma affects fibroblasts, a specific type of cell (Constable et al., 2017). Epithelial-origin tumors are the most frequently occurring tumor type in Ethiopia (Mathewos et al., 2020). A study conducted in Wolayita Sodo indicated that young animals are more susceptible to bovine papilloma than mature animals (Feyisa, 2018). This increased susceptibility has resulted in early culling or slaughtering of affected animals, leading to significant economic implications. As indicated in Figure 19, gross papilloma lesion on the mandibular, ocular, teat, shoulder, and cutaneous region (Mathewos et al., 2020).

The periocular squamous cell carcinoma (SCC) is located on the third eyelid of a bull (Mathewos et al., 2020). It can be grossly identified by its raised, irregular surfaces, with some portions being friable and soft, while most of the tumor is hard in consistency. The lesion is ovoid and varies in color from white to gray (Feyisa, 2018; Moharram et al., 2019). As indicated in Figure 20, both macroscopic and microscopic features of peri-ocular SCC (Mathewos et al., 2020). Figure 21, shows the Microscopic appearance of peri-ocular squamous cell carcinoma; Shows round masses of keratin pearls of peri-ocular SCC from the third eyelid of the local breed bull (Mathewos et al., 2020).

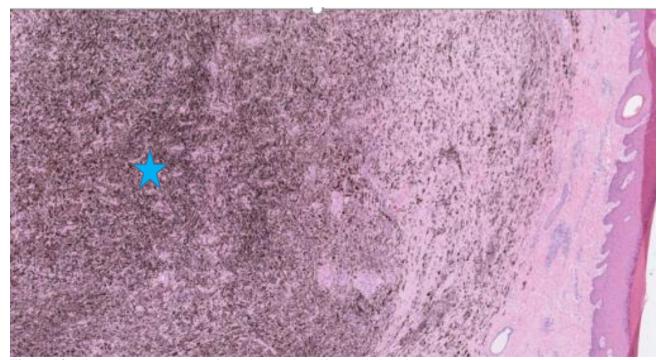


Figure 14. Microscopic appearance of a melanoma in heifer exotic breed. The Photomicrograph depicting a proliferation of spindle to round-shaped cells containing abundant black glandular pigment, 4x (blue star), Source: Khalid et al. (2020).

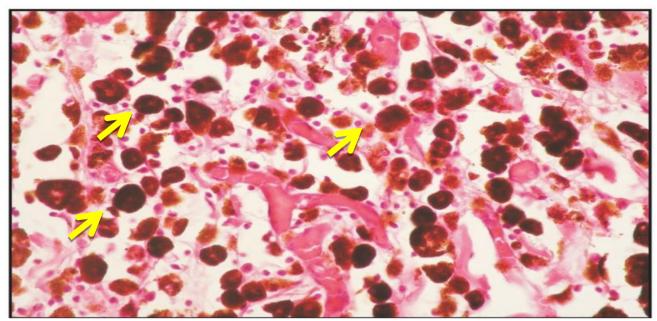


Figure 15. Histological appearances of dermal melanoma in cattle. Dermal melanocytoma, round cell type, 10x (yellow arrows), Source: Baba and Câtoi (2007).

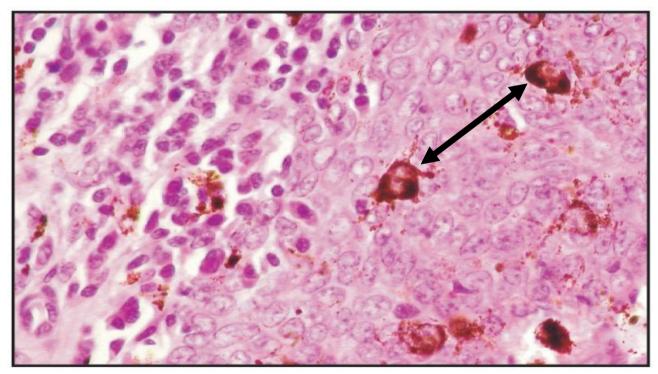


Figure 16. Histological appearances of melanoma in cattle. Photomicrograph depicting a proliferation of spindle to round-shaped cells (dendritic cell type) containing abundant brown glandular pigment, 10x (double black arrow), Source: Šitum et al. (2014).

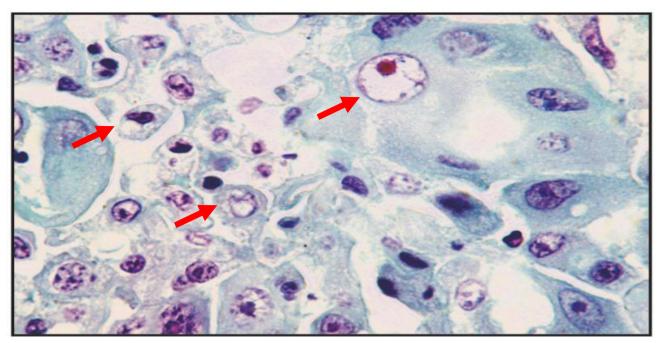


Figure 17. Histological appearances of melanoma in cattle. Photomicrograph depicting malignant melanoma, pleomorphic, and giant cell type, 10x (red arrows), Source: Goldschmidt (2016).

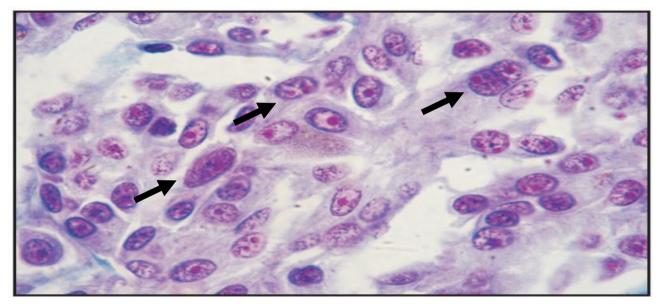


Figure 18. Histological appearances of melanoma in cattle. Photomicrograph depicting malignant melanoma, epithelioid cell type, 10x (black arrows), Source: Baba and Câtoi (2007).

Table 3. Frequencies of tumors of epithelial origin among domestic animals

Number	Tumor Type	Number of Cattle affected	Percent
1	Squamous cell papilloma	20	33.3
2	Ocular SCC	1	1.7
3	Basal Cell Carcinoma	-	
	Mammary Gland Tumor	-	-
4	Papillary Mammary Adenocarcinoma	-	-
4	Carcinoma Mixed Type	-	-
	Mammary Adenocarcinoma		-
5	Sertoli Cell Tumor	-	-
6	Atypical Anal Sac Adenocarcinoma	-	-
Total		21	35

Source: Feyisa (2018); Mathewos et al. (2020)



Figure 19. Gross appearance of papilloma on different body parts of cattle. **A**: gross papilloma lesion on the mandibular region (white arrow). **B**: gross papilloma lesion on the ocular region (white arrow). **C**: gross papilloma lesion on the leg region (white arrow). **D**: gross papilloma lesion on the teat region (white arrow). **E**: gross papilloma lesion on the shoulder region (white arrow). **F**: gross papilloma lesion on the cutaneous region (white arrow), Source: Mathewos et al. (2020).

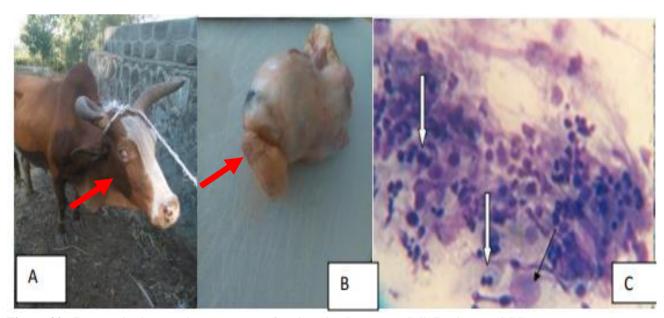


Figure 20. Gross and microscopic appearance of peri-ocular Squamous Cell Carcinoma (SCC). **A** and **B**: Represent the macroscopic features of peri-ocular SCC (red arrows). **C**: Shows the microscopic appearance of peri-ocular SCC from the third eyelid of the bull (white arrows), Source: Mathewos et al. (2020).



Figure 21. Microscopic appearance of peri-ocular squamous cell carcinoma. **D:** Show round masses of keratin pearls of peri-ocular SCC from the third eyelid of the local breed bull, 10x (black arrow), Source: Mathewos et al. (2020).

2.5. Cause and risk factors of skin tumor

Most tumors are linked to problems in gene expression (Mathewos et al., 2020). However, the specific causes of various skin tumors remain largely unknown (Hunt, 2017). Despite decades of research and advancements in early identification and therapy, the actual causes of most skin malignancies often associated with gene expression issues are still not fully understood (Kiupel, 2020). Some known tumor-inducing factors include excessive exposure to UV light, exposure to chemical toxins, for example; aflatoxins and reactive oxygen radicals, oncogenic viruses, and spontaneous changes in DNA replication (Habte, 2022). More than 90 percent of skin tumors are associated with UV radiation (Moharram et al., 2019). Additionally, factors such as skin color, species, lack of pigment, age, breed, sex, immune system disorders, and inherited conditions that increase sensitivity to sunlight all contribute to DNA mutations, potentially leading to either benign or malignant tumors (Moharram et al., 2019). In particular, cattle with white coats are more severely affected by skin tumors (Habte, 2022). Furthermore, exotic cattle breeds are more prone to tumors than indigenous breeds (Crespo et al., 2019).

Pathogenesis and development of skin tumor

Development

The mechanisms that lead to the formation and development of skin tumors are similar to those of other cancer types. Damage to a cell's genetic machinery is widely recognized as the etiology of all types of cancer (Khalid et al., 2020). Generally, the development of tumor lesions is a complex and often unpredictable phenomenon (Moharram et al., 2019). However, three phases are typically recognized in many tumors, including mutation (altered DNA replication), promotion, and irreversible tumor growth (Hunt, 2017).

The physiological process of tissue repair can sometimes lead to abnormal processes, especially when multiple foci of microdamage occur within the organism (Crespo et al., 2019). The pathophysiological mechanism involves multiple permanent (long-lasting) tissue microdamage, which, combined with sympathetic dominance, can sustain cell proliferation while inhibiting anti-tumor immunity (Hunt, 2017). The cancer reparative trap represents a resistant pathophysiological condition that contributes to the appearance, development, and progression of cancer (Bukhtoyarov and Samarin, 2015).

Skin tumor metastasis

Metastasis refers to the development of secondary cancer in locations other than the primary tumor (Khalid et al., 2020). The cells continue to proliferate in these new areas, eventually forming additional tumors composed of cells that

resemble the tissue of origin (Mathewos et al., 2021). The spread of cancerous cells from a primary tumor to the skin is specifically referred to as skin metastasis (Kumar et al., 2015).

Genetic modifications in cancer cells provide advantageous properties, such as the ability to adhere to other cells, degrade the extracellular matrix, induce the secretion of normal cell-killing factors, increase cell motility, and spread through tissues (Timurkan and Alcigir, 2017). Additionally, these modifications allow for growth autonomy, resistance to anti-growth signals, evasion of apoptosis (immortality), and angiogenesis, all of which contribute to further cancer growth and dissemination in various tissues (Kumar et al., 2015). Several classes of molecules are involved in the metastatic cascade, including those that govern invasion (such as degradative enzymes and motility factors) and adhesion (including integrins, selectins, and cadherins, Khalid et al., 2020).

Diagnosis of skin tumor

Diagnosis of skin tumors can be performed clinically and histopathologically. Clinically, skin tumors can be identified through palpation and percussion of a raised, swollen, firm, nodular mass or any non-healing growth or lesion (Vasconcelos et al., 2023). Additionally, growths that are profusely bleeding should also be suspected as tumors (Habte, 2022). Histopathological diagnosis involves tissue biopsy and Fine Needle Aspiration Cytology (Khalid et al., 2020).

Histopathological examination

Histopathology is a method used to diagnose and understand abnormal cellular characteristics such as anaplasia, invasion, extreme mitosis, metastasis, and loss of polarity, all of which indicate malignancy (Timurkan and Alcigir, 2017). It is employed to differentiate between benign and malignant tumors (Vasconcelos et al., 2023). A skin or tissue biopsy is utilized to perform cytological and histological studies, and it is typically regarded as the gold standard for making a final diagnosis (Khalid et al., 2020).

Characteristics of cancer cells include high cellularity, cellular enlargement, an increased nuclear-to-cytoplasmic ratio, nuclear hyperchromasia, cell dis cohesiveness, and prominent, large nucleoli (Thomas et al., 2016). Additionally, cellular and nuclear pleomorphism, along with background tumor necrosis, are also important histological features of tumor cells (Mithila et al., 2020).

Application of immunohistochemistry for tumor diagnosis

Immunohistochemistry is the process of selectively identifying antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Mathewos et al., 2020). After tissue sections are incubated with the relevant antibodies, positive reactions (tumor antigen-antibody binding) are identified through the application of a detection system (Kabiraj et al., 2015).

This technique is used to understand the distribution and localization of biomarkers and differentially expressed proteins in various parts of biological tissue (Khalid et al., 2020). Tumor markers are biochemical indicators used to identify the presence of tumors (Ugochukwu et al., 2019). These markers can include cell surface proteins, cytoplasmic proteins, enzymes, or hormones (Mathewos et al., 2021). The system is highly sensitive because it allows for the attachment of a relatively large number of enzyme molecules, such as peroxidase, at the antigen site (Kabiraj et al., 2015). Specific molecular markers are characteristic of particular cellular events, such as proliferation or cell death (apoptosis), and examples include keratinocytes and various tumor antibodies and markers (Mithila et al., 2020).

Visualizing the antibody-antigen interaction can be accomplished in several ways. One method is chromogenic immunohistochemistry, where an antibody is conjugated to an enzyme, such as peroxidase (the combination is termed immunoperoxidase), which catalyzes a color-producing reaction (Mathewos et al., 2021). Common substrates for this reaction include diaminobenzidine (producing a brown color) or aminoethylcarbazole (producing a red color), with which the enzyme reacts (Kabiraj et al., 2015). Another method is immunofluorescence, where the antibody is tagged with a fluorophore, such as a fluorescent dye (Khalid et al., 2020).

Cytological examination

Cytological examination of tumors can be performed using the fine needle aspiration cytology (FNAC) technique (Ugochukwu et al., 2019). Cytology is an important tool that helps veterinarians distinguish tumors from inflammatory lesions (Mathewos et al., 2021). This process involves cell sampling by inserting a thin hollow needle into the tissue mass, followed by staining the collected cells and examining them under a microscope (Thomas et al., 2016). Other diagnostic techniques may include dermoscopy and various imaging modalities such as X-rays, computed tomography (CT) scans, positron emission tomography (PET), ultrasound, and magnetic resonance imaging (MRI, Mithila et al.,

2020). Additionally, tests such as urine and blood analyses can provide valuable information for tumor diagnosis (Timurkan and Alcigir, 2017).

The economic impact of skin tumor

In Ethiopia, skin and hide account for 14–16% of all export revenue annually (Nigussu, 2014). However, the quality of raw materials poses significant challenges that hamper the leather industry (Vasconcelos et al., 2023). One of the primary causes of poor-quality skin is skin tumors, which lead to substantial economic losses (Kumar et al., 2015). Over time, the market share value of skin and hides has declined due to a decrease in leather prices on the global market, stemming from deteriorating skin quality (Mathewos et al., 2021). The economic losses associated with skin tumors are linked to the morbidity and mortality of domestic animals (Mathewos et al., 2020). Moreover, the morbidity of affected animals results in decreased body weight, reduced productivity and reproduction, difficulties during calving or milking, carcass condemnation, treatment costs, compromised skin quality, and decreased market value, as well as suckling problems and increased mortality (Kumar et al., 2015).

Periocular squamous cell carcinoma (SCC) can cause blindness in cattle, leading to early culling and accounting for approximately 12% of carcass condemnations (Mathewos et al., 2020). Another common skin tumor, lymphosarcoma, leads to extensive organ condemnation, contributing to 13.5% of beef cattle and 26.9% of dairy carcass condemnations (Mauldin, 2019). The estimated annual financial loss from carcass condemnations due to all types of tumors is approximately \$2.2 million (Mathewos et al., 2020), highlighting the significant economic impact of skin diseases.

Skin tumors in the cattle industry lead to an increased culling rate due to metastasis to critical organs such as the lungs, liver, and draining lymph nodes, resulting in severe complications (Kumar et al., 2015; Mauldin, 2019). In the context of healthcare management for skin cancer, data from over 880,000 healthcare providers indicated that they received \$77 billion in Medicare payments (Chen et al., 2016).

Management and medication of skin tumor

Prevention and management of tumors are challenging due to numerous environmental influences that can alter normal DNA replication (Mauldin, 2019). However, early diagnosis and treatment remain crucial for effective management before metastasis occurs (Khalid et al., 2020). Additionally, strengthening national policies and programs to raise awareness and reduce exposure to risk factors, such as prolonged UV radiation, is important (Achalkar, 2019).

The management and treatment methods for skin tumors may depend on the cancer's stage, the type of tumor, and the likelihood of cancer spreading or regressing (Thomas et al., 2016). Common management approaches for skin tumors include radiation therapy, chemotherapy, and surgical removal (Ugochukwu et al., 2019). Other methods may include electroporation, gene therapy, cryotherapy (using liquid nitrogen or carbon dioxide to freeze and destroy cancer cells, (Timurkan and Alcigir, 2017), and immunotherapy through the administration of inactivated tumor cells (Bukhtoyarov and Samarin, 2015). If the tumor is localized and confined to a specific area, surgery is prioritized; conversely, if it has widely spread, chemotherapy and radiotherapy become more significant (Xiao et al., 2020).

Chemotherapy

Chemotherapy can be administered subcutaneously, orally, or intravenously (Lotfalizadeh et al., 2022). Topical therapy drugs used for non-melanoma skin cancers, such as SCC and papilloma, include imiquimod and 5-fluorouracil (Timurkan and Alcigir, 2017). Systemic chemotherapy employs anticancer (cytotoxic) drugs that circulate through the bloodstream to target and destroy cancer cells (Ugochukwu et al., 2019). Common chemotherapy agents used to treat cancers like melanoma or to slow their growth include cisplatin and paclitaxel (Taxol, Xiao et al., 2020). Intravenous infusions of magnesium chloride have been reported to be effective in treating bovine cutaneous papillomatosis when lesions are few; this treatment is often combined with Ivermectin (Lotfalizadeh et al., 2022).

Drug therapy plays a crucial role in destroying cancer cells, slowing or halting their growth and spread (Khalid et al., 2020). It can also shrink tumors before surgical or radiation treatments, eliminate cancer cells that may remain post-surgery, and relieve or manage symptoms of advanced non-melanoma skin cancer (Mithila et al., 2020).

Nanophytosomes

Lipid-based nanocarriers called phytosomes combine phytoconstituents or plant extracts with phospholipids (Saeed et al., 2022). Through this conjugation, a complex with enhanced solubility, stability, and bioavailability is formed (Barani et al., 2021). The potential of phytosomes in cancer treatment has been documented in previous studies (Dubey et al., 2022). Curcumin, resveratrol, and quercetin phytosomes, for example, have demonstrated anticancer effects in a variety of *in vitro* and *in vivo* settings (Patra et al., 2021). Additionally, chemotherapeutic drugs including Paclitaxel, Docetaxel, and Camptothecin have been delivered via phytosomes with increased efficacy and decreased toxicity

(Alharbi et al., 2021). Several methods, such as solvent evaporation, thin-film hydration, and coacervation, can be used to create phytosomes (Alharbi et al., 2021).

A promising medication delivery method for enhancing the bioavailability and effectiveness of anticancer substances produced from plants is nanophytosomes (Alharbi et al., 2021). Compared to conventional delivery methods, these lipid-based nanocarriers have several benefits, including enhanced cellular absorption, higher bioavailability, and decreased toxicity (Saeed et al., 2022). The effectiveness of nanophytosomes in delivering phytoconstituents to cancer cells with little harmful effects on healthy cells has been demonstrated by recent research that has investigated the potential of nanophytosomes in cancer therapy (Saeed et al., 2022).

Silibinin has efficient anticancer properties against a variety of cancer cells, such as hepatocellular carcinoma, prostate cancer, breast cancer, and lung cancer, have drawn a lot of interest in recent years (Barani et al., 2021). The anticancer properties of silibinin have been explained by several different mechanisms (Saeed et al., 2022). One of the main strategies is to stop the growth of cancer cells by causing cell cycle arrest and death (Dubey et al., 2022). By targeting multiple signaling pathways, silibinin has also been demonstrated to prevent the angiogenesis, invasion, and metastasis of cancer cells (Saeed et al., 2022).

One of the most common forms of cancer is skin cancer, which is a serious global public health concern (Patra et al., 2021). An appealing method of treating skin cancers is the use of wound-healing substances that also have cytotoxic effects on cancer cells (Saeed et al., 2022). It has been demonstrated that sinigrin, a naturally occurring substance in the Brassicaceae family, has anticancer properties (Barani et al., 2021). The anticancer effects of Sinigrin's phytosomal formulation on A-375 melanoma cells and its wound-healing properties on normal human keratinocyte cells (HaCaT) were examined by Mazumder et al. in a novel study (Alharbi et al., 2021). The study found that when it came to cytotoxicity against A-375 cell lines, Sinigrin-loaded phytosomes significantly outperformed free Sinigrin (Saeed et al., 2022). Furthermore, normal cells (HaCaT) showed only mild cytotoxic effects (Patra et al., 2021). 50% more wound closure was observed at different doses and periods in the *in vitro* wound healing study using HaCaT cells (Dubey et al., 2022). These findings imply that phytosomes loaded with sinigrin might be a viable option for treating malignant wounds and cancer therapy (Saeed et al., 2022). It has been demonstrated that phytosomes, a novel delivery technology, increase the bioavailability of natural substances, including anticancer drugs (Barani et al., 2021). The lipid-based Sinigrin found in phytosomes is encased in a phospholipid bilayer (Dubey et al., 2022). This encapsulation improves the compound's stability and solubility, which raises its bioavailability (Saeed et al., 2022).

An innovative method of treating skin malignancies is the use of phytosomes loaded with Sinigrin as a therapeutic agent (Patra et al., 2021). It has been demonstrated that Sinigrin has anticancer effects by causing apoptosis and preventing cell division (Dubey et al., 2022). Sinigrin's phytosomal form may make it possible to deliver the drug to cancer cells more effectively while reducing its impact on healthy cells (Alharbi et al., 2021).

Mitomycin C (MMC) has demonstrated encouraging outcomes in the treatment of a variety of malignancies in addition to its strong anticancer properties (Patra et al., 2021). However, a significant barrier to MMC's therapeutic use has been its quick absorption into the systemic circulation (Barani et al., 2021). As a result, the drug's plasma concentration in the pertinent areas drops, which reduces the effectiveness of treatment (Saeed et al., 2022).

Turmeric, or *Curcuma longa*, is a long-used traditional herb that has been utilized for ages for its therapeutic qualities (Barani et al., 2021). It contains chemical substances known as curcuminoids and naturally occurring hydrophobic polyphenols, which have been demonstrated to have a wide range of pharmacological activity, including positive benefits in the treatment of various malignancies (Dubey et al., 2022). However, curcumin's poor oral solubility and bioavailability frequently impede its medicinal potential (Patra et al., 2021). To overcome these challenges and make its clinical application easier, several formulation techniques and other strategies have been developed (Saeed et al., 2022).

Vaccination

Vaccination remains the best prevention method against papilloma (Vasconcelos et al., 2023). Bovine papillomavirus (BPV) vaccine strategies typically involve the use of recombinant BPV L1 protein vaccines and the intradermal administration of autogenously produced wart vaccines (Ugochukwu et al., 2019). Due to the lack of available vaccinations, efforts to mitigate the risk of enzootic bovine lymphosarcoma focus on controlling the spread of the bovine leukemia virus and, in sporadic cases, reducing risk factors (Achalkar, 2019). Unfortunately, there is no curative treatment for viral infections or lymphosarcoma in cattle (Habte, 2022).

Radiation therapy

Radiation therapy involves exposing the cancerous part of the body to high doses of radiation, which can destroy rapidly growing cells and shrink tumors. This treatment is targeted specifically to the area where the cancer is developing

(Hennequin et al., 2016; Low and Sahi, 2016). Radiation can also be applied after surgery as an adjuvant therapy to eliminate any small areas of remaining cancer cells that may not have been visible during the procedure (Lotfalizadeh et al., 2022).

CONCLUSION

Skin tumors are the most frequently diagnosed neoplastic disorders in bovines, with bovine papilloma and squamous cell carcinoma being particularly prevalent. Bovine papilloma tumors, in particular, are among the most commonly diagnosed and economically significant tumors in cattle. Although the specific causative agent remains unknown, most skin tumors are associated with prolonged exposure to ultraviolet radiation. These tumors lead to substantial economic losses due to reduced productivity, decreased reproduction, calving, and milking difficulties, treatment costs, suckling problems, early culling, carcass condemnation, and management expenses. Given the economic significance of skin tumors in Ethiopia, skin tumors warrant greater attention from researchers and control centers. Early diagnosis and management of skin tumors are critical issues that need to be addressed.

DECLARATIONS

Competing interests

The authors declared that there is no conflict of interest.

Author's contributions

Mengesha Ayehu Getnet wrote the draft of the paper. Asnakew Mulaw Berihun supervised the drafts and revised the draft of the manuscript. All authors checked and approved the final draft of the manuscript.

Ethical considerations

All authors have contributed to the preparation of this paper. The authors observed the final version of the finished paper and evaluated any corrections and updates. They also checked the similarity index of article.

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Availability of data and materials

Since this manuscript has been prepared as a review article it has only used different journal papers that have been done on bovine skin tumors. Therefore, there is no Excel available data.

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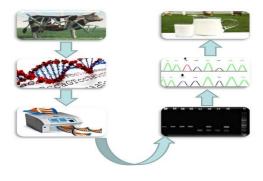
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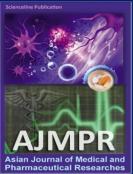
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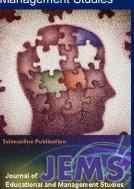
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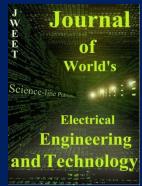
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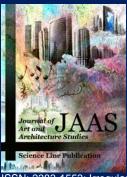
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